Two-Dimensional Visualization of Cholesterol and Cholesteryl Esters Within Human Coronary Plaques by Near-Infrared Fluorescence Angioscopy

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ABSTRACT

Background: Cholesterol (C) and cholesteryl esters (CE) within coronary plaques are minimally visualized directly by any of the available imaging modalities in vivo. If they are rendered visible in vivo, the progression of coronary plaques and the effects of respective therapies on these plaques can be objectively evaluated.

Hypothesis: The C and CE within human coronary plaques can be visualized by near-infrared fluorescence angioscopy (NIRFA).

Methods: By exciting at 710 ± 25 nm and emitting at 780 nm, near-infrared fluorescence (NIRF) of lipid components was examined by microscopy in vitro. Lipid components in 49 plaques of 32 excised human coronary arteries were examined by NIRFA in vitro. Coronary plaques were examined by NIRFA in 25 patients with coronary artery disease.

Results: C, CE, and calcium (Ca) individually did not exhibit NIRF but did in the presence of β -carotene, which is known to coexist with lipids in the vascular wall. Other substances that are contained in atherosclerotic plaques did not.² The excised human coronary plaques were classified as those with NIRF and those without. The former plaques were classified into homogenous, doughnut-shaped, and spotty types. Histological examinations revealed that these image patterns were determined by the differences in the locations of C, CE, and Ca, and that those deposited within 700 μ m in depth from the plaque surface were imaged by NIRFA. Homogenous, doughnut-shaped, or spotty NIRFA images were also observed in patients.

Conclusions: NIRFA is feasible for 2-dimensional imaging of C and CE deposited in human coronary plaques.

Introduction

Although a wide variety of imaging modalities is applied for imaging of coronary plaques angiography, intravascular ultrasonography (IVUS),^{1,2} optical coherence tomography (OCT),^{3,4,5} virtual histology,⁶ near-infrared spectroscopy (NIRS),^{7,8} computed tomography (CT),⁹ magnetic resonance imaging (MRI),¹⁰ conventional angioscopy using white light,^{11–15} positron-emission computed tomography,¹⁶ intravascular radiation detectors,¹⁷ near-infrared Raman spectroscopy,¹⁸ radioisotope-labeled antibodies,¹⁹ and intravascular MRI²⁰ direct and 2-dimensional imaging of individual lipid components such as cholesterol (C) and cholesteryl esters (CE) deposited inside the atherosclerotic coronary plaques is clinically not successful.

If the individual components of the lipids deposited inside the plaques become visible in vivo, the progression

of plaques and the effects of medical and interventional therapies on them can be evaluated more objectively.

Near-infrared light penetrates deeper into the tissues than visual light and evokes near-infrared fluorescence (NIRF) of the targeted substances. Detection of C and CE by NIRS in atherosclerotic plaques has been attempted in vitro but not clinically.^{21–23} NIRS shows the substance as spectra, and therefore is not suitable for direct and 2-dimensional imaging of the substances.

Therefore, the present study was carried out to obtain direct and 2-dimensional images of C and CE within human coronary plaques by a near-infrared fluorescence angioscopy (NIRFA) system in human coronary plaques in vitro and in vivo.

Methods

Search for the NIRF Characteristic of Lipid Components by Microscopy

NIRF of the major substances that are contained in atherosclerotic plaque (Table 1)²⁴ was examined by NIRF

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microscopy (NIRFM) using a microscope (IX 70; Olympus, Tokyo, Japan) and a chilled charge-coupled device (CCD) camera (C5985; Hamamatsu Photonics, Hamamatsu, Japan). A band-pass filter (BPF) of 710 ± 25 nm, a dichroic membrane (DM) of 780 nm, and a band-absorption filter (BAF) of 780 nm were used for NIRF imaging because a certain group of lipid components was most clearly discriminated by combination of these wavelengths in a preliminary study. One of the major substances (crystal, powder, or liquid) comprising atherosclerotic plaques was mounted on a glass deck for NIRFM, and the obtained images were recorded by a DVD.

Beta-carotene is lipotropic, binding to lipids and coexisting with lipids in human atherosclerotic lesions, and produces the yellow color of atherosclerotic plaque.²⁵ Therefore, we compared the yellow color of human coronary plaques to the yellow color of various concentrations of β -carotene. As a result, yellow color depth of 10^{-5} M of this substance corresponded to the darkest yellow color of the plaques. Therefore, β -carotene was diluted in glycerin at a concentration of 10^{-5} M and was mixed with each substance for NIRFM imaging.

