Fluorescence Analysis of Biochemical Constituents Identifies Atherosclerotic Plaque With a Thin Fibrous Cap

Koh Arakawa, Kikuo Isoda, Toshimitu Ito, Kei Nakajima, Toshio Shibuya, Fumitaka Ohsuzu

Abstract—Vulnerable plaque generally contains a thin fibrous cap, lipid pools, and reduced internal plaque collagen. Arterial fluorescence analysis can differentiate atherosclerotic lesions from normal arteries; however, the contribution of the lipid core to atherosclerotic arterial fluorescence remains controversial. This study aimed to identify lipid core fluorophores and to differentiate the lipid core from normal artery and atheroma. The helium-cadmium laser–induced fluorescence spectra of cadaveric arteries and known chemical constituents were recorded. Lipid core fluorescence spectra exhibited marked red shifts and broadening compared with the fluorescence spectra of normal tissue and atheroma. Similar fluorescence spectra were obtained for lipid core and oxidized low density lipoprotein, for atheroma and collagen, and for normal artery and elastin. A classification based on collagen, elastin, and oxidized low density lipoprotein spectral decomposition could discriminate the lipid core (n = 29), normal artery (n = 74), atheroma (n = 73), and preatheroma (n = 10) with 86% accuracy. Fibrous cap thickness was correlated with the spectral collagen content index (r = 0.65, P < 0.0001), especially at a thickness of <200 μm. We conclude that a classification algorithm based on chemical spectral decomposition can accurately classify the fluorescence spectra of normal artery, atheroma, and lipid core and may be useful in identifying vulnerable atheroma in vivo. (Arterioscler Thromb Vasc Biol. 2002;22:1002-1007.)

Key Words: lasers ■ plaque ■ spectroscopy ■ lipids ■ oxidized LDL

Atherosclerotic plaque consists of a lipid-rich core covered with a collagen-rich fibrous cap, varying widely in thickness. Plaque disruption is associated with varying degrees of internal hemorrhage and luminal thrombosis because the lipid core and exposed collagen are thrombogenic. Acute coronary syndrome usually occurs as a consequence of such disruption or ulceration of vulnerable plaque. Vulnerable plaque is characterized by lipid pools, a thin fibrous cap, and reduced internal plaque collagen. The ability to characterize and detect vulnerable plaque would contribute to the treatment of plaque and prevention of acute coronary syndrome.

Laser-induced fluorescence spectroscopy of the cardiovascular system has been used not only to identify atheroma but also to measure intimal thickness. Many classification indexes for differentiating plaque types and measuring intimal thickness have been derived from empirical studies of fluorescence spectra. Therefore, understanding the nature of arterial fluorophores is important for accurate classification of arterial tissues. A classification algorithm based on fluorescence spectral decomposition of elastin and collagen can be used to discriminate between normal and atherosclerotic arterial tissues. Knowing the major fluorophores of the lipid core is essential for identifying vulnerable plaque. Fluorescence intensities of 500 and 600 nm characterize fatty plaque; whether the lipid core (lipids) contributes to atherosclerotic and normal arterial fluorescence is controversial.

The present study was undertaken to (1) observe laser-induced fluorescence spectra from normal artery, atherosclerotic plaque, and ulcerated plaque (lipid core); (2) clarify the fluorophores of the lipid core and derive a classification algorithm to distinguish these 3 tissue types on the basis of arterial fluorophores; and (3) characterize changes in fluorescence spectra during fibrous cap removal and estimate fibrous cap thickness.

Methods

The present study involved several steps. First, laser-induced fluorescence spectra were recorded from purified chemical samples of known constituents of normal artery, atheromatous plaque, and lipid core and were compared with intact artery fluorescence. Second, a spectral algorithm was developed to classify normal arteries, lipid core, and atheromatous plaque spectra. Finally, spectral changes during plaque removal were recorded and evaluated to estimate fibrous cap thickness.

