Localization of Native High-Density Lipoprotein and Its Relation to Plaque Morphology in Human Coronary Artery

Yasumi Uchida, MD, Nobuyuki Hiruta, MD, Yasuto Uchida, MD, and Ei Shimoyama, MD

Summary

High-density lipoprotein (HDL) plays a key role in reverse cholesterol transport, and halts the progression of atherosclerosis. However, its localization in human vascular wall is not well understood. We discovered that by exciting at 470-nm and emitting at 515-nm light wavelengths, Fast green dye (FG) elicits brown fluorescence characteristic of HDL only. Therefore, the localization of native HDL in normal segments and plaques in excised human coronary artery was investigated by scanning their transected surface with color fluorescent microscopy (CFM) using FG as a biomarker, and the relationships between the localization of HDL and morphology of plaques and normal segments classified by conventional angioscopy and histology were examined. The % incidence of HDL in 13 normal segments (NS) with thin (≤ 200 µm) intima, 28 NS with thick (200 µm <) intima, 41 white plaques (early stage of plaque growth), 15 yellow plaques (Y) without necrotic core (NC), and 20 Y with NC (advanced stage of plaque growth), was 30, 71 (P < 0.05 versus NS with thin intima and Y with NC), 83 (P < 0.05 versus NS with thin intima and Y with NC), 60, and 35, respectively. HDL begins to deposit in human coronary arterial wall in the early stage of atherosclerosis and deposits increase with plaque growth, but HDL decreases in plaques at an advanced stage of growth. (Int Heart J 2013; 54: 348-354)

Key words: Color fluorescent microscopy, Fast green dye, Human coronary plaques, Low-density lipoprotein, Lyso-phosphatidylcholine

A high plasma level of high-density lipoprotein (HDL) is associated with decreased risk of coronary heart disease. Elevating the plasma level of HDL by fibrates is associated with a lower incidence of coronary heart disease. Mechanisms by which antiatherogenic HDL reduces atherosclerosis include facilitating cholesterol uptake from cholesterol-loaded macrophage-foam cells in plaques for transport back to the liver, a role as an anti-inflammatory lipoprotein, decreasing oxidized low-density lipoprotein (oxLDL), increasing nitric oxide synthesis, improving endothelial function, and as an antiatherogenic agent. Infusions of HDL or its major component, apolipoprotein A-I (Apo A-I), halt the progression or induce the regression of atherosclerosis.

The relationship between the plasma HDL levels and atherosclerotic lesions has been extensively investigated. Trafficking HDL movement was performed using gadolinium methanethiosulfonate-labeled ApoA-I in animal liver and kidney, and using nanocrystals such as Au-HDL and FeO-HDL in the mouse aorta, but imaging of native HDL in the human vascular wall has not been successful to date. Therefore, it is not known in what type(s) of atherosclerotic plaques native HDL deposits, whether this substance deposits in the normal vascular wall, how this substance acts against atherosclerotic plaque growth and destabilization, and whether or not its concentration changes in parallel with that in the plasma. If HDL could be visualized in the vascular wall, the molecular mechanisms by which HDL causes regression of atherosclerosis and whether or not the amount of HDL in the vascular wall is increased by HDL-raising compounds could be clarified more objectively.

We recently discovered that Fast green dye (FG), which is used clinically to stain oral mucosal micronuclei for detection of the genotoxicity of antihyperglycemic drugs in patients with diabetes mellitus, in intraocular membrane staining, and subchondral bone staining, elicits a brown fluorescent color from HDL when fluorescence is excited at 420 ± 20-nm and emitted at 515-nm light wavelengths. Therefore, in the present study, color fluorescent microscopic (CFM) scanning of the transected surface of the excised human coronary artery (both plaques and normal segments) was performed to clarify the localization of native HDL using FG as a biomarker, and to correlate it to plaque morphology studied by conventional angioscopy.

Methods

Classification of coronary plaques by conventional angioscopy:
In the present study, conventional angioscopy using white light as a light source was used to classify the coronary plaques and normal segments because their angioscopic appearances represent their macroscopic pathologic changes, and this technology is widely used clinically for detection of vulnerable coronary plaques. The details of the system are described elsewhere.

