BIOPHYSICAL IMAGING AND COMPUTATIONAL BIOLOGY

Spatial Distributions of Lipids in Atherosclerosis of Human Coronary Arteries Studied by Time-of-Flight Secondary Ion Mass Spectrometry

Satu Lehti,^{*} Peter Sjövall,[†] Reijo Käkelä,[‡] Mikko I. Mäyränpää,^{§¶} Petri T. Kovanen,^{*} and Katariina Öörni^{*}

From the Wihuri Research Institute,^{*} Helsinki, Finland; the SP Technical Research Institute of Sweden,[†] Borås, Sweden; the Department of Biosciences[‡] and the Department of Pathology,[§] Haartman Institute, University of Helsinki, Helsinki, Finland; and the Division of Pathology,[¶] HUSLAB, Meilahti Laboratories of Pathology, Helsinki University Central Hospital, Helsinki, Finland

Accepted for publication
January 27, 2015.

Address correspondence to
Katariina Öörni, Ph.D., Wihuri
Research Institute, Haartma-
ninkatu 8, 00290 Helsinki,
Finland. E-mail: kati.oorni@wri.fi.

The accurate spatial distribution of various lipid species during atherogenesis has remained unexplored. Herein, we used time-of-flight secondary ion mass spectrometry (TOF-SIMS) to analyze the lipid distribution in human coronary artery cryosections. The images from the TOF-SIMS allowed visualization of ions derived from individual species of cholesterol esters, phospholipids, and triacylglycerols in the context of lesion characteristics and severity. In addition, cholesterol-containing crystal-like structures were seen in high-resolution images of advanced lesions. The ratio of cholesterol fragment ions (m/z 385: m/z 369) was found to differentiate unesterified cholesterol from cholesterol esters. This ratio changed during atherogenesis and in different areas of the lesions, reflecting differences in the accumulation of the two forms of cholesterol. Thus, atheromas were characterized by accumulation of cholesterol esters with apolipoprotein B near the intima-media border, whereas in the complicated lesions, unesterified cholesterol dominated in neovessel-containing areas enriched in glycoporphin A. Interestingly, triacylglycerols were found in areas surrounding neovessels and lacking either form of cholesterol. The lipid composition of the tunica media reflected the alterations observed in the intimal lipids, yet being more subtle. The detailed molecular information obtained by TOF-SIMS revealed unanticipated differences in the type and composition of the accumulating lipids in different stages of atherogenesis, notably the spatial segregation of cholesterol and triglycerides in the advancing lesions. (*Am J Pathol* 2015; 185: 1216–1233; <http://dx.doi.org/10.1016/j.ajpath.2015.01.026>)

The ground to formation of atherosclerotic lesions is set when circulating apolipoprotein B (apoB)—containing lipoproteins begin to accumulate in the arterial intima. The proteoglycan-rich arterial intima retains the lipoproteins, which are then exposed to modifications by oxidative agents and by lipolytic and proteolytic enzymes.^{1,2} On modification, the lipoproteins become unstable, and easily aggregate or fuse to form extracellular lipid droplets.^{3–7} The modified apoB-containing lipoprotein particles, whether present as individual particles, aggregates, or droplets, are readily absorbed by macrophages, and also by smooth muscle cells, which then become filled with cholesteryl ester (CE) droplets and are converted into foam cells.^{8–11} When numerous foam cells form clusters, they have a yellow appearance visible to the naked eye, and the site appears as a fatty dot or

a fatty streak.¹² Lipoproteins not absorbed by the macrophages are retained within the extracellular matrix¹³ and, as such, contribute to the lipid load of the arterial intima. In addition to the lipoproteins, red blood cells may act as a source of the intimal cholesterol and phospholipids (PLs), particularly in advanced atherosclerotic lesions in which leaky and fragile neovessels are present.¹⁴ Fundamentally, progressive extracellular accumulation in the arterial intima

Supported by grants from the Magnus Ehrnrooth Foundation, the Finnish Foundation for Cardiac Research, the Finnish Concordia Fund, the Veritas Foundation, the Paulo Foundation, the Aarne Koskelo Foundation, and the Ida Montin Foundation (all to S.L.); and the Academy of Finland (265940 to K.Ö.). Wihuri Research Institute is maintained by the Jenni and Antti Wihuri Foundation.

Disclosures: None declared.

of cholesterol and other lipids of apoB-containing lipoprotein particles produces the conversion of fatty streaks into more advanced types of atherosclerotic lesions (ie, atheromas, fibroatheromas, and, ultimately, complicated lesions).¹⁵

The overall lipid composition of human atherosclerotic plaques has been determined in many studies over a period of more than half a century.^{16–19} In these studies, the lipids have been extracted from arterial segments, isolated plaques, or extracellular lipid droplets, and then subjected to thin-layer chromatographic or, more recently, mass spectrometric analysis. The cholesterol in the lesions (total cholesterol) is present in both esterified and unesterified form, and the relative proportions of these two forms have been found to vary in relation to the degree of the plaque development. Thus, the proportion of CE of total cholesterol is highest in fatty streaks and decreases as the plaques progress.^{4,7,18,20–22} Also, other lipid ratios have been found to alter during the course of the disease, including the ratio of total cholesterol to PLs that is the highest in the fatty streaks, and the sphingomyelin (SM) to phosphatidylcholine (PC) ratio (SM/PC-ratio) that has been found to increase as the disease progresses.^{6,18,20–22}

Recently, it has become possible to examine the spatial distribution of lipids with the aid of imaging mass spectrometry methods, such as time-of-flight secondary ion mass spectrometry (TOF-SIMS), with which the lipids on a tissue section can be detected, identified, and localized with a spatial resolution down to $<1\ \mu\text{m}$. During analysis, the tissue sections are bombarded with high-energy (primary) ions, typically cluster ions, such as Au_3^+ , Bi_3^+ , or C_{60}^+ .^{23–25} The collisions of the primary ions results in emission of atoms, molecular fragments, and intact molecules, which can be either neutral or ionized (positive or negative). The emitted (secondary) ions are then separated according to mass per charge (m/z) and detected in a TOF analyzer, producing a mass spectrum that contains detailed molecular information about the sample surface. By focusing and scanning the primary ion beam over the analysis area, separate mass spectra are acquired from many raster points (typically 256×256 pixels), allowing for the generation of ion-specific images, which represent the spatial distribution of the corresponding molecular species within the analysis area or mass spectra from selected regions of interest on the sample surface.^{23,26} The spatial molecular distributions have been studied with other imaging mass spectrometry methods, such as desorption electrospray ionization and matrix-assisted laser desorption ionization. However, an important advantage of SIMS over these methods for imaging of lipids in tissue samples is the high spatial resolution.²⁷

Herein, we examined the spatial distribution of lipids in the intima of human coronary arteries by imaging TOF-SIMS, to link differences in the lipid distribution pattern to protein markers of atherosclerosis and the progression of atherosclerosis. We found that cholesterol colocalized with apoB-100 in the atheromas and, in addition, with red blood cell surface protein glycophorin A (GpA) in the complicated lesions. Cholesterol and triacylglycerols (TAGs) rarely accumulated in

the same areas of the plaques: cholesterol accumulated largely near the intima-media border, whereas TAGs were detected mainly subendothelially and in the vicinity of neovessels of complicated lesions. By using the TOF-SIMS tool, we also found that the lipid composition of the tunica media alters with the severity of the atherosclerosis in the intima. Taken together, this study identifies spatial relationships between various molecular species characteristic of human coronary atherogenesis, and reveals previously unrecognized heterogeneity in the distribution of cholesterol and TAGs in the lesions.

Materials and Methods

Ethics Statement

Human coronary arteries were acquired from hearts obtained from heart transplant recipients and from donor hearts not suitable for transplantation because of tissue type mismatch ([Supplemental Table S1](#)). The use of human material conforms to the principles outlined in the Declaration of Helsinki, and the study was approved by the Ethics Committee of Helsinki University Central Hospital (Helsinki, Finland) and the National Authority for Medicolegal Affairs. All transplant patients had given informed consent. Human plasma was obtained from several healthy blood donors (Finnish Red Cross Blood Service, Helsinki, Finland), who had signed an informed consent document. The plasma samples were by-products from the preparation of blood products for clinical use. The use of plasma samples in lipoprotein isolation was approved by the Finnish Red Cross Blood Service.

Preparation of Sections for Mass Spectrometric and Microscopic Imaging

Human coronary arteries were stored at -80°C after detaching the arteries from the heart. Segments of the coronary arteries (10 mm long) were selected by a pathologist (M.I.M.) with a visual inspection under a dissection microscope (model C-DSS230; Nikon, Tokyo, Japan). Four normal and four atherosclerotic coronary arteries were chosen on the basis of visual assessment of the severity of the lesions.

