Detection of Ceramide, a Risk Factor for Coronary Artery Disease, in Human Coronary Plaques by Fluorescent Angioscopy

Yasumi Uchida, MD; Yasuto Uchida, MD; Takanobu Kobayashi, MD; Seiichiro Shirai, MD; Nobuyuki Hiruta, MD; Ei Shimoyama, MD; Tsuyoshi Tabata, PhD

Background: The presence of ceramide in human coronary plaques is a risk factor for ischemic heart disease, but its visualization in the human vessel wall is currently beyond the scope of any available imaging techniques.

Methods and Results: Deposition of ceramide was examined by fluorescent angioscopy (FA) and microscopy (FM) using golden fluorescence (Go) as a specific marker of ceramide in yellow plaques, which were obtained from 23 autopsy subjects and classified by conventional angioscopy and histology. Ceramide was observed by FM in 34 of the 41 yellow plaques with a necrotic core (NC) but rarely in the 28 without. Ceramide and macrophages/foam cells co-deposited mainly in the border zone of the NC and fibrous cap (FC). The Go of ceramide was seen when the fibrous cap thickness was ≤100μm. FA was performed to detect coronary plaques exhibiting Go in patients with coronary artery disease. Ceramide was also detected by FA in 6 of 18 yellow plaques (33.3%) in 8 patients with stable angina and in 18 of 24 yellow plaques (75.0%, P<0.05 vs. stable angina) in 8 patients with old myocardial infarction.

Conclusions: The Go of ceramide in human coronary plaques is detectable by FA and Go could be used as a marker of vulnerable plaque (i.e., thin FC with NC).

Key Words: Ceramide; Color fluorescent angioscopy; Golden fluorescence; Macrophages; Vulnerable coronary plaque

It is reported that ceramide is synthesized in macrophages stimulated by oxidized low-density lipoprotein (oxLDL) during apoptosis. Ceramide has crucial significance in cell functions such as apoptosis, cell growth, senescence and cell cycle control; it induces oxidative stress in human endothelial cells, thereby reducing levels of bioactive nitrous oxide; plasma levels are elevated in patients with acute coronary syndrome (ACS) and ceramide is associated with an increased risk of myocardial infarction (MI). Thus, the presence of ceramide in plaque would indicate vulnerability of the plaque and if ceramide in coronary plaques could be detected, the fate of the plaque, and subsequent coronary events, could be predicted. However, detection of ceramide is currently beyond the scope of any available imaging techniques used in vivo.

In a previous study of the constituents of atherosclerotic plaque, we discovered that ceramide exhibits golden fluorescence (Go) in the presence of β-carotene, which co-deposits with lipotrophic substances, including ceramide, in the vascular wall (i.e., in atherosclerotic plaque). In the present study, fluorescent angioscopy (FA) and microscopy (FM) were performed to clarify whether Go is characteristic of ceramide only and in what type(s) of human coronary plaque it deposits.

Methods

Color Definition of the Target

Because there are no definite border between colors, their definition differs among observers, influenced by experience and visual sense. For our studies, we developed a more objective method of color definition. A color spectrum chart was arbitrarily divided into sections, and each section was recorded by an AquaCosmos analyzer (C7746, Hamamatsu Photonics, Hamamatsu, Japan) using conventional angioscopy (CA). The obtained images were divided into 3 primary colors and the intensity ratio of red:green:blue was calculated to define each color. A golden color (i.e., glittering brownish-yellow color) is not shown in the standard color spectrum cart, so we imaged pure gold powder. The calculated intensity ratio of red:green:blue for each respective color is shown in Figure 1A. White is
also not shown in the color chart, so white light generated by a Xenon lamp was used to calculate its intensity ratio (1.0:0.9–1.1:0.9–1.1).

The colors of substances in coronary plaques obtained using CA, FM or FA were similarly separated through the image analyzer and the intensity ratios of the 3 primary colors were compared with those obtained from the standard color spectrum to determine each color.