Plaque is defined as a non-mobile, protuberant or lining mass that is clearly demarcated from the adjacent normal wall and the shape, location and color of which do not alter under the influence of a saline solution flush. Plaque is further classified as white or yellow based on its surface color. A normal segment is defined as milky-white and smooth-surfaced without any protrusions.

Images of the plaque obtained by conventional angioscopy were classified as white or yellow using an AquaCosmos image analyzer (C7746, Hamamatsu Photonics), which sets a window on an appropriate portion of an image and separates the color within the window into three primary colors, namely red, green and blue. Plaque was defined as “white” when the intensity ratio of red: green = 1.0 : 0.9–1.1 : 0.9–1.1, respectively, and as “yellow” when it was 1.0 : 0.8–1.2 : 0.3–0.6, respectively.

Observation of coronary plaques by conventional angioscopy:

Ethical statements

This *in vitro* study was carried out after approval of the Ethical Committees of the Japan Foundation for Cardiovascular Research, Funabashi-Futawa Hospital, and Toho University, and after obtaining written informed consent from the families concerned on the use of excised coronary artery for pathological study to clarify the mechanisms of atherosclerosis.

Twenty-four proximal to middle segments of coronary arteries (8 left anterior descending arteries, 8 left circumflex arteries, 8 right coronary arteries) were removed from 8 cadavers [60.0 ± 3.6 years; 3 females, 5 males; death due to acute myocardial infarction (3), diabetic nephropathy (2), cerebral arteries, 8 right coronary arteries) were removed from 8 cadavers].

Excised coronary artery was perfused with saline solution at a rate of 10 mL/min and then an angioscope was introduced through the connector into the artery to detect plaques. Because the light from the tip of the angioscope was visible through the coronary wall, the site of the targeted plaque could be confirmed.

**Imaging of HDL by CFM:**

**CFM apparatus**

Using a fluorescent microscope (IX 70, Olympus, Tokyo), the fluorescence of the target was obtained by excitation at 470 ± 20-nm and emission at 515-nm (details of the apparatus are described elsewhere). The intensity of fluorescence was categorized as strong, weak or absent when the exposure time required for imaging was ≤ 1 s, > 1 s and ≤ 5 s, and > 5 s, respectively.

**Table. Autofluorescence and fluorescent color of the major substances comprising atherosclerotic plaques when excited by fast green (FG) dye and imaged with color fluorescent microscopy**

<table>
<thead>
<tr>
<th>Substances</th>
<th>Autofluorescence</th>
<th>Fluorescence in the presence of FG (10⁻⁵ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-density lipoprotein</td>
<td>no</td>
<td>Br</td>
</tr>
<tr>
<td>Oxidized low-density lipoprotein</td>
<td>no</td>
<td>BB</td>
</tr>
<tr>
<td>Low-density lipoprotein</td>
<td>no</td>
<td>R</td>
</tr>
<tr>
<td>Very low-density lipoprotein</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine</td>
<td>no</td>
<td>R</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Apolipoprotein A-1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Apolipoprotein E-2</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Matrix metalloproteinase-1,-9</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Y</td>
<td>no</td>
</tr>
<tr>
<td>Cholesteryl oleate</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Cholesteryl linoleate</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>7-Keto cholesterol</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Collagen I</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>LG</td>
<td>G</td>
</tr>
<tr>
<td>Collagen III, V</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Albumin</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Globulins</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Ceramide</td>
<td>Y</td>
<td>no</td>
</tr>
<tr>
<td>Elastin</td>
<td>LY</td>
<td>R</td>
</tr>
<tr>
<td>Hydroxypatite</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Y, Y-to-W</td>
<td>no</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>O</td>
<td>no</td>
</tr>
</tbody>
</table>

Band-pass filter = 470-nm. Band-absorption filter = 515-nm. B indicates blue; BB, black brown which masks other fluorescence including background fluorescence; Br, brown; G, green; LY, light yellow; O, orange; W, white; Y, yellow; and no, no fluorescence.