Subjects

In Vitro Tissue Samples

Segments of human femoral and coronary arteries containing atherosclerotic plaque and normal tissue were obtained from 15 adult cadavers within 12 hours of death. The tissues were rinsed in saline. The vessels were opened longitudinally to expose the intimal surface.
for fluorescence measurements. Each sample was classified by gross inspection as normal artery (n = 74) or protruded plaque, which was subclassified as preatheroma (Stary type III, n = 10), atheroma (Stary types IV and V, n = 73), or ulcerated plaque (plaque with an exposed lipid core and without a fibrous cap or thrombus, Stary type VI, n = 29). The classification was confirmed by histological examination. Each sample was placed in a tissue holder and submerged in a saline bath. During fluorescence measurements, a fused silica optical fiber tip was positioned at the middle of the plaque and kept in constant contact with it by a specially designed apparatus that allowed exact placement of the tip. Samples were fixed in formalin after use. Specimens were stained with hematoxylin-eosin and elastica-Masson; frozen specimens were fixed in formalin after use. Specimens were stained with elastin, and those not suited for histological analysis were excluded before examination. Each sample was placed in a tissue holder and kept in constant contact with it by a specially designed apparatus that allowed exact placement of the tip. Samples were fixed in formalin after use. Specimens were stained with hematoxylin-eosin and elastica-Masson; frozen specimens were stained with oil red O as necessary. Samples showing calcification and those not suited for histological analysis were excluded before the correlation studies.

**In Vivo Human Coronary and Femoral Arteries**
During surgical vascular repairs in an operating room, laser-induced fluorescence spectra were recorded at the anastomotic site of native coronary and femoral arteries in 13 patients by use of the same system that was used for the in vitro study. Fluorescence was measured at sites clearly classified by the surgeon as white normal artery and to assess variations in spectral features according to tissue type.

**Development of a Discriminative Algorithm**
The spectra of 186 atherosclerotic and normal arterial specimens were recorded to identify spectral features that characterize preatheroma, atheroma, ulcerated plaque (exposed lipid core), and normal artery and to assess variations in spectral features according to tissue type.

Because of their major contributions to the tissue contents of normal and atherosclerotic arteries, collagen, elastin, and lipid were evaluated. The fluorescence spectra of LDLs and oxidized LDLs (oxLDLs) were prepared as follows: Blood obtained from healthy blood donors after an overnight fast was collected in tubes containing EDTA (0.25 mmol/L). Lipoproteins were separated by sequential preparative ultracentrifugation as described by Havel et al. Fractions with a density of 1.019 to 1.063 were pooled as LDL. LDLs were extensively dialyzed against PBS without EDTA. OxLDLs were obtained by dialysis against PBS supplemented with 5 mmol/L CuSO₄ at 37°C for 3, 16, 24, and 48 hours (3h-oxLDL, 16h-oxLDL, 24h-oxLDL, and 48h-oxLDL, respectively). The reactions were stopped by the addition of EDTA (final concentration 0.25 mmol/L). The protein contents of native LDLs and oxLDLs were determined by the method of Lowry et al., with BSA used as a standard, and were then diluted with PBS to 1 µg of protein per milliliter. The extent of lipoprotein modification was assessed by electrophoretic mobility. Collagen I, elastin, cholesteryl linoleate, cholesteryl oleate, chondroitin sulfate, heparan sulfate, and lysophosphatidylcholines were obtained from Sigma Chemical Co (Sigma-Aldrich Japan K.K.).