For TOF-SIMS imaging and immunohistochemistry, the coronary artery samples were prepared by cryosectioning. Segments of bare deep-frozen human coronary arteries (4 mm long) were attached to the cryotome specimen disks with cryoembedding medium (Tissue-Tek O.C.T. Compound; Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands). To prevent this medium from contaminating the tissue surface, which is the critical layer for TOF-SIMS analysis, the samples were cut without surrounding support of the medium.²⁸ After cutting the samples into sections (10 μm thick), the sections were attached on $1.5 \times 1.5\text{-cm}$ silica wafers for TOF-SIMS and kept at -80°C until analyzed.

For immunohistochemistry, the sectioning of the coronary arteries was continued after the sections for TOF-SIMS had been prepared. The serial sections were mounted onto

Table 1 Assignment of Negative and Positive Ions in the Acquired TOF-SIMS Spectra of Coronary Artery Samples

Observed mass	Assignment	Ion	Formula	Theoretical mass	Reference
Negative ions					
253.22	16:1	(M-H) ⁻	C ₁₆ H ₂₉ O ₂ ⁻	253.22	32,33
255.23	16:0	(M-H) ⁻	C ₁₆ H ₃₁ O ₂ ⁻	255.23	32,33
279.23	18:2	(M-H) ⁻	C ₁₈ H ₃₁ O ₂ ⁻	279.23	32,33
281.24	18:1	(M-H) ⁻	C ₁₈ H ₃₃ O ₂ ⁻	281.25	32,33
283.26	18:0	(M-H) ⁻	C ₁₈ H ₃₅ O ₂ ⁻	283.26	32,33
303.2	20:4	(M+H) ⁻	C ₂₀ H ₃₁ O ₂ ⁻	303.24	32,33
327.22	22:6	(M+H)	C ₂₂ H ₃₁ O ₂ ⁻	327.22	32,33
616.50	SM (16:0)	(M-C ₂ H ₂ NC ₃ H ₁₀) ⁻	C ₃₄ H ₆₇ NO ₆ P ⁻	616.47	33
642.50	SM (16:0)	(M-NC ₃ H ₁₀) ⁻	C ₃₆ H ₆₉ NO ₆ P ⁻	642.48	33
640.49	SM (16:1)	(M-NC ₃ H ₁₀) ⁻	C ₃₆ H ₆₇ NO ₆ P ⁻	640.48	33
687.56	SM (16:0)	(M-CH ₃) ⁻	C ₃₈ H ₇₆ N ₂ O ₆ P ⁻	687.54	33
685.53	SM (16:1)	(M-CH ₃) ⁻	C ₃₈ H ₇₄ N ₂ O ₆ P ⁻	685.53	33
715.6	SM (18:0)	(M-CH ₃) ⁻	C ₄₀ H ₇₈ O ₆ P	715.56	33
713.5	SM (18:1)	(M-CH ₃) ⁻	C ₄₀ H ₈₀ O ₆ P	713.54	33
885.60	PI (38:4)	(M-H) ⁻	C ₄₇ H ₈₂ O ₁₃ P ⁻	885.54	33
Positive ions					
55.94	Fe		Fe ⁺	55.94	
184.08	PC fragment	Fragment	C ₅ H ₁₅ NPO ₄ ⁺	184.06	34
369.35	Cholesterol	(M-OH) ⁺	C ₂₇ H ₄₅ ⁺	369.35	23
385.34	Cholesterol	(M-H) ⁺	C ₂₇ H ₄₅ O ⁺		
430.34	Vitamin E	(M+H) ⁺	C ₂₉ H ₅₀ O ₂ ⁺	430.34	
496.33	LPC (16:0)	(M+H) ⁺	C ₂₄ H ₅₀ NO ₇ P ⁺	496.38	
524.36	LPC (18:0)		C ₂₆ H ₅₄ NO ₇ P ⁺	524.36	
547.49	DAG (32:2)	(M-OH) ⁺	C ₃₅ H ₆₃ O ₄ ⁺	547.47	32,33
549.51	DAG (32:1)	(M-OH) ⁺	C ₃₅ H ₆₅ O ₄ ⁺	549.49	32,33
551.52	DAG (32:0)	(M-OH) ⁺	C ₃₅ H ₆₇ O ₄ ⁺	551.52	
575.52	DAG (34:2)	(M-OH) ⁺	C ₃₇ H ₆₇ O ₄ ⁺	575.50	32,33
577.54	DAG (34:1)	(M-OH) ⁺	C ₃₇ H ₆₉ O ₄ ⁺	577.52	32,33
601.54	DAG (36:3)	(M-OH) ⁺	C ₃₉ H ₆₉ O ₄ ⁺	601.52	32,33
603.56	DAG (36:2)	(M-OH) ⁺	C ₃₉ H ₇₁ O ₄ ⁺	603.54	32,33
734.59	PC (32:0)	(M+H) ⁺	C ₄₀ H ₈₁ NO ₈ P ⁺	734.56	34
758.59	PC (34:2)	(M+H) ⁺	C ₄₂ H ₈₁ NO ₈ P ⁺	758.57	34
760.60	PC (34:1)	(M+H) ⁺	C ₄₂ H ₈₃ NO ₈ P ⁺	760.58	34
782.60	PC (34:1)	(M+Na) ⁺	C ₄₂ H ₈₂ NO ₈ PNa ⁺	782.57	
786.63	PC (36:2)	(M+H) ⁺	C ₄₄ H ₈₅ NO ₈ P ⁺	786.60	34
788.62	PC (36:1)	(M+H) ⁺	C ₄₄ H ₈₇ NO ₈ P ⁺	788.61	34
798.57	PC (34:1)	(M+K) ⁺	C ₄₂ H ₈₂ NO ₈ PK ⁺	798.54	
806.58	PC (36:3)	(M+Na) ⁺	C ₄₄ H ₈₂ NO ₈ PNa ⁺	806.57	
808.61	PC (36:2)	(M+Na) ⁺	C ₄₄ H ₈₄ NO ₈ PNa ⁺	808.58	
810.62	PC (36:1)	(M+Na) ⁺	C ₄₄ H ₈₆ NO ₈ PNa ⁺	810.60	

DAG, diacylglycerol; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; TOF-SIMS, time-of-flight secondary ion mass spectrometry.

Superfrost slides (Menzel-Gläser, Braunschweig, Germany), and the slides were stored at -20°C until stained. Before the stainings, frozen sections were fixed with 4% formaldehyde for 10 minutes, and washed with Tris-buffered saline (TBS) before use for histology or immunohistochemistry. For histological detection of the neutral lipids, the sections were stained with Oil Red O (ORO) with hematoxylin counterstain.

Histology and Immunohistochemistry

ORO staining for neutral lipids was performed on formalin-fixed sections, as follows. Sections were rinsed with 60% isopropanol and stained with freshly prepared ORO working

solution for 15 minutes, after which the sections were again rinsed with 60% isopropanol. The nuclei were counterstained with Mayer’s hematoxylin for 30 seconds, and the sections were rinsed under cold running tap water until no extra stain was found in the water. The slides were mounted with water-based mounting medium.

ORO and standard hematoxylin and eosin stainings were used to classify the arteries using the classification by the American Heart Association (AHA).¹² The AHA class 1 corresponds to a normal intima; 2, an adaptive intimal thickening; 3, fatty streak; 4, atheroma; 5, fibroatheroma; and 6, complicated plaque. The classification was determined according to the worst affected area in each coronary sample.

Figure 1 Cholesterol and phosphocholine head group (m/z 184) in atherosclerotic human coronary arteries. **A–D:** Optical micrographs with superimposed cholesterol time-of-flight secondary ion mass spectrometry images of human coronary arteries having different degrees of atherosclerosis. The **boxed areas** in **A–D** indicate areas shown in the high-resolution images in **E–P**, respectively. Phosphocholine head group (m/z 184; **E–H**) and total cholesterol (**I–L**) were detected in 256×256 - μm areas of normal intima (**A**, **E**, **I**, and **M**), fatty streak (**B**, **F**, **J**, and **N**), atheroma (**C**, **G**, **K**, and **O**), and complicated plaque (**D**, **H**, **L**, and **P**). **M–O:** The generated images were overlaid to view the colocalization of cholesterol and phospholipid. Mc, maximum number of detected ions (counts) per pixel.