**Color Fluorescence of Substances Comprising Plaque**

The color fluorescence of each of the major substances that constitute atherosclerotic plaque (Table 1) was examined by FM before and after the addition of β-carotene. Ceramide (Tocris, Bristol, UK) and other commercially available substances were used in the examination. The FM system (IX70, Olympus Co., Tokyo, Japan) used for fluorescent imaging consisted of a band-pass filter (BPF) of 340±15 nm and a band-absorption filter (BAF) of 420 nm. The intensity of fluorescence was categorized as strong, weak or absent when the exposure time required for imaging was ≤1, 1–5, and >5 s, respectively.

**CA System**

The CA system consisted of an angioscope (modified VecMover, Clinical Supply Co., Gifu, Japan), a light source and a 3-coupled chilled device digital camera (Clinical Supply Co.), which enabled imaging of the target similar to the naked eye. The details of the system are described elsewhere. Plaque was defined by CA as a nonmotile and protruding or lining mass clearly demarcated from the adjacent normal wall and whose shape, location and color did not alter after saline solution flush. Plaques were further classified as protruding white or yellow according to their surface color as defined by the image analyzer. A normal segment was defined as a white and smooth-surfaced portion of the vessel without any protrusion.

**FA System**

The FA system consisted of a fluorescence excitation unit with a BPF (345±15 nm), an angioscope as used for CA, a fluorescence emission unit with a BAF (420 nm) and a camera. The system has been approved for clinical use on a commercial basis in Japan by the Japanese Ministry of Health and Labor, supported by National Insurance.
Experimental Procedures

Ethics The ex vivo study of coronary arteries obtained from autopsy cases was carried out with the approval of the ethical committees of the Japan Foundation for Cardiovascular Research, Tsukuba Memorial Hospital, Funabashi-Futawa Hospital, Chiba-kensei Hospital, and Toho University, and after obtaining informed consent from the families concerned.

Subjects From April 1, 2010 to June 30, 2016, proximal to middle segments of coronary arteries 5–12 cm in length (23 left anterior descending arteries, 22 left circumflex arteries, 21 right coronary arteries) were excised from 23 successive autopsy cases [age 62.2±6 (mean±SD) years, 17 males; underlying disease: coronary artery disease (CAD: 7), ventricular arrhythmia (2), aortic disease (2), cerebrovascular disease (1), Parkinson’s disease (1), diabetic nephropathy (3), gastric carcinoma (1), hepatocellular carcinoma (2), interstitial pneumonia (1), urinary bladder cancer (1), breast cancer (1)]. Autopsy was performed within 5–12 h of death and the study was started within 6–12 h thereafter.

FA Procedure CA followed by FA was performed as follows. A Y-connector was introduced into the proximal portion of each coronary artery for perfusion with saline solution at a rate of 10 mL/min and then the angioscope was introduced through the connector for observation of the artery. Initially, CA was carried out to detect plaques and because the light irradiated from the angioscope tip was visible through the coronary wall, the location of the target plaque could be confirmed. After observation by CA, the light guide and the image guide were connected to the fluorescence excitation and emission units, respectively. The BPF and BAF were set and images were obtained under perfusion with saline solution. Angioscopic images were analyzed by 2 independent observers who did not participate in the angioscopic studies.

FM Procedure A total of 69 yellow plaques, 53 white plaques and 31 normal segments were confirmed by CA. The 4–5-mm long portion of the vessel in which the observed plaque was located was isolated by transecting the proximal and distal ends at the shorter axes to avoid any damage to the plaque. Subsequently, the isolated segments were each transected across the center into 2 parts. One part was fixed with formaldehyde for histological examination and the other part was mounted on a deck glass in such a way that the luminal surface of the plaque faced the deck glass. The luminal surface was scanned and the transected surface was then scanned by FM at x10 or x40 magnification using light wavelength filters similar to those used for FA. The plaques classified by CA as yellow