The NIRFA System

The NIRFA system was composed of a fluorescence-excitation unit, an angioscope, a fluorescence-emission unit, and a camera. The fluorescence-excitation unit (Olympus) was composed of a mercury-xenon lamp and a 710 ± 25 nm BPF disc. The angioscope (modified VecMover; Clinical Supply, Gifu, Japan) was composed of a 2.5-F fiberscope incorporated in a 5-F guiding balloon catheter.

The fluorescence-emission unit (Olympus) was composed of a 780-nm DM and a 780-nm BAF. This unit was connected to an intensified CCD (ICCD) camera (C3505; Hamamatsu) for imaging.

Conventional Angioscopy

The conventional angioscopy system was composed of the excitation unit and angioscope used for NIRFA, in addition to a color CCD camera (CSVEC-10; Clinical Supply). A disc of the excitation unit without filter was selected, and the image guide of the angioscope was connected to the CCD camera for imaging. Before observation, the white balance was adjusted for color correction. The plaques were classified into white and yellow ones according to the criteria described elsewhere.²⁶

Observation of the Excised Human Coronary Artery by NIRFA

This in vitro study was carried out with the approval of the institutional review board of Toho University Sakura Hospital and Chiba-kensei Hospital, where autopsy was carried out. Thirty-two coronary arteries were excised

Table 1. NIRF of the Major Substances Contained in Atherosclerotic Plaques

Substances	NIRF		
	Autofluorescence With β -Carotene		
Cholesterol	c	—	+
Cholesteryl oleate	c	—	+
Cholesteryl linoleate	c	—	+
5-cholesten-3 β -ol	l	—	+
7-ketocholesterol	c	—	—
Oleic acid	c	—	—
Linoleic acid	c	—	—
Oxidized LDL	l	—	—
LDL	l	—	—
VLDL	l	—	—
HDL	l	—	—
apo B ₁₀₀	c	—	—
apo AI	c	—	—
apo E2	c	—	—
MMP-9	c	—	—
Phosphatidylcholine	c	—	—
L(α)-lysophosphatidylcholine	c	—	—
TG	l	—	—
Collagens I, III, IV, V	c	—	—
Hyaluronic acid	c	—	—
Ceramide	c	—	±
HS	c	—	—
Albumin	c	—	—
Globulins	c	—	—
Ca phosphate	c	—	+
β -carotene	l	—	—

Abbreviations: apo AI, apolipoprotein AI; apo B₁₀₀, apolipoprotein B₁₀₀; apo E2, apolipoprotein E2; Ca, calcium; HDL, high-density lipoprotein; HS, heparan sulfate; LDL, low-density lipoprotein; MMP-9, matrix metalloproteinase-9; NIRF, near-infrared fluorescence; TG, triglyceride; VLDL, very low-density lipoprotein.
NIRF findings: c, crystal; l, liquid; —, absent; ±, weak; +, present.

from 20 cadavers (12 males and 8 females; mean age, 62.2 ± 2.8 years) after the informed consent of the

concerned families. A Y-connector was introduced into the proximal portion of a coronary artery for perfusion with saline solution. Then, an angioscope was introduced through the connector into the artery for observation. Initially, conventional angioscopy was carried out to detect a plaque.

The plaque and its color were defined as described elsewhere.²⁶ After observation by conventional angioscopy, the image guide was connected to a fluorescence-emitter unit for NIRFA imaging without changing the position of the angioscope tip.

After NIRFA, the 4- to 5-mm-long portion of the artery in which the plaque was located was isolated by transecting both the proximal and the distal portions at the shorter axes. Then, the isolated segment was cut longitudinally to open the lumen. After confirmation of the plaque, the center of the plaque was cut into 1-mm-thick slices at the shorter axis and the cut surface of a slice was scanned by NIRFM using the same wavelengths as those in the NIRFA study. The remaining portions were cut into successive slices, 10 μm in thickness, for histological study by staining lipids in red and Ca in black with Oil Red-O and methylene blue dye.