The tissue sections were also stained with immunohistochemical antibodies against human apoB-100 [10 $\mu\text{g/mL}$ mouse IgG_{2b} (Lifespan Biosciences, Seattle, WA), catalog number LS-C35039], CD163 [1 $\mu\text{g/mL}$ IgG₁ (Acris Antibodies, Herford, Germany), catalog number SM4041, clone 5D6-FAT], CD34/31 [0.5 $\mu\text{g/mL}$ CD34 mouse IgG_{1k} (Novocastra, Newcastle upon Tyne, UK), catalog number NCL-L-END, clone QBEnd; and 10 $\mu\text{g/mL}$ CD31 mouse IgG_{1k} (Dako, Glostrup, Denmark), catalog number M0823, clone JC70A], and GpA [mouse IgG_{2b} (Dako), catalog number M0819; and 32 $\mu\text{g/mL}$ mouse IgG₁].

The fixed sections were washed twice with TBS with 0.5% Tween 20 to remove the 4% phosphate-buffered paraformaldehyde, and the non-specific labeling was prevented

by blocking the tissues with 0.5% bovine serum albumin in TBS for 30 minutes, after which the sections were incubated with the primary antibody for 1 hour at room temperature. After washing with TBS with 0.5% Tween 20, horseradish peroxidase-labeled anti-mouse secondary antibody conjugated with polymer beads [anti-mouse EnVision Detection system horseradish peroxidase (diaminobenzidine) K4007; Dako] was incubated for 1 hour at room temperature. After the incubation, the sections were washed with TBS with 0.5% Tween 20. Endogenous peroxidases were blocked with sodium azide provided in the EnVision Kit for 30 minutes at room temperature, after which the sections were washed and the horseradish peroxidase substrate was administered on the sections and

incubated for 2 minutes. Next, the sections were washed with distilled water and the nuclei were stained with hematoxylin. The sections were then rinsed with tap water until no color was released.

TOF-SIMS Analysis

TOF-SIMS analysis was conducted in a TOF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) using

25 keV Bi_3^+ primary ions and electron flooding for charge compensation. High-mass resolution data were acquired with the instrument in the bunched mode ($m/\Delta m \approx 5000$; lateral resolution, 3 to 4 μm) at a pulsed primary ion beam current of 0.1 pA over analysis areas of $500 \times 500 \mu\text{m}^2$ using 256×256 pixels. High spatial resolution images were obtained with the instrument in unbunched mode ($m/\Delta m \approx 300$; lateral resolution, 200 to 300 nm) at a pulsed primary ion beam current of 0.04 pA. The primary ion dose density was always kept $< 3.8 \times 10^{10} \text{ cm}^{-2}$ and $1.8 \times 10^{12} \text{ cm}^{-2}$ in the bunched and unbunched modes, respectively, ensuring static SIMS conditions.

Two to eight sample areas of $500 \times 500 \mu\text{m}$ were analyzed from each of the eight coronary artery sections by TOF-SIMS. After data acquisition, the analyzed 23 square areas were further divided into 46 smaller subareas, from which separate TOF-SIMS spectra were reconstructed from the acquired data (Supplemental Figure S1). Eight of the subareas were tunica media, whereas the rest were intimal areas. The intimal subareas were separately graded into six different grades (1 to 6) by a pathologist (M.I.M.), using the classification by AHA^{12,15} as a guideline (Supplemental Tables S2 and S3).

The TOF-SIMS ion intensities from the subareas were normalized to the number of pixels of the subarea. The signal intensities reflect relative concentrations of the various ions, but because there are differences in the ionization of the various lipids, matrix effects are likely to underestimate the concentration of the neutral lipids that do not ionize easily, and overestimate the amount of easily ionizable PLs. The ion images generated from mass spectrometric ion intensities were transformed into monochromatic tagged image file format images with Photoshop software (Adobe Photoshop CS3 version 10.0.1; San Jose, CA), and assembled into composite images and composite image pixel colocalizations with Image Pro version 6.2.0.424 (Media Cybernetics Inc., Rockville, MD). The images were overlaid to analyze colocalizations of the various lipid

Figure 2 Area-normalized intensities of phosphocholine and cholesterol ions, and the cholesterol ion intensity ratio 385:369 for standard samples and for subareas in tissue sections. **A:** Phosphocholine ion (m/z 184) intensities, normalized to the number of pixels in the media and in the six grades of subareas (Supplemental Table S2). **B:** The normalized intensities of cholesterol-derived ions m/z 369 and m/z 385 (shown enlarged in the inset). **C:** The intensity ratio of the two cholesterol-derived ions (385:369) in reference time-of-flight secondary ion mass spectrometry spectra recorded from standard samples of unesterified cholesterol and cholesteryl linoleate. **D:** The ratio 385:369 calculated from the spectra of the media and the subareas of the intima. As statistics, Kruskal-Wallis nonparametric analysis of variance was applied ($P < 0.05$). This analysis was followed by Mann-Whitney test, one pair at a time. Guide to the lower case letter code: The values with no common letter differ at the $P < 0.05$ level (no statistical differences, no code). As a default, the highest mean was marked with letter a. Grades 1 to 6 denote subareas of the intima. Groups 1 and 5, $n = 3$; group 2, $n = 4$; group 3, $n = 10$; group 4, $n = 9$; group 6 and media (M), $n = 8$.

Figure 3 Colocalization of cholesterol with linoleic acid, oleic acid, palmitic acid, and stearic acid. **A–P:** Overlay ion images of cholesterol (red; m/z 369) with the fatty acids (green) linoleic acid (18:2), oleic acid (18:1), palmitic acid (16:0), and stearic acid (18:0) in atherosclerotic lesions. **Dotted lines** mark the endothelium; **solid lines**, the intimal-medial border; and **dashed lines**, the medial-adventitial border. Field of view, 500 μm . A, adventitia; I, intima; M, media; R, degree of colocalization.

species. Colocalization of coefficient R showed the degree of pixel colocalization.^{29,30}

Statistical Analysis

For univariate statistical comparisons, we used Kruskal-Wallis nonparametric analysis of variance, followed by a post hoc Mann-Whitney test available in the software package SPSS statistics version 22 (IBM, Armonk, NY). For multivariate statistical comparisons, principal component analysis (PCA)³¹ was performed by using Sirius software version 8.5 (Pattern Recognition Systems, Bergen, Norway). Before the PCA, the ion intensity data were logarithmically transformed and

subsequently standardized (variable deviations homogenized) to prevent the abundant components with large variance from dominating the analysis. In PCA, the samples originally positioned in multidimensional space (as many coordinates as ion intensities of chemical components analyzed) were plotted in a newly formed two-coordinate system of principal components 1 and 2, which accounted for as much of the original data variability as possible. Biplot graphs were generated to illustrate the compositional similarities/differences among the graded samples and to indicate correlations between the variables. For quantification of the compositional differences between the sample classes, soft independent modeling of class analogies was used.

Figure 4 Area-normalized ion intensities of linoleic acid, oleic acid, palmitic acid, and stearic acid. **A–D:** The ion intensities of the same fatty acid ions in the intimal regions representing different classes. As statistics, Kruskal-Wallis nonparametric analysis of variance was applied ($P < 0.05$). This analysis was followed by Mann-Whitney test, one pair at a time. Guide to the lower case letter code: The values with no common letter differ at $P < 0.05$ (no statistical differences, no code). As a default, the highest mean was marked with letter a. Grades 1 to 6 denote subareas of the intima. Groups 1 and 5, $n = 3$; group 2, $n = 4$; group 3, $n = 10$; group 4, $n = 9$; group 6 and media (M), $n = 8$.

Results

Cryosections (10 μm thick) of human coronary arteries in different stages of atherogenesis were selected for this study, showing grossly normal intimas, minimal intimal thickening or fatty streaks, atheromas, or complicated lesions. Several $500 \times 500\text{-}\mu\text{m}$ areas from the intima and the media were analyzed by TOF-SIMS, in both positive and negative ion mode, and spatial distributions of the most relevant ions were studied. Ions that were analyzed by TOF-SIMS are listed in Table 1, and histological images of the arterial sections are shown in Supplemental Figure S2. In all of the studied eight coronary arteries, cholesterol was found in close proximity to the endothelium (Supplemental Figure S2, A–H). Elsewhere in the normal intima (Supplemental Figure S2, A and B), most of the cholesterol was evenly distributed and the intensities of cholesterol-derived ions were low. In the thick intima of the advanced atherosclerotic lesions, large amounts of cholesterol were detected near the border between the intima and the media (Supplemental Figure S2, E and F). In the complicated lesions, several distinct areas with cholesterol accumulation were present (Supplemental Figure S2, G and H). The cholesterol distribution pattern detected by TOF-SIMS was similar to the pattern of neutral lipids visualized by ORO staining in adjacent sections of the same arteries (Supplemental Figure S3).