<table>
<thead>
<tr>
<th>Table 1. Fluorescence of the Substances Comprising Atherosclerotic Plaques Before and After the Addition of β-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substances</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>β-carotene</td>
</tr>
<tr>
<td>Ceramide</td>
</tr>
<tr>
<td>Ceramide+Collagen I</td>
</tr>
<tr>
<td>Cholesterol+Collagen I</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
</tr>
<tr>
<td>Cholesteryl oleate</td>
</tr>
<tr>
<td>Cholesteryl linoleate</td>
</tr>
<tr>
<td>Oleic acid</td>
</tr>
<tr>
<td>Linoleic acid</td>
</tr>
<tr>
<td>TG</td>
</tr>
<tr>
<td>OxLDL</td>
</tr>
<tr>
<td>LDL</td>
</tr>
<tr>
<td>VLDL</td>
</tr>
<tr>
<td>HDL</td>
</tr>
<tr>
<td>ApoA1</td>
</tr>
<tr>
<td>ApoE2</td>
</tr>
<tr>
<td>ApoB-100</td>
</tr>
<tr>
<td>LPC</td>
</tr>
<tr>
<td>Elastin</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>Ferritin</td>
</tr>
<tr>
<td>Apoferritin</td>
</tr>
<tr>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td>γ-Globulin</td>
</tr>
<tr>
<td>Collagen I</td>
</tr>
<tr>
<td>Collagen II–V</td>
</tr>
</tbody>
</table>

Light wavelengths for fluorescent excitation and emission are 345±15 and 420 nm, respectively. Golden fluorescence (Go) was emitted by ceramide in the presence of β-carotene but not by any of the other major constituents of plaque. B, blue; Br, brown; Go, golden; LB, light-blue; LBr, light-brown; LG, light-green; LY, light-yellow; No, no significant fluorescence; O, orange; P, purple; W, white.

Figure 2. Fluorescent angioscopy (FA) and microscopy (FM) imaging of a yellow plaque with necrotic core (NC). Yellow plaque on CA (arrow in A) exhibiting golden fluorescence (Go) on FA (arrow in A-1), Luminal surface scan by FM also revealed Go (arrow in A-2), indicating deposition of ceramide. Ceramide deposited mostly in the border zone of the NC (arrow in A-3) and the fibrous cap (arrowheads in A-3). Collagen fiber staining revealed a thin fibrous cap (arrowheads in A-4).
were also classified as those with or without a necrotic core (NC). By scanning the transected surface by FM, the presence or absence of ceramide and its relation to fibrous cap (FC) thickness was examined in order to compare the FA and FM images. Ruptured plaques and totally occluded segments were excluded.

**Histological Examinations**

**Staining of Ceramide and Collagen Fibers** The part of each vessel fixed with formaldehyde was cut into successive 5–10 µm sections and used for collagen fiber staining with silver stain (which maintains plaque structure), and with Ziel-Neelsen stain for ceramide.

**Measurement of Fibrous Cap Thickness** Using the collagen fiber stained slides, the thinnest part of the collagen fiber layer of the FC was measured as the cap’s thickness. Plaque debris attached to the collagen fibers was excluded from the measurement because it is the collagen fibers not the debris that protects the plaque against mechanical stress.

**Immunohistochemistry of Macrophages** CD68(+) macrophages were also stained immunohistochemically because they may participate in plaque progression. Briefly, a section was fixed with 4% paraformaldehyde solution for 7 min at 4°C, incubated with a mixture of 1% hydrogen peroxide in methanol for 30 min, reacted with anti-CD68 antibody mouse monoclonal NCL-CD 68-KP1 (antigen lysosomal granules from human lung macrophages; Leica Biosystems Newcastle Ltd, Newcastle, UK) diluted 200-fold, the peroxidase reaction was developed by 3,3’-diaminobenzidine tetrahydrochloride using an Envision kit (code no. K4061, DAKO Co., Glostrup, Denmark) for 30 min, and finally the cell nuclei were stained with hematoxylin.