Fast green dye (FG; 10⁻⁵ M) was added to each substance to elicit color fluorescence. Brown fluorescence was evoked by adding FG to HDL. This brown fluorescence was not evoked in any other known substances that comprise atherosclerotic plaques listed in this table, indicating that this brown fluorescence is characteristic of only HDL. Red fluorescence characteristic of LDL and LPC was also elicited by FG.
During a search for a biomarker which elicits fluorescence characteristic of HDL, we found that FG elicits a brown fluorescence from human HDL (Merck Millipore Ltd., Temecula, CA) and a red fluorescence from human LDL (Merck Millipore Ltd.) and LPC (L-α-lysophosphatidylcholine, Wako Co, Osaka, Japan). Because these fluorescent colors were not exhibited by any other known substances that comprise atherosclerotic lesions, we postulated that the brown fluorescence is characteristic of HDL only and the red fluorescence characteristic of LDL and LPC only (Figure 1 and Table).

FG (Wako Co., Osaka) was diluted in distilled water to a concentration of 2% and was used in the following study.

**Figure 2.** Deposition of High-density Lipoprotein (HDL) in Deep Layers of a Coronary Artery Segment Visualized by Color Fluorescent Microscopy (CFM). A: Normal coronary segment imaged by conventional angioscopy. Arrow: portion observed by CFM transected surface scan. B: CFM image of the transected surface of the same segment before administration of Fast green dye (FG). The segment showed green fluorescence, indicating the existence of collagen I and absence of β-carotene. B-1: CFM image of the same segment after administration of FG. A brown fluorescence is observed in deep layer of the intima, indicating the existence of HDL. C: Histology of the same segment after Oil Red-O and methylene blue dye staining. Lipids (cholesterol and/or cholesteryl esters) in a spotty pattern in deep layer of intima (red spots). L, I and M: lumen, intima and media, respectively. Spotty deposition of lipids (red portion) in deep layer. Bar = 100 µm.

**Figure 3.** Diffuse Deposition of High-density Lipoprotein (HDL). A: A white plaque imaged by conventional angioscopy (arrow). B: CFM image of the transected surface of the same segment before administration of Fast green dye (FG). The segment showed green fluorescence, indicating the existence of collagen I and absence of β-carotene. B-1: CFM image of the same segment after administration of FG. Brown fluorescence is observed diffusely in entire intima and media (arrow), indicating diffuse deposition of HDL. C: Histology of the same segment after Oil Red-O and methylene blue dye staining. Deposition of lipids is not seen. Bar = 100 µm.

**Scanning the transected surface by CFM**

In the 41 white plaques, 35 yellow plaques and 41 normal segments, the center of each plaque or segment was transected and one half was mounted on a deck glass such that the transected surface faced the glass. After obtaining a control image by CFM at × 40 magnification, the specimen was immersed in 2% FG saline solution for 5 minutes. The specimen was then observed again by CFM.

The plaques were classified into two layers, namely superficial (∼200 μm from the luminal surface) and deep (200–μm < from the luminal surface), and the relationships between the deposition site of HDL and LDL/LPC and plaque mor-
Phenology studied by conventional angioscopy were examined. Histology: After CFM scanning, each sample was cut into slices along the shorter axis and stained with Oil Red O and methylene blue (MB), which stains lipids red, calcium black, and collagen fibers and smooth muscles blue. In a preliminary study, cholesterol and cholesteryl esters and ceramide were stained red with Oil Red O, while none of the other substances, including apolipoproteins and lipoproteins, listed in Table were stained.

Statistical analysis: The data obtained were tested by χ²-formulae. A value of P < 0.05 was considered to be statistically significant. The percent (%) incidence and deposition patterns of HDL were compared between the different categories of plaques and normal segments.

Results

HDL in excised human coronary artery observed by CFM: Representative Examples

Figure 2 shows a normal segment studied by conventional angioscopy. CFM showed the transected surface of the plaque exhibited green fluorescence, which indicated the presence of collagen I. After administration of FG, brown fluorescence appeared in a deep layer of intima and also in media of the same segment, indicating the existence of HDL. Histologically, intima was thickened more than 200 µm with proliferated collagen fibers but deposition of lipids was localized to the deep layer of intima.

Figure 3 shows a white plaque studied by conventional angioscopy. Before administering FG, the entire intima and media were stained brown, indicating diffuse deposition of HDL. Histologically, lipid deposition was not observed.

Figure 4 also shows a white plaque. After administration of FG, brown fluorescence appeared in the deep layer of intima and media, and red fluorescence appeared in the superficial layer of intima, revealing co-deposition of HDL and substances that exhibit red fluorescence, ie, LDL/LPC.