Colocalization of Cholesterol with PLs

We further analyzed selected $256 \times 256\text{-}\mu\text{m}$ areas of each sample with high spatial resolution. Representative images of each plaque type are shown in Figure 1. In Figure 1, A–D, the boxed areas depict photomicrographs of high resolution areas in E–P. The samples were analyzed for the PLs with phosphocholine head group (m/z 184) (Figure 1, E–H) and total cholesterol (m/z 385 plus m/z 369) (Figure 1, I–L), where m/z 385 is the $(\text{M-H})^+$ -ion and m/z 369 is the $(\text{M-OH})^+$ -ion (Table 1). Phosphocholine head group is a characteristic of PC, lysophosphatidylcholine (LPC), and SM, which together represent 99% of all PLs in low-density lipoprotein (LDL)³⁵ and 50% to 65% of the PLs in mammalian nucleated cells.³⁶ The images were combined to visualize the colocalization of these two types of lipids (Figure 1, M–P). The PLs were distributed more evenly than cholesterol, which showed distinct sites of accumulation, particularly in the atheromas and in the complicated lesions. The PL signal was the highest in fatty streaks, whereas the cholesterol signal increased from normal intima to fatty streaks and further to atheromas. In the complicated lesions, the cholesterol signal was again lower. Cholesterol colocalized only minimally with the PLs.

To compare the amounts of lipids in the individual areas of the arteries, we divided the areas analyzed by TOF-SIMS (Supplemental Figure S1, A–C) separately into subareas of intima and areas containing only media (Supplemental Figure S1, D and E). The intimal subareas were further

graded into six grades of atherosclerosis according to their neutral lipid accumulation, neovascular and macrophage distribution, and morphology using the AHA classes 1 to 6 as a guideline. The amounts of PLs and cholesterol were then determined in the media and in the intimal subareas. The phosphocholine head group ion (Figure 2A) had the highest intensity in the medial layer of the normal coronary arteries and the lowest intensity in the intimal subareas graded as grade 6.

Cholesterol was detected in the positive ionization mode with two ions, m/z 385 and m/z 369, with the latter having higher intensity (Figure 2B). The intensities of both cholesterol ions were the highest in grade 4 subareas, followed by the grade 6 subareas. Interestingly, the cholesterol intensities in grade 5 subareas were relatively low. As expected, the lowest cholesterol intensities were found in grade 1 subareas. Although cholesterol is known to accumulate in the arterial intima in esterified and unesterified forms, the TOF-SIMS spectra did not show any molecular peaks from CEs, most likely due to their strong fragmentation by ionization during the analysis process. Therefore, we investigated whether esterification changes the relative intensities of the secondary ions (m/z 385 and m/z 369) derived from cholesterol in TOF-SIMS. We, therefore, analyzed standard samples of cholesterol and cholesteryl linoleate and calculated their ion intensity ratios of 385:369, which were found to be 0.3 for unesterified cholesterol and 0.04 for cholesteryl linoleate, respectively (Figure 2C). Thus, the ratio 385:369 appears to be a good indicator of the presence of esterified cholesterol, the value being lower if cholesterol is in esterified form. However, regarding the specificity of these findings, other matrix effects may affect the 385:369 ratio. Among the various subareas of the arteries, the values for the ratio 385:369 were found to decrease threefold to fourfold along with the increasing lesion severity: from 0.3 in the media and grade 1 intimal subareas to 0.1 in grade 4 and 6 subareas. As an interesting exception, in grade 5 subareas, the ratio was significantly higher than in the grade 4 or 6 subareas (Figure 2D). The result points to the possibility that most of the cholesterol in grade 4 and 6 subareas is in lipoprotein particles, the cores of which are filled with CE, or in foam cells, in which CE is stored as intracellular lipid droplets. In contrast, in grade 5 subareas, more cholesterol is unesterified.

Colocalization of Cholesterol with FAs

The colocalizations of cholesterol ion m/z 369 and fatty acids (FAs) were investigated by generating overlay images and calculating the colocalization coefficients for cholesterol and the various FAs (Figure 3, A–P). In the normal intima, the ion intensities of FAs in general were low and their spatial distributions showed no correlation with those of cholesterol. In the fatty streaks, all of the FAs colocalized with cholesterol, and their ion intensities in the intima were much higher than in the

normal intima. In the atheromas, the distribution of FA fragment ion 18:0 (Figure 3L) differed remarkably from the distribution of the other FAs, and only minimal amounts of 18:0 were seen in the intima. The FAs 18:2 and 18:1 colocalized significantly with cholesterol and the signals accumulated near the internal elastic lamina (Figure 3, I and J). In the complicated lesions, the degree of colocalization of 18:2 and 18:1 with cholesterol was slightly decreased compared to that seen in the atheromas (Figure 3, M and N, and I and J, respectively). Interestingly, a striking colocalization of 16:0 and cholesterol was seen in the complicated lesions (Figure 3O). The most common FAs in lipoprotein CEs (18:2) had the highest intensities in the grade 4 subareas and in the grade 6 subareas (Figure 4). The intensity of cholesterol was also the highest in grade 4 and 6 subareas (Figure 2B). The FA with the highest total intensity in all of the subareas was 16:0 (Figure 4), a common FA in membrane PLs. This observation prompted us to next analyze the colocalization of various FAs and PLs in the intima.

PCs, LPCs, and SMs

Supplemental Figure S4, A–P, shows strong colocalization of FAs and the choline head group of PLs in the normal intima and in the fatty streaks, suggesting that the FA ions in these sections largely represent fragments of the PLs formed during the TOF-SIMS analysis.

We also analyzed the presence of PC, LPC, and SM in the media and in the intimal subareas of the lesions (Supplemental Figure S5). The intensities of PC showed a generally decreasing tendency from the media toward grade 6, whereas LPC, a product of PC hydrolysis by phospholipase A₂ (PLA₂) or LDL oxidation,^{37,38} had low intensities in the media and in grade 1 and 2 subareas, but was increased in subareas graded 3 to 6, reaching its highest values in grade 4 subareas (Supplemental Figure S5A). In fact, the intensity of the LPC signal was almost as high as the signal of PC in grade 4 subareas. Accordingly, the ratio of LPC/PC (Supplemental Figure S5B) had its highest value in grade 4 subareas. The ratios of cholesterol to sum of PC and SM [Chol/(SM+PC)] were significantly higher in grade 4 and 6 subareas than in the media and subareas with other grades (Supplemental Figure S5C).

Analysis by TOF-SIMS of Native and Lipolytically Modified LDL

To determine whether the above-described changes in the choline PLs could reflect lipase-induced changes in the accumulating lipoprotein particles, we hydrolyzed isolated human plasma LDL particles with PLA₂, sphingomyelinase, or cholesteryl esterase (CEase), and analyzed the native and the lipolyzed LDL particles by TOF-SIMS. As expected, the CEase treatment increased the ratio 385:369 (Supplemental Figure S6A) fourfold compared to the native LDL. Thus, in the native LDL containing mainly CEs, the ratio of m/z 385/ m/z 369 was 0.03, whereas in the CEase-treated LDL sample enriched in unesterified cholesterol, the ratio had

increased to 0.1. Similarly, the other lipolytic modifications of LDL induced changes in the detected ratios of the various lipids: PLA₂ treatment increased the Chol/(SM+PC) and the LPC/PC ratios, and decreased the PC/SM ratio, whereas sphingomyelinase treatment of LDL increased its PC/SM ratio (Supplemental Figure S6, B–D).

Immunohistochemical Findings in Comparison with TOF-SIMS

The mass spectrometric images of cholesterol and vitamin E were compared with images of adjacent sections stained with anti-apoB-100 antibody (Figure 5, A–L). In the normal intima, the cholesterol signal was present mainly in the endothelium and the vitamin E signal was diffuse and low (Figure 5, A and B); the apoB-100 staining was strongest in the subendothelial layer of the intima (Figure 5C). In the fatty streak, cholesterol was accumulated in the thickened intima, where increased signals from vitamin E could be observed with 1.5-fold higher ion intensity than in the normal intima (Figure 5, B–H). ApoB-100 staining was found near the endothelium. In the atheromas, the accumulation patterns of cholesterol, vitamin E, and apoB-100 were similar, and showed strong accumulation, particularly in the deep intimal layer near the internal elastic lamina. In the complicated lesion, a widespread accumulation of cholesterol and apoB-100 could be detected in the intima, but the signal of vitamin E was only half of that seen in the atheromas. In general, the ion intensities for vitamin E reflected those arising from esterified cholesterol, both peaking in grade 4 subareas (Figures 2B and 5M).