To develop constituent indexes, each spectrum was normalized to peak fluorescence intensity. The peak fluorescence intensity was adjusted to 10,000, and fluorescence intensities at each wavelength were multiplied by the adjusted ratio and decomposed into a linear combination of pure elastin, collagen, and 3h-oxLDL spectra by multiple regression analysis (SPSS statistical analysis software, SPSS Japan; sample spectrum = a×collagen spectrum + b×elastin spectrum + c×3h-oxLDL spectrum + constant, a, b, c: coefficient). Spectral standardized coefficients for collagen, elastin, and 3h-oxLDL were obtained as constituent indexes. For negative values, the standardized coefficient was presumed to be 0. The collagen index (CI) was calculated as the standardized coefficient of collagen divided by the sum of the standardized coefficients of collagen, elastin, and 3h-oxLDL.

**Changes in Laser-Induced Fluorescence During Plaque Excision**
Eight specimens (~8×8 mm) of atheroma with a lipid core covered by a fibrous cap were obtained from 8 autopsy sources and were frozen and stored at −80°C until use. The adventitia from these specimens was removed to facilitate mounting in a cold microtome (Cryostats, OT/FAS/MR, Bright Inc). Each sample was frozen in OCT compound (which does not fluoresce; Sakura Finetechical) and was placed in the microtome to obtain atheroma slices. Any OCT compound covering the tissue was removed by the microtome before tissue slice preparation. During plaque removal with the microtome, fluorescence spectra were recorded as previously described, but the optical fiber tip was held in manual contact with the tissue. The microtome removed atheroma in 10-µm increments, and spectra were recorded after the removal of each layer. All plaque specimens were thawed and analyzed histologically to evaluate the composition of the fibrous cap, lipid core, and media. Fibrous cap thickness was calculated by totaling the number of tissue slices removed until histological and spectroscopic examinations revealed a lipid core.
Fluorescence Spectra of Known Constituents and Plaque

After normalization of the fluorescence spectrum to peak intensity, similarities in fluorescence spectra were evaluated among different chemicals and types of atherosclerotic lesions. Similar spectral patterns and wavelengths at peak intensity were observed for collagen and heparan sulfate, for elastin, cholesteryl linoleate, and chondroitin sulfate, and for 3h-oxLDL, cholesteryl oleate, and lysophosphatidylcholine (Figure 2A, 2B, and 2C, respectively). The fluorescence spectrum of 3h-oxLDL was similar to the spectra of LPC and cholesteryl oleate. Representative laser-induced fluorescence spectra of normal artery, atheroma, and ulcerated plaque (lipid core) were compared with those of elastin, collagen, and 3h-oxLDL. There were similarities between the fluorescence spectra of atheromatous plaque and collagen (D), normal artery and elastin (E), and ulcerated plaque and 3h-oxLDL (F). Spectra are normalized to peak intensity.

Statistical Analysis

All values are expressed as mean±SD, and differences among the tissue types in standardized coefficients for chemical components were assessed by ANOVA. The significance of differences in unpaired measurements was assessed by least significant difference tests. Characterization of plaque type based on a standardized coefficient for each chemical component was performed by discriminant analysis. A logarithmic curve estimation regression model was used for comparisons of fibrous cap thickness and CI. All statistical analyses were performed with SPSS statistical analysis software. A value of P<0.05 was considered to indicate statistical significance.

Results

Fluorescence Spectra of Known Constituents and Plaque

One hundred eighty-six arterial samples were evaluated histologically and by fluorescence spectroscopy. When the fluorescence spectra of the arteries were decomposed into linear combinations of elastin, collagen, and 3h-oxLDL spectra, the standardized coefficients showed that the main chemical components were elastin in normal artery, collagen in atheromatous plaque, and 3h-oxLDL in ulcerated plaque and preatheroma (Table 1). When the collagen, elastin, and 3h-oxLDL spectrum coefficients were subjected to discrimi-

Ex vivo Collagen Elastin 3h-oxLDL

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>Elastin</th>
<th>3h-oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal artery</td>
<td>0.053±0.154††</td>
<td>0.689±0.296††</td>
<td>0.299±0.279††</td>
</tr>
<tr>
<td>Preatheroma</td>
<td>0.297±0.252††</td>
<td>0.231±0.239‡</td>
<td>0.753±0.201†</td>
</tr>
<tr>
<td>Atheroma</td>
<td>0.775±0.196‡</td>
<td>0.242±0.220*</td>
<td>0.186±0.175‡*</td>
</tr>
<tr>
<td>Ulcerated plaque</td>
<td>0.171±0.193†</td>
<td>0.061±0.133†</td>
<td>0.885±0.193†</td>
</tr>
</tbody>
</table>

Data are expressed as means±SD. Differences in standardized coefficients were significant in all four tissue types (P<0.001, ANOVA).