Double immunohistochemical staining of ceramide and CD68(+) macrophages was also performed to examine whether the ceramide was contained in the macrophages. Briefly, after washing with phosphate-buffered saline, a section was reacted with anti-CD68 antibody as described before for 60 min, and with anti-mouse Alexa555 (Alexa Fluoro555 goat anti-mouse IgG, code no. A21422, Molecular Probes Ltd, CA, USA) for 30 min to elicit the red fluorescence of CD68. Next, the section was reacted with DAPI (4’,6-diamidino-2-phenylindole; Life Technologies, Carlsbad, CA, USA) to elicit the blue fluorescence of cell nuclei.

**Histological Examinations**

**Staining of Ceramide and Collagen Fibers** The part of each vessel fixed with formaldehyde was cut into successive 5–10 µm sections and used for collagen fiber staining with silver stain (which maintains plaque structure), and with Ziel-Neelsen stain for ceramide.

**Measurement of Fibrous Cap Thickness** Using the collagen fiber stained slides, the thinnest part of the collagen fiber layer of the FC was measured as the cap’s thickness. Plaque debris attached to the collagen fibers was excluded from the measurement because it is the collagen fibers not the debris that protects the plaque against mechanical stress.

**Immunohistochemistry of Macrophages** CD68(+) macrophages were also stained immunohistochemically because they may participate in plaque progression. Briefly, a section was fixed with 4% paraformaldehyde solution for 7 min at 4°C, incubated with a mixture of 1% hydrogen peroxide in methanol for 30 min, reacted with anti-CD68 antibody mouse monoclonal NCL-CD 68-KP1 (antigen lysosomal granules from human lung macrophages; Leica Biosystems Newcastle Ltd, Newcastle, UK) diluted 200-fold, the peroxidase reaction was developed by 3,3’-diaminobenzidine tetrahydrochloride using an Envision kit (code no. K4061, DAKO Co., Glostrup, Denmark) for 30 min, and finally the cell nuclei were stained with hematoxylin.

Double immunohistochemical staining of ceramide and CD68(+) macrophages was also performed to examine whether the ceramide was contained in the macrophages. Briefly, after washing with phosphate-buffered saline, a section was reacted with anti-CD68 antibody as described before for 60 min, and with anti-mouse Alexa555 (Alexa Fluoro555 goat anti-mouse IgG, code no. A21422, Molecular Probes Ltd, CA, USA) for 30 min to elicit the red fluorescence of CD68. Next, the section was reacted with DAPI (4’,6-diamidino-2-phenylindole; Life Technologies, Carlsbad, CA, USA) to elicit the blue fluorescence of cell nuclei. Macrophages and their nuclei thus stained were photo-
The data obtained were tested by Fisher’s exact test or Statistical Analysis CA. The BPF and BAF were then set for imaging by FA.

To observe the targeted plaque by wavelengths as used in the ex vivo study, the patients underwent CA followed by FA using BPF and BAF with the same light wavelengths as used in the ex vivo study. After confirming plaque location by CAG, an angioscope was introduced into the artery for observation of the targeted plaque by CA. The BPF and BAF were then set for imaging by FA.

Results

Fluorescence of Major Substances Comprising Atherosclerotic Plaque

Ceramide exhibited purple autofluorescence, but exhibited Go on FA when mixed with β-carotene (Figure 1B). The mixture also exhibited Go on CA. Further, the fluorescence of the major substances that comprise atherosclerotic plaques did not exhibit Go after the addition of β-carotene, indicating that Go is characteristic of β-carotene-conjugated ceramide only (Table 1).

Deposition of Ceramide and Plaque Morphology

Figure 2 shows a demonstrable example of Go visualized by FA and FM in a plaque with a NC. A yellow plaque on CA exhibited Go on FA. Also, Go was observed by FM scan of the luminal or transected surface of the plaque. Ceramide deposited in the NC, especially in the border zone of the NC and FC. Of 69 yellow plaques on CA, 41 had a NC. Ceramide as identified by Go was observed by FM in 34 and by FA in 16 of the 41 yellow plaques with a NC, but rarely in yellow plaques without a NC or in white plaques, and in none of the segments classified by CA and histology as normal (Table 2A).