Incidence of HDL and its relation to plaque morphology

The % incidence of HDL in 13 normal segments with thin (≤ 200 µm) intima, 28 normal segments with thick intima (200 µm >), 41 white plaques (early stage of plaque growth) and 15 yellow plaques without necrotic core (NC) and 20 yellow plaques with NC (advanced stage of plaque growth), was 38, 71, 83, 60 and 35, respectively. Thus, the % incidence of HDL in normal segments with thin intima was low, and that in white plaques was significantly higher than that in normal segments with thin intima and yellow plaques with NC. There were no significant differences in the % incidence between white plaques and normal segments with thick intima and yellow plaques without NC (Figure 5).

Deposition sites of HDL and their relation to plaque morphology

Figure 4. Co-deposition of High-density Lipoprotein (HDL) with Low-density Lipoprotein (LDL) and/or Lysophosphatidylcholine (LPC). A: Yellow plaque imaged by conventional angioscopy. Arrow: observed portion by CFA. B: CFA image before administration of Fast green dye (FG), shows yellowish green, indicating existence of collagen I and β-carotene. B-1: CFA image after administration of FG, shows brown (arrow) deep layer and red fluorescence in superficial layer (arrowhead), indicating co-deposition of HDL and LDL/LPC. C: Histological examination shows deposition of lipids (arrow). Bar = 100 µm.
Figure 6. Deposition Patterns of High-density Lipoprotein (HDL) and Low-density Lipoprotein (LDL) and/or Lysophosphatidylcholine (LPC). A: Deposition of HDL in deep layer (arrow). B: Deposition in superficial layer (arrow). C: Deposition in both deep and superficial layers (arrow). D: Deposition in a necrotic core (white arrows) with LDL/LPC (yellow arrow) and calcium compounds (white arrowhead). E: Deposition in media (arrow). F: Deposition of HDL in deep layer of intima and media (arrow) with LDL/LPC in superficial layer (arrowhead).

Figure 7. Relationship between Deposition Sites of High-density Lipoprotein and Plaque Morphology. n indicates number of preparations in which HDL deposited; Diffuse, deposition of HDL in superficial and deep layers of intima with or without its deposition in media; *, P < 0.05 versus yellow plaques; †, P < 0.001 versus normal segments; ‡, P < 0.05 versus superficial layer; §, P < 0.001 versus diffuse deposition; ‖, P < 0.01 versus deep layer; #, P < 0.001 versus superficial layer; and ¶, P < 0.05 versus deep or superficial layer. HDL deposited frequently in deep layer of intima in normal segments, less frequently in white plaques, whereas diffusely in white and yellow plaques. Deposition of HDL in superficial layer of intima was observed although infrequently in normal segments with thick (200 μm <) intima and white plaques but in none of the yellow plaques.

Figure 8. Relationship between Plaque Morphology and Deposition of High-density Lipoprotein (HDL), Low-density Lipoprotein (LDL) and/or Lysophosphatidylcholine (LPC). n indicates number of preparations examined; LDL/LPC, LDL and/or LPC; *, P < 0.05; and **, P < 0.01. HDL deposited frequently in normal segments, less frequently in white plaques, and rarely in yellow plaques. HDL co-deposited with LDL/LPC in white and yellow plaques but less frequently in normal segments. LDL/LPC deposited alone frequently in yellow plaques, rarely in white plaques, and never in normal segments.
As shown in Figure 4 A-C and E, HDL deposited in the deep layer of intima (200 µm < from luminal surface), superficial layer of intima (200 µm from luminal surface), media, or diffusely in both deep and superficial layers of intima (with or without deposition in media). HDL also deposited in NC of yellow plaques, often with LDL/LPC (Figure 6).

HDL deposited frequently in deep layer of intima in normal segments, whereas diffusely in both deep and superficial layers in white and yellow plaques. HDL localized to superficial layer was infrequent (Figure 7).

**Relationship between HDL and LDL/HDL deposition**

Deposition was classified as HDL alone, HDL with LDL/LPC, and LDL/LPC without HDL.