*P<0.01 vs normal artery, †P<0.05 vs atheroma, ‡P<0.001 vs preatheroma.
The boundary between the fibrous cap and the lipid core was defined as the point at which the 3h-oxLDL coefficient reached its plateau during plaque removal. Fibrous cap thickness was determined by calculating the thickness of all plaque removed up to this boundary. The standardized coefficients for collagen and 3h-oxLDL and the CI were plotted retrospectively for each 10 µm of fibrous cap thickness. The standardized coefficients for collagen and 3h-oxLDL and the CI markedly changed at a fibrous cap thickness of <200 μm (Figure 4). There was a significant correlation between the fibrous cap thickness and the CI (r = 0.65, P < 0.001; Figure 4) on logarithmic curve estimation regression.

**Discussion**

We found that (1) the fluorescence spectrum of the lipid core was similar to the spectrum of 3h-oxLDL; (2) the analysis based on collagen, elastin, and 3h-oxLDL spectral decomposition successfully distinguished normal tissue, atheroma, and ulcerated plaque (lipid core); and (3) the CI in atheroma accurately reflected fibrous cap thickness.

**Fluorescence Source of Lipid Core**

The lipid core contains many extracellular matrices and lipids but little collagen and elastin and few vessels and cell components. Sixty percent of atheromatous plaque in dry weight is lipid. More than 50% of the lipids accumulated in atherosclerotic plaque are cholesterol esters, and 15% to 20% are phospholipids. According to the oxidized LDL hypothesis, oxLDL plays a pivotal role in atherosclerosis. Phagocytosis of oxLDL in the arterial wall leads to foam cell formation and atherosclerotic progression. Therefore, oxLDL contributes to the representative fluorophore in the lipid core.

The classification algorithm based on elastin and collagen spectral decomposition accurately differentiated atherosclerotic plaque from normal tissue, despite the moderate lipid

---

**TABLE 2. Discrimination of Plaque Types In Vitro**

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Fluorescence, % (n)</th>
<th>Collagen</th>
<th>Elastin</th>
<th>3h-oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal artery</td>
<td>86.5 (64)</td>
<td>1.4 (1)</td>
<td>1.4 (1)</td>
<td>10.8 (8)</td>
</tr>
<tr>
<td>Preatheroma</td>
<td>10 (1)</td>
<td>60 (6)</td>
<td>0 (0)</td>
<td>30 (3)</td>
</tr>
<tr>
<td>Atheroma</td>
<td>0 (0)</td>
<td>2.7 (2)</td>
<td>95.9 (70)</td>
<td>1.4 (1)</td>
</tr>
<tr>
<td>Ulcerated plaque</td>
<td>3.4 (1)</td>
<td>27.6 (8)</td>
<td>0 (0)</td>
<td>69 (20)</td>
</tr>
</tbody>
</table>

**TABLE 3. Standardized Coefficients of Collagen, Elastin, and 3h-oxLDL for Coronary and Femoral Arteries In Vivo and In Vitro**