Influence of Fibrous Cap Thickness on Ceramide Imaging by FA

Among the plaques in which ceramide was deposited, those with FC thickness ≤50 µm and the majority of those with a FC ≤100 µm exhibited Go on FA, whereas plaques with a FC between 100 and 250 µm in thickness exhibited light-yellow fluorescence, and those with a FC thickness ≥250 µm showed a white to light-blue or blue color on FA (Table 2B); plaques without ceramide exhibited a white to light-blue or blue color and did not exhibit either Go or light-yellow color, irrespective of FC thickness (Table 2C).

Colocalization of Ceramide and Macrophages

Because it has been reported that ceramide is produced by macrophages,2 the localization of ceramide and of CD68(+) macrophages/foam cells was compared, which revealed that on FM the Go was located in the same place as ceramide stained by Ziel-Neelsen stain and the macrophages/foam cells stained by immunohistochemistry (Figure 3).

Ceramide in CD68(+) Macrophages

Because it is believed that macrophages produce ceramide at their death,2 we examined whether ceramide production is confined to macrophages within the plaques. CD68(+) macrophages that contained ceramide were observed not only in the plaque but also in the media, adventitia and pericoronary adipose tissues, but fragmentation of the ceramide-containing macrophages was observed only beneath the FC (Figure 4).

Ceramide Observed by FA in Coronary Plaques of Patients With CAD

A total of 16 white and 18 yellow plaques, and 22 white and 24 yellow plaques, were observed on CA in 8 patients with stable angina and in 8 patients with OMI, respectively. Go was observed in yellow plaques as in ex vivo study, but the incidence of Go in yellow plaques in the OMI group was significantly higher than in yellow plaques in the stable angina group (Table 3). No obvious complications were noted during or after CAG, CA and FA (Figure 5).
Ceramide in Coronary Plaques

during or after NC formation. It remains obscure why ceramide did not deposit in a certain group of yellow plaques with NC.

Masking of Ceramide by the FC
Go characteristic of ceramide was visualized by FA when the FC thickness was ≤100 µm, but as light-yellow or light-blue to white, or blue when the FC was thicker. In our preliminary and previous studies, collagen I exhibited blue fluorescence and a mixture of cholesterol or cholesteryl esters with collagen I and β-carotene exhibited a light-blue to white color (Table 1). It is conceivable that the failure to detect the Go of ceramide in plaque with a FC thicker than 100 µm was because of masking of the Go by these other substances.

Role of Macrophages in Ceramide Production
It is reported that macrophages produce ceramide during apoptosis. In the present study, ceramide visualized by FM, ceramide stained by standard Ziel-Neelsen stain and

Discussion

Characteristic Fluorescence of Ceramide in the Presence of β-Carotene
In the present study, β-carotene, which deposits in lipid-laden atherosclerotic plaques, evoked Go by ceramide, enabling visualization of ceramide in the human coronary arterial wall. As in case of Evans blue dye, which elicits the fluorescence of oxLDL, the mechanism by which β-carotene evokes fluorescence by ceramide is conjugation with ceramide to form an adduct that emits Go. Because there were no other major substances in plaques that exhibited Go, we believe it is characteristic of ceramide only, and that β-carotene can be used as its biomarker.