Images of consecutive sections stained for GpA, CD163, and CD31/34 were compared with the iron signal in the intima (Figure 6). GpA is a surface protein specific for red blood cells,³⁹ CD163 is a scavenger receptor for hemoglobin in macrophages,⁴⁰ and CD31 and CD34 are endothelial proteins and, when used in combination, particularly suitable for detection of neovascularization.⁴¹ In the normal intima (Figure 6, A–D) and in the fatty streak (Figure 6, E–H), no red blood cells, macrophages, or neovessels could be detected. Also, in the atheromas (Figure 6, I–L), no red blood cells or neovessels were present, and only a few CD163-positive macrophages could be detected. In the complicated lesion, however, strong signals for GpA, CD163, and CD31/34 were detected (Figure 6, M–P). Iron, an integrate ion of hemoglobin and thus a marker of red blood cells in TOF-SIMS, was found only in low intensities,

except in the complicated lesions where its average intensity was fivefold higher than in the media (Figure 6Q). Half of the subareas in the complicated lesions had high ion intensity of iron, and in these lesions, the intensity localized in the same areas as the red blood cell membrane protein GpA, and so reflected local microhemorrhages.

Lipoproteins, TAGs, and Cholesterol Crystals

TAG-rich lipoprotein particles (chylomicron remnants, very low density lipoproteins, and intermediate-density lipoproteins) have been suggested to contribute to lipid accumulation during atherogenesis.⁴² In the TOF-SIMS analysis, TAG is detected with high sensitivity by the diacylglycerol ion, into which TAG is efficiently fragmented during the ionization process.³² In the lesions, TAG was seen mainly near the luminal endothelium, and occasionally also in the media and in the adventitia (Figure 7, C and D). Interestingly, in the complicated lesions, TAG also surrounded the neovascular structures, but seldom colocalized with cholesterol (Figure 7P), a finding suggesting differences in the accumulation and/or metabolism of TAGs and cholesterol during atherogenesis. Interestingly, spike-like formations in which cholesterol appears not to colocalize with choline PLs or any other lipids were observed in a high-resolution image of atheromas (Figure 7, Q–T), suggesting the presence of cholesterol crystals in the intima.

Lipid Composition in the Tunica Media

We also found differences in the lipid composition of tunica media, which were linked to the progression of the atherosclerotic changes in the studied coronary arteries. The changes were, however, much more subtle than those in the corresponding segments of the intima (Supplemental Figure S7).

Multivariate Comparisons

The multivariate PCA comparisons of the secondary ions emitted by the tissue sections showed that when the ions from the main lipid classes and those from vitamin E and iron were used as loadings, the main separation of the media and intima samples occurred on the PC-cholesterol axis (Figure 8A). The principal component 1 explained 39% of the data variation. As judged from the ion intensities, the media and grade 1 to 2 subareas were

Figure 5 Cholesterol and vitamin E ion distributions and apolipoprotein B (apoB)-100 immunohistochemistry. Coronary artery sections were analyzed with time-of-flight secondary ion mass spectrometry (TOF-SIMS) for cholesterol and vitamin E. ApoB-100 was immunohistochemically stained from the adjacent sections. Normal intima (A–C), fatty streak (D–F), atheroma (G–I), and complicated lesion (J–L). **White dotted line** marks the endothelium; **white and black solid lines**, the internal elastic lamina; and **white and black dashed lines**, the external elastic lamina. **M**: Vitamin E intensities in the media (M) and intimal subareas, graded 1 to 6. Kruskal-Wallis nonparametric analysis of variance was applied ($P < 0.05$). This analysis was followed by Mann-Whitney test, one pair at a time. Guide to the lower case letter code: The values with no common letter differ at $P < 0.05$ (no statistical differences, no code). As a default, the highest mean was marked with letter a. Groups 1 and 5, $n = 3$; group 2, $n = 4$; group 3, $n = 10$; group 4, $n = 9$; group 6 and media (M), $n = 8$. A, atheroma; I, intima; Mc, maximum number of detected ions (counts) per pixel; Tc, total number of detected ions.

Figure 6 Red blood cells, macrophages, neovessels, and iron (Fe) in the arteries. Images showing immunohistochemically labeled glycoprotein A (GpA) for red blood cells, CD163 for macrophages, and CD31/34 for neovessels in the sections (10 μ m thick), and time-of-flight secondary ion mass spectrometry (TOF-SIMS) images of Fe in the corresponding areas. Normal intima (A–D), fatty streak (E–H), atheroma (I–L), and complicated lesion (M–P). **Arrowheads** point to areas in which apolipoprotein B 100 accumulates (Figure 4, I–L). **White** and **black dotted lines** mark endothelium; **white** and **black solid lines**, internal elastic lamina; and **white** and **black dashed lines**, the adventitial-medial border. **Q:** Fe intensities in the media and the intimal subareas, graded from 1 to 6. Grades 1 to 6 denote subareas of the intima. As statistics, Kruskal-Wallis nonparametric analysis of variance was applied ($P < 0.05$), but for Fe, Kruskal-Wallis nonparametric analysis of variance did not show differences at $P < 0.05$. The total numbers of subareas are shown in Supplemental Table S2. Groups 1 and 5, $n = 3$; group 2, $n = 4$; group 3, $n = 10$; group 4, $n = 9$; group 6 and media (M), $n = 8$. A, atheroma; I, intima; Mc, maximum number of detected ions (counts) per pixel; Tc, total number of detected ions.

characterized by relatively high PC contents. In contrast, the grade 6 and 4 subareas were enriched in cholesterol, CEs, and vitamin E. Interestingly, the TAG contents of the studied samples tended to correlate negatively with their contents of both forms of cholesterol and vitamin E. One grade 6 subarea with neovessels contained high abundance of iron. Soft independent modeling of class analogies analyses (Figure 8B) confirmed that the grade 4 and 6 subareas had significantly different secondary ion distributions (related to composition) from the media and grade 1 to 2 subareas.

When the major fatty acyl fragment ions were used as loadings for PCA (Figure 8C), large individual sample variation was detected in each subarea category, and thus the media and the grade of intima subareas were not separated because of principal component 1, on which axis the FAs 18:0 and 20:4, common in PLs, were placed on left and the 16:1, 16:0, 18:2, and 18:1, common FAs in neutral lipids, on right. However, the principal component 2 (explaining 38% of the data variation) clear-cut separated the grade 4 and 6 subareas from the media and grade 1, 2, and 5 subareas. The FA 18:2, abundant in CEs of lipoprotein particles, pulled the grade 4 and 6 subareas toward low positions on the biplot. Thus, the grade 4 and 6 samples were outstanding in terms of their fatty acyl fragment distributions, and several statistically significant differences were found between the sample categories by soft independent modeling of class analogies (Figure 8D).

Discussion

Our aim was to investigate the precise distributions of various lipid species in human coronary arteries during the progression of atherosclerosis. We analyzed coronary artery sections with TOF-SIMS and immunohistochemistry, which together provide a detailed analysis on how the lipid composition of the arterial intima changes with regard to other markers of atherosclerosis in the various stages of atherogenesis. In this study, we advanced from sole lipid analysis of a coronary artery segment to determining the distribution of individual lipid species within atherosclerotic lesions. Previously, cholesterol and other neutral lipids had been detected from frozen sections with fluorescent filipin staining⁴³ and ORO staining, respectively, but these methods were unable to detect other, more polar, lipids in the same sections. The whole lipid composition of an arterial segment has previously been analyzed with thin-layer chromatography, and more recently and in more detail, with mass spectrometry, especially electrospray ionization of the whole tissue extracts.^{3,44} With these methods, again, information on the spatial distribution of the lipids remains lacking. Herein, we were able to detect the accurate location of various individual lipid species in the coronary arteries with TOF-SIMS. We found that the atherosclerotic plaques contained domains with distinct lipid composition,

and the domains showing strong signal for CE were also enriched with the acyl chains 18:2 and 18:1, the two FAs characteristic for CEs in lipoproteins and foam cells. In the atheromas, the CE-derived ions were found in the same areas where the apoB-100 was found in the adjacent sections, whereas in the complicated lesions, high amounts of red blood cell surface protein were found in the areas where high iron ion intensities and unesterified cholesterol (UC) were found in the adjacent sections. In addition, cholesterol crystals were visualized in the atherosclerotic plaques. In all lesion types, UC and CEs seldom colocalized with TAGs. Also, we found that the degree of atherosclerosis in the intima may slightly affect the lipid load and composition of the adjacent media.