Observation of the Coronary Plaques in Patients With Coronary Artery Disease by NIRFA

Twenty-five patients (age 61.8 ± 2.6 years; 17 males and eight females; 14 with stable angina pectoris due to organic coronary artery disease, 5 with vasospastic angina pectoris, and 6 with prior myocardial infarction) underwent conventional angioscopy and NIRFA during routine coronary angiography. All patients provided informed consent for the procedures.

After coronary angiography, an angioscope as described above was introduced carefully over a guidewire into the artery. The balloon of the angioscope was inflated with carbon dioxide to stop blood flow, and heparinized saline solution (10 IU/mL) was infused manually to displace the blood for observation. Subsequently, the light and image guides of the angioscope were connected to a fluorescence-excitation and fluorescence-emission unit, respectively, for NIRFA imaging.

Statistical Analysis

The data obtained were analyzed using the Fisher exact test. A P value <0.05 was considered significant.

Results

NIRFM and NIRFA Studied on the Substances That Are Contained in Human Atherosclerotic Plaques

None of the major substances that are contained in the atherosclerotic plaques displayed near-infrared autofluorescence. However, β -carotene (10^{-5} M) evoked NIRF of C, CE

(cholesteryl oleate, cholesteryl linoleate, and 5-cholesten-3 β -ol), and calcium phosphate but not the NIRF of other substances (Figure 1 and Table 1).

NIRFA and NIRFM Scanned Images of Excised Human Coronary Plaques and Their Corresponding Histological Changes

White plaques studied by conventional angioscopy were classified as those with NIRF and those without. The entire former group comprised NIRF-positive layers covered by NIRF-negative layers when observed by NIRFM and lipid layers were observed to be covered by nonlipid layers by histology. In addition, the majority of the former group presented homogenous NIRF, indicating homogenous deposition of lipid components such as C and CE (Figure 2, and Tables 2A and 2B). The latter group of white plaques was devoid of lipid deposition by histology (Table 2B). A small number of apparently normal coronary segments, as determined by conventional angioscopy, also presented NIRF. Thick intima and deposition of lipids beneath them were observed by histology in all these segments (Table 2B).

All the yellow plaques observed by conventional angioscopy showed NIRF on observation by NIRFA. Panels B to D in Figure 2 show yellow coronary plaques, their corresponding NIRFA and NIRFM scanned images, and the histological changes. NIRFA images of the yellow plaques were classified into those with homogenous NIRF occupying the entire plaque (homogenous type; "b" in Figure 2), those with NIRF-absent portion surrounded by NIRF ring (doughnut-shaped type; "c" in Figure 2), and those with NIRF-absent portion distributed by strong NIRF spots (spotty type; "d" in Figure 2). Using NIRFM scanning, NIRF-positive materials were found to occupy the entire plaque in the homogenous type, a NIRF-absent necrotic core was surrounded by NIRF layers in doughnut-shaped type, and strong NIRF spots clearly demarcated from the surrounding tissues were dispersed within a fibrous cap in spotty type (β , γ and δ of Figure 2, respectively). By histology, the plaques had no necrotic cores and lipids occupied the entire plaques in the homogenous type; a lipid-deposited fibrous cap with an underlying necrotic core was observed in the doughnut-shaped type; and a lipid-laden and Ca-disseminated fibrous cap with a necrotic core below was observed in the spotty type. In addition, normal collagen fibers were not observed in the fibrous cap in doughnut-shaped and spotty types (2, 3, and 4 in Figure 2; Table 2B).

Relationships Between the NIRFA Images and Depth From Plaque Surface to the Lipids

The capability of the NIRFA system to visualize lipids deep within the plaques was examined by correlating whether the

NIRF images can be obtained and the shortest depth from the plaque surface to the deposited lipids as determined by histology. It was revealed that this system can visualize lipids deposited within a depth of 700 μm from the plaque surface.

NIRFA in Patients With Coronary Artery Disease

Figure 3 shows a demonstrable example of yellow plaque by conventional angioscopy and homogenous type by NIRFA in a patient with stable angina pectoris.

As summarized in Table 2C, white plaques were classified into NIRF-present and NIRF-absent groups, as in the case of excised coronary arteries. The majority of the former presented homogenous NIRF. The majority of yellow plaques showed NIRF. Three NIRF-image patterns were also observed in this group, similar to those in the *in vitro* study.

The time required for conventional angioscopy and NIRFA was 10–12 minutes and 5–10 minutes, respectively. No complications were noted during the observation.