Ceramide Detected by FM and FA
Using Go as a marker, ceramide was detected in the NC of the majority of yellow plaques with a NC, but rarely in yellow plaques without a NC, as classified by CA and histology, suggesting that ceramide deposits in the NC either

Table 3. Coronary Plaques With Go Detected by FA in Patients With CAD

<table>
<thead>
<tr>
<th>Plaque color on CA</th>
<th>No. of patients</th>
<th>SAP</th>
<th>OMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque color on FA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) Golden</td>
<td></td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>(B) Light-yellow</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>(C) Light-blue to white</td>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>(D) Blue</td>
<td></td>
<td>14†</td>
<td>17‡</td>
</tr>
</tbody>
</table>

**P<0.01 vs. white plaques in SAP and those in OMI. †P<0.05 vs. yellow plaques in SAP. ‡P<0.05 vs. white plaques in SAP and OMI. The incidence of plaques exhibiting Go in patients with OMI was higher than that in patients with SAP. CAD, coronary artery disease; OMI, old myocardial infarction; SAP, stable angina pectoris. Other abbreviations as in Table 2.
CD68(+) macrophages all co-localized mainly beneath the FC, which is the site of tissue degeneration in progress, indicating that ceramide was produced by macrophages.

Double immunohistochemical staining revealed that although ceramide-containing macrophages were densely distributed beneath the FC, they were also distributed in the adventitia, media and pericoronary adipose tissue, suggesting that macrophages synthetized ceramide outside of the NC (i.e., in the adventitia or pericoronary adipose tissue, distributed beneath the FC, they were also distributed in the superficial layers.

Role of Ceramide in Atherosclerosis
Ceramide, the backbone of sphingolipids, is the key component affecting atherosclerotic changes. Cheng et al observed that ceramide is involved in obesity-induced endothelial dysfunction.

Plaque Vulnerability
It is generally believed that a plaque with a thin FC and a voluminous NC is a vulnerable plaque (i.e., rupture prone).

Ceramide Deposits in Coronary Plaques in Patients
Coronary plaques exhibiting Go were more frequently observed in patients with OMI than in those with stable angina, suggesting that the former group of patients has a more frequent risk of recurrent ACS.

Clinical Implications
CA and FA are routinely performed during CAG or coronary interventions in selected institutions in Japan. Because FA can be performed simply by changing the BPF and BAF without changing the angioscope, FA could be used for detection of the Go of ceramide and accordingly for detection of vulnerable coronary plaques in the clinical setting. Catheter-based treatment targeting the plaques that exhibit Go could be performed simply by replacing the angioscope with interventional catheters (i.e., FA-guided intervention) The effects of medical therapy targeting these plaques could also be directly evaluated by FA.

Study Limitations
Firstly, although the present study revealed that ceramide located ≤100 μm below the surface of a plaque can be visualized by FA, that located deeper could be not determined because of the interposed thicker FC. Secondly, the number of patients was small, so differences in the incidence of Go in plaques on FA could not be compared between the underlying diseases. Finally, because real-time visualization of the movement of the ceramide-carrying macrophages was beyond the scope of current immunohistochemical techniques, whether ceramide is conveyed by macrophages from the adventitia or pericoronary adipose tissue to plaque remains to be elucidated.

Conclusions
Ceramide deposits in human coronary plaques obtained from autopsy subjects were visualized by FA and FM using Go as a marker. FM could visualize ceramide located in the NC of yellow plaques detected on CA, but rarely in other types of plaques. Ceramide depositad ≤100 μm in depth from the luminal surface was visualized by FA. Ceramide was also detected in coronary plaques during CAG in patients with stable angina. The findings suggested that ceramide in human coronary plaques is detectable by FA using Go as a marker when the FC thickness is ≤100 μm, and that Go could be used as a marker of vulnerable plaques (i.e., thin FC with NC). This study appears to
be the first to visualize ceramide in human coronary plaques both ex vivo and in vivo.

**Author Contributions**

Conceived and designed the study: Yasumi U. and Yasuto U. Performed the in vitro study; Yasumi U. and Yasuto U. Performed autopsy and immunohistochemical staining: E.S. and N.H. Performed microscopic photography: T.K. Performed fluorescent angioscopy in patients: Yasuto U., S.S. and T.K. Statistical analysis: T.T. Wrote the paper: Yasumi U. All authors edited the manuscript.

**Disclosure**

The authors have no conflicts of interest to declare.

**Relationships With Industry**

The authors have no relationships with industry.

**Funding**

No external funding was received for this study.

**References**