TOF-SIMS is a method well suited for analysis of tissue lipids because the mass range extends up to m/z 1500, covering the full range needed to detect lipids (herein, cholesterol, PC, SM, LPC, and diacylglycerol), their fragments, and small molecules of the low-mass region (eg, phosphocholine head group and iron).⁴⁵ Because the lateral resolution of the technique can reach $<1\ \mu\text{m}$, SIMS has been used to analyze lipids and their lateral distribution in cells.⁴⁶ However, ion images with such spatial resolution can only be obtained for ions that generate sufficient signal per area unit, which is normally the case only for fragment ions characteristic for lipid classes, whereas imaging of specific lipid molecules is typically only possible at spatial resolutions in the 5 to 10 μm range. Furthermore, optimal lateral resolution normally requires that the mass resolution need to be compromised, which sometimes can lead to reduced specificity of the probed ion, although new methods greatly have improved the mass-resolving capacity in the high lateral resolution.^{23,46–52} As usual for the mass spectrometry of natural lipid mixtures, the PLs that are prone to accept or lose protons are detected more sensitively than the neutral lipids that become ionized less efficiently, thus generating a matrix effect where neutral ions gain weaker visibility. Because in TOF-SIMS, all of the lipids available on the surface of the tissue section are desorbed simultaneously, the PLs tend to suppress the signals from the neutral lipids,⁵³ such as cholesterol. Hence, to produce sufficient signal in the ion images, high quantities of neutral lipids may be required, whereas even small amounts of PLs will compete efficiently for the charge during the ionization process and thus gain intensity in the ion images. However, because atherosclerotic lesions are characterized by excess accumulation of CEs and TAGs, the amounts of these lipids in the tissue are clearly sufficient to admit SIMS detection with proper ion intensities.

With electrospray ionization-MS, derivatized UC and CEs can be detected as molecular ions,⁵³ but with TOF-SIMS, the CEs become fragmented and thus cannot be detected as such. Previously, TOF-SIMS has been used to compare the localization of two fatty acyl ions, 18:1 and 16:0, in aortas of *apoE*^{−/−} mice fed with a high-fat diet, and

Figure 8 Principal component analysis (PCA) biplots and class distance [soft independent modeling of class analogies (SIMCA)] tables of main lipid classes, vitamin E (vit E), and fatty acids. PCA biplots describing the compositional similarities and differences between the artery samples. First, time-of-flight secondary ion mass spectrometry total intensities for the main lipid classes, vitamin E, and iron (Fe) were used as loadings. After this PCA, SIMCA quantified the compositional distances for any two experimental groups, one pair at a time (**A**), and the significantly ($P < 0.05$) different compositions found in these paired comparisons were indicated by X in a contingency table ($P < 0.05$; **B**). Second, the fatty acyl fragment ion intensities were used as loadings for the PCA (**C**), and this analysis was followed by the paired quantifications of compositional difference by SIMCA ($P < 0.05$; **D**). The total numbers of subareas are shown in [Supplemental Table S2](#). Groups 1 and 5, $n = 3$; group 2, $n = 4$; group 3, $n = 10$; group 4, $n = 9$; group 6 and media (M), $n = 8$. CE, cholesteryl ester; SM, sphingomyelin; TAG, triacylglycerol; UC, unesterified cholesterol.

these ions were suggested to be derived from cholesteryl oleate and palmitate, respectively.⁵² Instead of the fatty acyl ions that may be emitted from lipids other than CEs, we calculated the ratio of two cholesterol-derived ions (m/z 385/ m/z 369) in the studied tissue sections^{23,26} and compared the values with those found in standard samples of cholesterol and cholesteryl linoleate. We found the ratio 385:369 to be 0.3 and 0.04 in cholesterol and cholesteryl

linoleate, respectively. In accordance with these results, native LDL showed a similar ratio of 385:369, as most of the cholesterol in LDL is esterified as cholesteryl linoleate, and when LDL was treated with CEase to unesterify CEs, the observed ratio was similar to that of UC. Thus, the ratio 385:369 appears to be a reliable way to detect CE, even when the molecular ion of a CE cannot be detected because of fragmentations during the analysis.

Figure 7 Colocalization of cholesterol, phosphocholine head group, and diacylglycerol (DAG). Visualization of cholesterol crystals in a high-resolution time-of-flight secondary ion mass spectrometry image combining cholesterol and phosphocholine. Colocalizations of cholesterol (first column, red in the composition image), phosphocholine head group (second column, blue in the composition image), and DAG (third column, green in the composition image) in normal intima (**A–D**), fatty streak (**E–H**), atheroma (**I–L**), and complicated lesion (**M–P**). Side of a square is 500 μm . **White solid line** indicates internal elastic lamina; **white dashed line**, external elastic lamina; **white dotted line**, endothelium. **Q–T**: High-resolution (square side, 100 $\times$ 100 μm) ion images of total cholesterol (**Q**), phosphocholine (**R**), and total ion image (**S**). **T**: An overlay of cholesterol (red) and phosphocholine (green). **Arrowheads** point to cholesterol crystals. Mc, maximum number of detected ions (counts) per pixel; TAG, triacylglycerol; Tc, total number of detected ions.

Figure 9 Schematic illustration of the main results of the study. Three different stages of atherosclerosis (fatty streak, atheroma, and complicated lesion), in which lipids begin to accumulate, are depicted. Of the three layers of a coronary artery, only intima and media, but not adventitia, are illustrated.

In the human coronary arteries with a normal intima, cholesterol was located mainly close to the luminal endothelium, and it appears to be on the luminal side of the endothelial cells, where the lipoproteins can attach to the proteoglycan glycocalyx of the cells, within the endothelial cells, and in the subendothelial space. However, the exact location of the signal is difficult to determine, particularly because it is possible that the endothelial lining breaks down after the organ is explanted. In the atheromas and complicated lesions, cholesterol was detected near the intimal-medial border, and in several layers of the intima. In these lesions, CEs (m/z 369) gave strong signals, and the ratio 385:369 was similar to the ratio detected in cholesteryl linoleate, approximately 0.05. The same areas had strong apoB-100 staining (Figure 5). Also, vitamin E detected by the TOF-SIMS was found in the same area. Because the apoB-containing lipoproteins are known to carry vitamin E as an antioxidant,³⁵ these findings suggest that in atheromas, cholesterol is carried in apoB-containing lipoprotein particles that have entered the arterial intima and become retained within the tissue.^{1,54}

In many domains of the complicated lesions rich in cholesterol, the 385:369 ratio (0.14 ± 0.03) was close to the ratio in UC and in isolated LDL hydrolyzed with CEase *in vitro*. In the same areas with high 385:369 ratio, intense staining for neovessels, the erythrocyte surface protein GpA, and a fivefold increased intensity of iron signal were found, which suggests that a significant proportion of the cholesterol in these areas was derived from extravasated erythrocytes. This idea was supported by the finding that the presence of macrophages with the scavenger receptor CD163, an endocytic receptor for hemoglobin-haptoglobin complexes, was high in these areas. The notion that a high proportion of UCs in the neovessel-containing complicated lesions may have been derived from microhemorrhages well agrees with the recent observations, according to which the neovessels in advanced lesions are leaky and fragile and easily rupture. This, thereby, provides a port of entry to the circulating erythrocytes,^{14,55} which are known to be rich in UCs.⁵⁶

TAGs, detected as diacylglycerol,^{33,57} were found to accumulate in the coronary arteries. Specifically, we found TAGs near the luminal endothelium of the artery in all stages of atherogenesis and, moreover, adjacent to the neovessels in

the advanced lesions. In contrast to cholesterol, TAG was not observed near the intimal-medial border of the atherosclerotic lesions. Because cholesterol is carried out to the intima mainly in LDL and TAGs mainly in chylomicron remnants, very low density lipoprotein, and intermediate-density lipoprotein, spatial differences in the accumulation of LDL and the triglyceride-rich lipoprotein in the lesions could explain the observed different spatial distributions of cholesterol and TAGs in the intima. Indeed, the size of the lipoprotein particles has been suggested to limit their passage through the network of extracellular matrix,^{58–60} and it is possible that the larger TAG-containing lipoproteins cannot reach the deep intima. It is also possible that the lack of TAGs in the deep intima results from their hydrolysis, either extracellularly or intracellularly in the tissue. Thus, the fate of the lipoprotein-carried TAGs differs from the fate of CEs: hydrolysis of TAGs leads to generation of FAs, whereas lipolysis of CEs leads to accumulation of UC in addition to the FAs and UC.