HDL deposited alone frequently in normal segments, less frequently in white plaques, and rarely in yellow plaques. HDL co-deposited with LDL/LPC in white and yellow plaques but less frequently in normal segments. LDL/LPC deposited alone frequently in yellow plaques, rarely in white plaques, and not at all in normal segments (Figure 8).

**Discussion**

In the present study, FG evoked a brown fluorescence when added to HDL, enabling both visualization of HDL in the human coronary arterial wall and analysis of the differences in its deposition patterns not only within plaques but also within normal segments. The mechanisms by which FG evoke fluorescence by HDL are that, as in the case of Evans blue dye which elicits fluorescence characteristic of oxLDL, FG conjugated to HDL to form an adduct to provoke a brown fluorescent color. Because there were no other major substances that exhibited a brown fluorescence color, we believed it to be characteristic of HDL alone, and that FG can be used as a biomarker of HDL.

By transected surface scanning with CFM, the % incidence of HDL deposition was low in normal segments with thin intima, increased in normal segments with thick intima and white plaques, but decreased in yellow plaques. In contrast, LDL/LPC which are well known to accelerate atherosclerosis, increased in the order of normal segments, white plaques, and yellow plaques.

It is well known that white plaques (early stage of plaque growth) mainly comprise proliferated collagen fibers, and become yellow plaques (advanced stage of plaque growth) with the accumulation of lipids and β-carotene.

The results of the present study indicate that HDL deposits infrequently in normal segments without intimal hyperplasia, but begins to deposit mainly in deep layers of normal segments when intima is thickened, extends to entire intima with plaque formation, but is decreased in plaques at an advanced stage of growth.

It should be clarified what factor(s) trigger HDL deposition when intimal thickening occurs and whether or not LDL/LPC increased secondary to a decreased supply of HDL to the coronary arterial wall. Also, it remains to be clarified why HDL begins to deposit in the deep layer of normal segments.

There were white or yellow plaques in which neither HDL nor LDL/LPC was detected. It remains to be elucidated what factor(s) determine their deposition.

Separate imaging of LDL and LPC was not successful in the present study.

Although we have successfully imaged LDL using Nile blue dye and LPC using Trypan blue dye as biomarkers, respectively, separate imaging of LDL and LPC has thus far been unsuccessful. Therefore, it is necessary to identify a biomarker that enables imaging of LDL and LPC separately.

Human HDL consists of subclass 2a, 2b, 3a, 3b and 3c. Patients with coronary heart disease are associated with lower plasma levels of large-sized HDL (2b) and higher plasma levels of small-sized HDL (3b). HDL (3b) is associated with an increased risk of metabolic syndrome.

Because biomarkers for individual HDL subclasses were not discovered, localization of HDL as a whole was examined in the present study. Therefore, it remains to be clarified whether deposition sites for individual HDL subclasses in human coronary plaques are different from each other.

Molecular targeting therapy with HDL or with a component of HDL (Apo A-1) has begun. Many attempts have been made with fibrates to elevate the serum levels of HDL in anticipation of its increase within the vascular wall. In fact, the stabilizing effect of fibrates on coronary plaques has been proven by a prospective follow-up study using conventional angioscopy. However, whether or not HDL is increased by fibrates in the targeted vascular wall remains unproven.

FG has been used clinically in the ophthalmologic and orthopedic fields. Recently, the present authors succeeded in imaging of HDL deposited in coronary artery in patients, as in the case of imaging of oxLDL using Evans blue dye. The present fluorescent microscopic study using FG as a biomarker appears to be the first of its kind to image native HDL in the human vascular wall.

However, there are some shortcomings; (1) Because biomarkers for individual subclasses of HDL have not yet been discovered, localization of individual subclasses in human coronary arterial wall remains to be elucidated.

(2) Also, LDL and LPC could not be discriminated from each other because both exhibited red fluorescence in the presence of FG.

**Conclusions:** Native HDL and LDL/LPC in excised human coronary plaques and normal coronary segments using FG as a biomarker of HDL were investigated by CFM.

HDL begins to deposit in coronary arterial wall at an early stage of atherosclerosis and deposits increase with plaque growth. However, there is a decrease in plaques at an advanced stage of growth, whereas LDL/LPC increased with plaque growth and increased further at an advanced stage of plaque growth.

**References**


3. Toth PP, Davidson MH. High-density lipoproteins: marker of car-