Discussion

In this study, C, CE, and Ca individually did not exhibit NIR autofluorescence. Any other substances composing atherosclerotic plaques²¹ did not exhibit autofluorescence. Ye et al found that β -carotene accumulates in atherosclerotic plaques and exhibits fluorescence in the range of visual light.²⁷ Blankenhorn et al found that this substance

exists in the human atherosclerotic plaques and exhibits fluorescence in the range of visual light²⁸; however, they did not examine NIRF of this substance.

In the present study, β -carotene did not exhibit NIR autofluorescence. However, the mixture of β -carotene and C, CE, or Ca exhibited NIRF. Because β -carotene is lipotrophic, it may have conjugated with these substances, forming an adduct and evoking a NIRF at wavelengths of ≥ 780 nm.

In the *in vitro* study on human coronary plaques, the NIRFA image patterns were well correlated with those obtained by NIRFM scanning of cut wall. Moreover, the location and patterns of NIRF by NIRFM scanning coincided with those of lipids and Ca determined by histology, indicating that the NIRF images by NIRFA were caused by the substances that evoke NIRF, such as C, CE, and Ca.

It was concluded that lipids deposited within a depth of 700 μm from the plaque surface could be imaged by the present NIRFA system. Although its potency is limited, it has the advantage that lipids inside the plaque can be visualized. The NIRFA images of the plaques were classified into homogenous, doughnut-shaped, and spotty types in both *in vitro* and *in vivo* studies.

In the yellow plaques observed by conventional angioscopy, the homogenous type showed deposition of lipids in the entire plaque histologically, indicating that C and CE were deposited throughout the entire plaque.

In the doughnut-shaped type, the NIRF-absent portion was surrounded by strong NIRF regions. Histological studies showed that a lipid-laden fibrous cap and necrotic core underneath existed in all samples of this type. The

Figure 1. NIRFM images of: (A) collagen I, (B) cholesterol, (C) cholesteryl oleate, and (D) Ca phosphate, respectively, before (upper panels, A–D) and after (lower panels, a–d) mixing with β -carotene (10^{-5} M). Horizontal bar in each panel represents 100 μm . Abbreviations: Ca, calcium; NIRFM, near-infrared fluorescence microscopy.

Figure 2. Relationships among scanned images using (A–D) conventional angioscopy, (a–d) NIRFA, and (α – δ) NIRFM of the cut wall surface, along with (1–4) histological changes after staining with Oil Red-O and methylene blue, in excised human coronary plaques. Findings in conventional angioscopy panel: (A) a white plaque; (B–D) yellow plaques. NIRFA findings: (a–b) homogenous type with arrows indicating homogenous NIRF; (c) doughnut-shaped type with arrow indicating NIRF-absent portion surrounded by a strong NIRF region; (d) spotty type with arrow indicating spots. NIRFM findings: (α – β) homogenous NIRF (arrows); (γ) necrotic core lacking NIRF (arrow) surrounded by a strong NIRF region; (δ) fibrous cap with strong NIRF spots (arrow). Histological findings, with red indicating lipids and black indicating Ca compounds: (1) homogenous deposition of lipids deep in the plaque (arrow); (2) lipid deposition throughout entire plaque; (3) lipid-deposited fibrous cap with a NC below; (4) Ca particles distributed within a lipid-laden fibrous cap. Horizontal bar represents 100 μ m. Abbreviations: Ca, calcium; NC, necrotic core; NIRFM, near-infrared fluorescence microscopy.

NIRF-absent portion featured yellow color when observed by conventional angioscopy, indicating existence of the lipids. Therefore, it is probable that the fibrous cap and necrotic core were filled with other NIRF-absent substances, such as oxidized low-density lipoprotein, triglycerides, and others.

In the spotty type, NIRF spots were observed in the NIRF-absent fibrous cap by NIRFM scanning and disseminated small Ca particles in lipid-laden thin fibrous cap by histology, indicating that Ca was disseminated in the fibrous cap.

In this study, not only C and CE, but also Ca exhibited NIRF. Calcium exhibited strong and clearly demarcated spotty NIRF even in the presence of C or CE in the *in vitro* study, probably due to its hydro- and lipophobic nature. Therefore, differentiation of Ca from C and CE was easy.