The glycerophospholipids (PC, SM, and LPC) were the lipid group that was detected with the highest intensities throughout the coronary artery series. However, their proportion of all of the lipid-derived ions was lower in the later stages of atherogenesis than in the normal intimas; in the intimas with a large atheroma, they seemed to totally disappear from the necrotic core area, a finding that can be explained by death of cells in the core.⁶¹ Moreover, the PL characteristics (PC/SM ratio, LPC/PC ratio, and cholesterol/PC ratio) of the analyzed coronary arteries were compatible with the hypothesis that the accumulating lipoproteins undergo a multitude of modifications that hydrolyze the PLs and neutral lipids of the lipoproteins in the arterial intima. The immunohistochemical stainings indicated that apoB-containing lipoproteins were present in the atheromatous intima in the areas where the ratios LPC/PC and cholesterol/PL had their highest values. The low PC/SM ratio in the advanced lesions, when compared to the normal intima or to isolated nonmodified LDL, suggests that the PC of the accumulating lipoproteins was hydrolyzed by PLA₂ or via oxidation, thus producing LPC and unesterified FAs. This idea is further supported by the differences in the LPC/PC ratio, which changed from 0.2 in the normal artery to 0.9 in the atherosclerotic lesions.

Lipoprotein modifications by lipolysis, proteolysis, or oxidation have all been suggested to be driving forces in the development and progression of atherosclerotic lesions.^{2,62} Lipoprotein-modifying enzymes, such as lysosomal acid lipase,⁶³ PLA₂,⁶⁴ sphingomyelinase,⁶⁵ and lipoprotein lipase,⁶⁶ are secreted by intimal cells into the extracellular compartment, and such modifications, with the contribution of proteolytic modifications and oxidation, can induce aggregation and fusion of the lipoprotein particles² and may even lead to formation of cholesterol crystals.⁶⁷

Herein, we show that the lipids present in the human coronary arterial intima are differentially distributed in different stages of atherogenesis (Figure 9). Although atherosclerosis has been considered a disorder solely affecting the arterial intima, we found signs suggesting that the media may also be affected, yet much less than the intima. It may be possible that the lipid profile of the smooth muscle cell layer adjacent to the atherosclerotic lesions is also affected by the lipids in the intima. The distribution pattern of cholesterol changes from evenly distributed in the normal intima to heavy aggregates in the advanced lesions. Because PLs are typically components of cellular membranes, their distribution patterns differ less between the normal intima and the lesions. Interestingly, a lesion in a coronary artery can contain several domains with different degrees of severity of atherosclerosis, and among them, the main differences were found in the cholesterol distribution and the ratio of UC/CE. Thus, in the early lesions, cholesterol appeared to be derived only from apoB-containing lipoproteins, and in the more advanced lesions, both lipoproteins and red blood cells appeared to contribute to the cholesterol load. Intriguingly, cholesterol and TAGs were found to accumulate in different areas of the lesions. This indicates major differences in the metabolism of the two lipid classes in the lesions and suggests that the site of accumulation and fate of TAG-enriched chylomicrons, very low density lipoprotein, and intermediate-density lipoprotein must differ from those of CE-enriched LDL particles.

In summary, TOF-SIMS did provide us with detailed information on the composition of the accumulated lipids found at different stages of atherosclerosis, from a normal intima to a complicated lesion, and also on the heterogeneity of the different domains found in the same atherosclerotic lesions. When combined with immunohistological analysis, TOF-SIMS imaging of the lipids in human coronary arterial lesions at different stages of atherogenesis also provided detailed spatial information about both native and modified lipids. Such composite lipid analysis at various stages of coronary atherosclerosis will help us to unravel not only the origin but also the fate of the lipoprotein-derived lipids at locations critically affecting the course of this disease.

Acknowledgment

We thank Suvi Sokolnicki for her invaluable technical expertise.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.01.026>.

References

- Williams KJ, Tabas I: The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995, 15:551–561
- Öörni K, Pentikäinen MO, Ala-Korpela M, Kovanen PT: Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions. *J Lipid Res* 2000, 41:1703–1714
- Lehti S, Käkälä R, Hörkkö S, Kumm O, Helske-Suihko S, Kupari M, Werkkala K, Kovanen PT, Öörni K: Modified lipoprotein-derived lipid particles accumulate in human stenotic aortic valves. *PLoS One* 2013, 8:e65810
- Chao FF, Amende LM, Blanchette-Mackie EJ, Skarlatos SI, Gamble W, Resau JH, Mergner WT, Kruth HS: Unesterified cholesterol-rich lipid particles in atherosclerotic lesions of human and rabbit aortas. *Am J Pathol* 1988, 131:73–83
- Schissel SL, Jiang X, Tweedie-Hardman J, Jeong T, Camejo EH, Najib J, Rapp JH, Williams KJ, Tabas I: Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH: implications for atherosclerotic lesion development. *J Biol Chem* 1998, 273:2738–2746
- Schissel SL, Tweedie-Hardman J, Rapp JH, Graham G, Williams KJ, Tabas I: Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein: proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *J Clin Invest* 1996, 98:1455–1464
- Guyton JR, Klemp KF: Development of the atherosclerotic core region: chemical and ultrastructural analysis of microdissected atherosclerotic lesions from human aorta. *Arterioscler Thromb* 1994, 14:1305–1314
- Allahverdiyan S, Pannu PS, Francis GA: Contribution of monocyte-derived macrophages and smooth muscle cells to arterial foam cell formation. *Cardiovasc Res* 2012, 95:165–172
- Hoff HF, O'Neil J, Pepin JM, Cole TB: Macrophage uptake of cholesterol-containing particles derived from LDL and isolated from atherosclerotic lesions. *Eur Heart J* 1990, 11(Suppl E):105–115
- Khoo JC, Miller E, McLoughlin P, Steinberg D: Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis* 1988, 8:348–358
- Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989, 320:915–924
- Sary HC, Chandler AB, Glagov S, Guyton JR, Insull W Jr, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW: A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1994, 89:2462–2478
- Pasquini G, Preda P, Vici M, Gargiulo M, Stella A, D'Addato M, Laschi R: Electron microscopy of lipid deposits in human atherosclerosis. *Scanning Microsc* 1989, 3:1151–1159
- Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulen TN, Wrenn SP, Narula J: Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* 2005, 25:2054–2061
- Sary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW: A definition of advanced types of atherosclerotic lesions and a