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>Elastin</th>
<th>3h-oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal coronary artery (n=14)</td>
<td>0.388±0.125*</td>
<td>0.652±0.125</td>
<td>0.089±0.106</td>
</tr>
<tr>
<td>Normal femoral artery (n=11)</td>
<td>0.072±0.151</td>
<td>0.782±0.179</td>
<td>0.198±0.183</td>
</tr>
<tr>
<td>Atherosclerotic coronary artery (n=6)</td>
<td>0.694±0.240†</td>
<td>0.169±0.189†</td>
<td>0.350±0.364</td>
</tr>
<tr>
<td>Atherosclerotic femoral artery (n=13)</td>
<td>0.559±0.290†</td>
<td>0.190±0.210†</td>
<td>0.540±0.319</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal coronary artery (n=20)</td>
<td>0.012±0.031</td>
<td>0.741±0.285</td>
<td>0.270±0.283</td>
</tr>
<tr>
<td>Normal femoral artery (n=54)</td>
<td>0.068±0.177</td>
<td>0.669±0.301</td>
<td>0.309±0.279</td>
</tr>
<tr>
<td>Atheroma coronary artery (n=16)</td>
<td>0.789±0.186†</td>
<td>0.247±0.234†</td>
<td>0.184±0.196</td>
</tr>
<tr>
<td>Atheroma femoral artery (n=57)</td>
<td>0.771±0.200†</td>
<td>0.241±0.218†</td>
<td>0.186±0.171</td>
</tr>
</tbody>
</table>

*P<0.05 vs normal femoral artery, †P<0.05 vs normal.
content of both tissues. Oraevsky et al. found that laser-induced fluorescence spectra of early lipid-rich noncollagenous lesions show the marked red shifts and broadening spectra compared with the spectra of nonatherosclerotic arteries. These former spectra are similar to the spectrum of oxLDL, with maximum fluorescence intensity at ~430 nm. In the present study, the fluorescence spectra of 16h-, 24h-, and 48h-oxLDL exhibited red shifts, with peak intensities at 430 and 460 nm, but that of 3h-oxLDL exhibited marked red shifts, with a peak intensity at 500 nm and a broadened spectrum. The lipid core also emitted a red-shifted and broadened fluorescence spectrum, which was closer to the spectrum of 3h-oxLDL than to the spectrum of 16h-, 24h-, or 48h-oxLDL.

Oxidized LDL consists of a cholesterol ester–rich core and a more polar coat containing unesterified cholesterol and phospholipids, including lysophosphatidylcholine, with 1 apoprotein B molecule embedded in the surface coat. The oxidative process begins at the surface coat of LDL particles. Therefore, the fluorescence spectrum of 3h-oxLDL resembles the fluorescence spectrum of oxidative products of phospholipids, such as lysophosphatidylcholine. Fluorescence spectra of other 16- to 48h-oxLDLs were not observed in necrotic cores. This extreme oxidation may not occur in vivo. Further study is needed to determine what chemical is responsible for the 3h-oxLDL fluorophore.

**Discrimination of Various Types of Plaques**

Usually, thin and thick intimal segments are both present in human coronary arteries. The thicker intimal segments result from adaptive intimal thickening and contain many smooth muscle cells and elastic fibers. In type IV lesions (atheroma), a dense accumulation of extracellular lipid initially occupies the intima affected by adaptive thickening. The intimal layer above the lipid core gradually changes and thickens by collagen deposition, and collagen becomes the predominant component of type IV lesions. These lesions have increased wall thickness with only a minimal reduction in lumen diameter. Type V lesions are characterized by additional increases in wall thickness, with a progressive reduction in the caliber of the lumen, in which layers of collagen are added to type IV lesions. Consequently, an elastin-dominant fluorescence pattern characterizes normal artery and intimal thickening of adaptation, whereas a collagen-dominant pattern characterizes type V lesions. In type IV lesions, variable amounts of collagen in the intimal layer above the lipid core are responsible for fluorescence spectral patterns. The internal and external elastica laminae also contribute to arterial fluorescence. Therefore, the positive predictive value of atheroma was 95% and that of normal artery was 86% in the present study. The accuracy of our method for distinguishing preatheroma from ulcerated plaque was somewhat low. This low accuracy is apparently related to the minimal thickness or low collagen content of the fibrous cap above the lipid accumulation in preatheroma.