Discrimination of C and CE by NIRS *in vitro* was reported by Weinmann et al.²³ These substances were not

discriminated by NIRFA in the present study. However, NIRFA has an advantage in that it can image 2-dimensional distribution of these substances.

These substances in the plaques can be discriminated by changing BPF and BAF of NIRFA in future. Thus, coronary plaques that were simply classified by their surface color by conventional angioscopy into white and yellow became classifiable into 4 image patterns by NIRFA. It was considered that these were determined by distribution patterns of C, CE, and Ca.

Study Limitations

Compared with NIRS, quantitative measurement of C, CE, and Ca deposited in the plaques was beyond the scope of the present NIRFA system because NIRF intensity measured by NIRFA was inversely correlated to the depth from the plaque surface to these substances. Although lipids and

Table 2. NIRFA of the Excised Human Coronary Arteries

A. Relationships Between Conventional Angioscopic Images and NIRFA Patterns				
Conventional Angioscopy	Normal Segments	White Plaques	Yellow Plaques	
No. of plaques or segments	9	25	24	
NIRFA study				
NIRF absent	6	13	0 ^{a,b}	
Homogenous	3	10	14	
Doughnut-shaped	0	2	6	
Spotty	0	0	4	
B. Relationships Among NIRFA Images, NIRFM Images, and Histological Changes				
NIRFA Images	Absent	Homogenous	Doughnut-Shaped	Spotty
No. of plaques	13	24	8	4
(1) NIRFM scanning homogenous NIRF	5	24	0 ^c	0
NIRF-absent portion surrounded by NIRF	0	0	8 ^{d,e}	4 ^{c,f}
NIRF spots in fibrous cap	0	0	0	4 ^{c,f}
(2) Histology				
Lipids present	0	24 ^g	8 ^d	4 ^f
Homogenous lipid deposition	0	24 ^g	0 ^h	0
Necrotic core surrounded by lipids	0	1	8 ^{c,d}	4 ^{f,h}
Ca particles in fibrous cap	0	0	0	4 ^{c,f}
C. NIRFA Patterns in Patients With Coronary Artery Disease				
Conventional Angioscopy	Normal Segments	White Plaques	Yellow Plaques	
No. of segments or plaques	25	18	15	
NIRF present	12 (48%)	10 (55%)	13 (86%) ⁱ	
Homogenous	10	8	5	
Doughnut-shaped	2	1	4	
Spotty	0	1	4 ⁱ	
Abbreviations: Ca, calcium; NIRF, near-infrared fluorescence; NIRFA, near-infrared fluorescence angioscopy; NIRFM, near-infrared fluorescence microscopy.				
^a <i>P</i> < 0.01 vs normal segments.				
^b <i>P</i> < 0.0001 vs white plaques.				
^c <i>P</i> < 0.01 vs homogeneous group.				
^d <i>P</i> < 0.01 vs absent group.				
^e <i>P</i> < 0.001 vs homogeneous group.				
^f <i>P</i> < 0.05 vs absent group.				
^g <i>P</i> < 0.001 vs absent group.				
^h <i>P</i> < 0.05 vs homogeneous group.				
ⁱ <i>P</i> < 0.05 vs normal segments.

Figure 3. NIRFA study in a 61-year-old male with stable angina. (A) angiogram, (B) conventional angioscopic image, and (C) NIRFA image of a plaque in the proximal segment of the left anterior descending coronary artery (arrow in A). The yellow plaque (arrow in B) presented a homogenous-type NIRF image (C). Arrowhead: guide wire. Abbreviations: NIRF, near-infrared fluorescence; NIRFA, near-infrared fluorescence angiography.

Ca were discriminated because the latter showed a clearly demarcated spotty configuration, discrimination of C and CE was beyond the scope of the present NIRFA system. Thus, these substances located $\geq 700 \mu\text{m}$ in depth from the plaque surface were not imaged.

Conclusion

Two-dimensional imaging of C and CE within human coronary plaques was accomplished by NIRFA. The plaques both in vitro and in vivo were classified into NIRF absent, homogenous, doughnut-shaped, and spotty types. Histological examinations showed that these image patterns were determined mainly by β -carotene-conjugated C, CE, and Ca deposited within $700 \mu\text{m}$ from the plaque surface. Thus, the technique of NIRFA is clinically feasible for the imaging of selected lipid components deposited not only in superficial layers, but also those deposited relatively deeply within coronary plaques.

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