- histological classification of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1995, 92:1355–1374
16. Smith EB, Evans PH, Downham MD: Lipid in the aortic intima: the correlation of morphological and chemical characteristics. *J Atheroscler Res* 1967, 7:171–186
 17. Smith EB, Slater RS: The microdissection of large atherosclerotic plaques to give morphologically and topographically defined fractions for analysis, 1: the lipids in the isolated fractions. *Atherosclerosis* 1972, 15:37–56
 18. Smith EB, Slater RS, Chu PK: The lipids in raised fatty and fibrous lesions in human aorta: a comparison of the changes at different stages of development. *J Atheroscler Res* 1968, 8:399–419
 19. Orekhov AN, Tertov VV, Novikov ID, Krushinsky AV, Andreeva ER, Lankin VZ, Smirnov VN: Lipids in cells of atherosclerotic and uninvolved human aorta, I: lipid composition of aortic tissue and enzyme-isolated and cultured cells. *Exp Mol Pathol* 1985, 42:117–137
 20. Katz SS, Shipley GG, Small DM: Physical chemistry of the lipids of human atherosclerotic lesions: demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J Clin Invest* 1976, 58: 200–211
 21. Lundberg B: Chemical composition and physical state of lipid deposits in atherosclerosis. *Atherosclerosis* 1985, 56:93–110
 22. Chao FF, Blanchette-Mackie EJ, Chen YJ, Dickens BF, Berlin E, Amende LM, Skarlatos SI, Gamble W, Resau JH, Mergner WT, Kruth HS: Characterization of two unique cholesterol-rich lipid particles isolated from human atherosclerotic lesions. *Am J Pathol* 1990, 136:169–179
 23. Sjövall P, Lausmaa J, Johansson B: Mass spectrometric imaging of lipids in brain tissue. *Anal Chem* 2004, 76:4271–4278
 24. Belu AM, Graham DJ, Castner DG: Time-of-flight secondary ion mass spectrometry: techniques and applications for the characterization of biomaterial surfaces. *Biomaterials* 2003, 24: 3635–3653
 25. Brunelle A, Touboul D, Laprevote O: Biological tissue imaging with time-of-flight secondary ion mass spectrometry and cluster ion sources. *J Mass Spectrom* 2005, 40:985–999
 26. Malmberg P, Börner K, Chen Y, Friberg P, Hagenhoff B, Mansson JE, Nygren H: Localization of lipids in the aortic wall with imaging TOF-SIMS. *Biochim Biophys Acta* 2007, 1771:185–195
 27. Watrous JD, Dorrestein PC: Imaging mass spectrometry in microbiology. *Nat Rev Microbiol* 2011, 9:683–694
 28. Vickerman JC: Molecular imaging and depth profiling by mass spectrometry—SIMS, MALDI or DESI? *Analyst* 2011, 136: 2199–2217
 29. Bolte S, Cordelieres FP: A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 2006, 224:213–232
 30. Yan D, Mäyränpää MI, Wong J, Perttilä J, Lehto M, Jauhiainen M, Kovanen PT, Ehnholm C, Brown AJ, Olkkonen VM: OSBP-related protein 8 (ORP8) suppresses ABCA1 expression and cholesterol efflux from macrophages. *J Biol Chem* 2008, 283:332–340
 31. Kvalheim OM, Karstang TV: A general-purpose program for multivariate data analysis. *Chemometr Intell Lab Syst* 1987, 2:235–237
 32. Debois D, Bralet MP, Le Naour F, Brunelle A, Laprevote O: In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF-SIMS imaging. *Anal Chem* 2009, 81:2823–2831
 33. Sjövall P, Johansson B, Belazi D, Stenvinkel P, Lindholm B, Lausmaa J, Schalling M: TOF-SIMS analysis of adipose tissue from patients with chronic kidney disease. *Appl Surf Sci* 2008, 255:1177–1180
 34. Prinz C, Hook F, Malm J, Sjövall P: Structural effects in the analysis of supported lipid bilayers by time-of-flight secondary ion mass spectrometry. *Langmuir* 2007, 23:8035–8041
 35. Esterbauer H, Gebicki J, Puhl H, Jurgens G: The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* 1992, 13:341–390
 36. Vance JE, Steenbergen R: Metabolism and functions of phosphatidylserine. *Prog Lipid Res* 2005, 44:207–234
 37. Parthasarathy S, Barnett J: Phospholipase A2 activity of low density lipoprotein: evidence for an intrinsic phospholipase A2 activity of apoprotein B-100. *Proc Natl Acad Sci U S A* 1990, 87:9741–9745
 38. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D: Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A* 1984, 81:3883–3887
 39. Grant CW, McConnell HM: Glycophorin in lipid bilayers. *Proc Natl Acad Sci U S A* 1974, 71:4653–4657
 40. Li W, Xu LH, Yuan XM: Macrophage hemoglobin scavenger receptor and ferritin accumulation in human atherosclerotic lesions. *Ann N Y Acad Sci* 2004, 1030:196–201
 41. Jeziorska M, Woolley DE: Neovascularization in early atherosclerotic lesions of human carotid arteries: its potential contribution to plaque development. *Hum Pathol* 1999, 30:919–925
 42. Chapman MJ, Ginsberg HN, Amarencu P, Andreotti F, Boren J, Catapano AL, Descamps OS, Fisher E, Kovanen PT, Kuivenhoven JA, Lesnik P, Masana L, Nordestgaard BG, Ray KK, Reiner Z, Taskinen MR, Tokgozoglu L, Tybjaerg-Hansen A, Watts GF: Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *Eur Heart J* 2011, 32:1345–1361
 43. Kruth HS: Localization of unesterified cholesterol in human atherosclerotic lesions: demonstration of filipin-positive, oil-red-O-negative particles. *Am J Pathol* 1984, 114:201–208
 44. Han X, Gross RW: Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J Lipid Res* 2003, 44:1071–1079
 45. Walker AV: Why is SIMS underused in chemical and biological analysis? challenges and opportunities. *Anal Chem* 2008, 80: 8865–8870
 46. Passarelli MK, Winograd N: Lipid imaging with time-of-flight secondary ion mass spectrometry (ToF-SIMS). *Biochim Biophys Acta* 2011, 1811:976–990
 47. Touboul D, Halgand F, Brunelle A, Kersting R, Tallarek E, Hagenhoff B, Laprevote O: Tissue molecular ion imaging by gold cluster ion bombardment. *Anal Chem* 2004, 76:1550–1559
 48. Sjövall P, Lausmaa J, Nygren H, Carlsson L, Malmberg P: Imaging of membrane lipids in single cells by imprint-imaging time-of-flight secondary ion mass spectrometry. *Anal Chem* 2003, 75:3429–3434
 49. Boxer SG, Kraft ML, Weber PK: Advances in imaging secondary ion mass spectrometry for biological samples. *Annu Rev Biophys* 2009, 38:53–74
 50. Wilson RL, Kraft ML: Quantifying the molar percentages of cholesterol in supported lipid membranes by time-of-flight secondary ion mass spectrometry and multivariate analysis. *Anal Chem* 2013, 85:91–97
 51. Kraft ML, Klitzing HA: Imaging lipids with secondary ion mass spectrometry. *Biochim Biophys Acta* 2014, 1841:1108–1119
 52. Lee ES, Shon HK, Lee TG, Kim SH, Moon DW: The regional ratio of cholesteryl palmitate to cholesteryl oleate measured by ToF-SIMS as a key parameter of atherosclerosis. *Atherosclerosis* 2013, 226:378–384
 53. Liebisch G, Binder M, Schifferer R, Langmann T, Schulz B, Schmitz G: High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *Biochim Biophys Acta* 2006, 1761:121–128
 54. Williams KJ, Tabas I: The response-to-retention hypothesis of atherogenesis reinforced. *Curr Opin Lipidol* 1998, 9:471–474
 55. Mäyränpää MI, Heikkilä HM, Lindstedt KA, Walls AF, Kovanen PT: Desquamation of human coronary artery endothelium by human mast cell proteases: implications for plaque erosion. *Coron Artery Dis* 2006, 17:611–621
 56. Dougherty RM, Galli C, Ferro-Luzzi A, Iacono JM: Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA. *Am J Clin Nutr* 1987, 45:443–455
 57. Sjövall P, Greve TM, Clausen SK, Møller K, Eirefelt S, Johansson B, Nielsen KT: Imaging of distribution of topically applied drug

- molecules in mouse skin by combination of time-of-flight secondary ion mass spectrometry and scanning electron microscopy. *Anal Chem* 2014, 86:3443–3452
58. Proctor SD, Vine DF, Mamo JC: Arterial permeability and efflux of apolipoprotein B-containing lipoproteins assessed by in situ perfusion and three-dimensional quantitative confocal microscopy. *Arterioscler Thromb Vasc Biol* 2004, 24:2162–2167
59. Proctor SD, Vine DF, Mamo JC: Arterial retention of apolipoprotein B(48)- and B(100)-containing lipoproteins in atherogenesis. *Curr Opin Lipidol* 2002, 13:461–470
60. Nordestgaard BG, Wootton R, Lewis B: Selective retention of VLDL, IDL, and LDL in the arterial intima of genetically hyperlipidemic rabbits in vivo: molecular size as a determinant of fractional loss from the intima-inner media. *Arterioscler Thromb Vasc Biol* 1995, 15: 534–542
61. Guyton JR, Klemp KF: Development of the lipid-rich core in human atherosclerosis. *Arterioscler Thromb Vasc Biol* 1996, 16:4–11
62. Tabas I, Williams KJ, Boren J: Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* 2007, 116:1832–1844
63. Hakala JK, Oksjoki R, Laine P, Du H, Grabowski GA, Kovanen PT, Pentikäinen MO: Lysosomal enzymes are released from cultured human macrophages, hydrolyze LDL in vitro, and are present extracellularly in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2003, 23:1430–1436
64. Öörni K, Kovanen PT: Lipoprotein modification by secretory phospholipase A(2) enzymes contributes to the initiation and progression of atherosclerosis. *Curr Opin Lipidol* 2009, 20:421–427
65. Marathe S, Kuriakose G, Williams KJ, Tabas I: Sphingomyelinase, an enzyme implicated in atherogenesis, is present in atherosclerotic lesions and binds to specific components of the subendothelial extracellular matrix. *Arterioscler Thromb Vasc Biol* 1999, 19: 2648–2658
66. Zilvermit DB: A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ Res* 1973, 33:633–638
67. Guarino AJ, Tulenko TN, Wrenn SP: Cholesterol crystal nucleation from enzymatically modified low-density lipoproteins: combined effect of sphingomyelinase and cholesterol esterase. *Biochemistry* 2004, 43:1685–1693