In a previous study of a canine model in which the total amounts of collagen and elastin in arterial segments were measured chemically, the collagen concentration was higher in the coronary artery than in the femoral artery. In our human fluorescence study, the elastin content was higher than the collagen concentration was measured chemically, the collagen concentration was higher in the coronary artery than in the femoral artery. The reason for this discrepancy is unknown. It may relate to differences between elderly human and young canine arteries (ie, as mentioned previously, thickened normal intima contains abundant elastin) or perhaps to the differences in analysis (ie, fluorescence versus chemical). Differences between the present results and those of previous studies may also reflect the presence of collagen-rich connective tissue behind the thin normal coronary arteries.

**Prediction of Fibrous Cap Thickness**

Analysis of chemical composition on the basis of fluorescence appears to depend primarily on brightness and chemical depth,
ie, the distance between the fiber tip and the chemical. The contribution of shallow layers to fluorescence is greater than that of deep layers, and the relationship between this contribution and depth is exponential. The fluorescence of collagen in the top layer of the fibrous cap is greater than that of oxLDL in the lipid pool. When the tissue is evoked by laser with an excitation wavelength of 325 nm, the tissue <200 μm from the surface does not contribute to fluorescence signals.27 Given this sampling depth, the gradual change from an atherosclerotic spectral pattern to a lipid core pattern should also have occurred at a residual fibrous cap thickness of <200 μm. Consistent with this hypothesis, in the present study the standardized coefficients of collagen and 3-h-oxLDL and the CI changed markedly within the fibrous cap at a thickness of <200 μm.

Although we found a correlation between fibrous cap thickness and the CI, there were large variations in the CI and residual fibrous cap thickness of pattern to a lipid core pattern should also have occurred at a residual fibrous cap thickness of <200 μm. Consistent with this hypothesis, in the present study the standardized coefficients of collagen and 3-h-oxLDL and the CI changed markedly within the fibrous cap at a thickness of <200 μm.

Many factors contribute to plaque instability, such as lipid pools, cap thickness, inflammation, and cap fatigue.7,28 A thin fibrous cap covering the lipid pool seems to play a central role in plaque vulnerability. At present, it is unknown to what degree reduction of the thin fibrous cap renders the plaque vulnerable.

**Clinical Implications**

Fluorescence absorption by hemoglobin and carotene may affect fluorescence spectral patterns. The accuracy of plaque differentiation based on 2 fluorescence intensity ratios was greatly affected by reabsorption properties. With the smart laser system, fluorescence recorded without correction for distortion resulted in few successful applications. Wu et al29 developed a sophisticated fluorescence analysis system to distinguish intrinsic fluorescence from distorted fluorescence on the basis of reabsorption. Use of their system with our diagnostic algorithm might improve diagnostic accuracy.

Angioscopy is currently the only technique available to directly observe the endoluminal surface of the coronary arteries in vivo, although there are several problems in clinical application, such as absorption by hemoglobin and distortion of the vascular surface. Technically, optical angioscopic filters can be used to measure fluorescence. A combination of angioscopy and fluorescence analysis, which would allow plaque type to be diagnosed by angioscopy and plaque vulnerability to be concurrently estimated on the basis of fibrous cap thickness, holds promise for clinical application.

**References**


Fluorescence Analysis of Biochemical Constituents Identifies Atherosclerotic Plaque With a Thin Fibrous Cap
Koh Arakawa, Kikuo Isoda, Toshimitu Ito, Kei Nakajima, Toshio Shibuya and Fumitaka Ohsuzu

Arterioscler Thromb Vasc Biol. 2002;22:1002-1007; originally published online April 4, 2002; doi: 10.1161/01.ATV.0000017461.79231.3D

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/6/1002

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/