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Objective—Foam cells perform critical functions in atherosclerosis. We hypothesize that coronary segments with superficial foam cells (SFCs) situated in a region of interest with a depth of 200 μm can be identified using intrinsic fluorescence spectroscopy (IFS) and diffuse reflectance spectroscopy (DRS). This is a key step in our ongoing program to develop a spectroscopic technique for real-time in vivo diagnosis of vulnerable atherosclerotic plaque.

Methods and Results—We subjected 132 human coronary segments to in vitro IFS and DRS. We detected SFCs in 13 thick fibrous cap atheromas and 8 pathologic intimal thickening (PIT) lesions. SFCs colocalized with accumulations of smooth muscle cells and proteoglycans, including hyaluronan ($P<0.001$). Two spectroscopic parameters were generated from analysis of IFS at 480 nm excitation and DRS. A discriminatory algorithm using these parameters identified specimens with SFC area >40%, 20%, 10%, 5%, 2.5%, and 0% of the region of interest with 98%, 98%, 93%, 94%, 93%, and 90% accuracy, respectively.

Conclusion—Our combined IFS and DRS technique accurately detects SFCs in thick fibrous cap atheromas and PIT lesions. Because SFCs are associated with histological markers of plaque erosion, our spectroscopic technique could prove useful in identifying vulnerable plaques. (***Arterioscler Thromb Vasc Biol*. 2006;26:1594-1600.)**

Key Words: foam cells ■ spectroscopy ■ atherosclerosis ■ coronary artery disease ■ human

As a continuation of our work of arterial fluorescence and Raman spectroscopy,[1] we developed an ongoing program targeting the diagnosis of vulnerable atherosclerotic plaques. The main objective of the current study was to demonstrate that a combination of intrinsic fluorescence spectroscopy (IFS) and diffuse reflectance spectroscopy (DRS) can accurately detect human coronary superficial foam cells (SFCs), a potential marker of vulnerable atherosclerotic plaques.

Fibrous cap rupture and plaque erosion are the 2 main causes of coronary thrombosis.[2] Eroded plaques are responsible for at least a third of sudden cardiac deaths, with increased prevalence among young subjects.[2,3] Several studies have demonstrated the presence of macrophages and foam cells in ruptured and eroded vulnerable plaques.[3–5]

Eroded plaques accumulate in their superficial layers macrophages, proteoglycans, smooth muscle cells (SMCs), and extracellular lipids.[2–7] However, whereas ruptured plaques feature a necrotic core and thin fibrous cap, these 2 structures are not necessarily present in eroded plaques.[2,3,6,7]

Hence, an ideal technique for diagnosis of vulnerable plaques prone to rupture or erosion should be able to accurately identify macrophages and macrophage-derived foam cells.

Although existing invasive diagnostic techniques such as intravascular ultrasound, elastography, and intravascular MRI can identify subsurface plaque features such as fibrous cap and necrotic core, it is only thermography and more recently optical coherence tomography that can identify areas of increased macrophage density.[8] However, none of these methods have focused specifically on foam cells, which, through their interaction with oxidized low-density lipoprotein (LDL) particles and complex inflammatory and plaque degrading functions, play a critical role in the vulnerable plaque.[9–12]

We and others have shown previously that laser-induced fluorescence can be used to diagnose atherosclerosis in the aorta and coronary arteries, and a recent study was able to identify macrophages in rabbit aortas.[1,13–15] Foam cells are macrophages with a large content of oxidized lipoproteins, which hold remarkable fluorescent properties[16] and thus are

reasonable targets for fluorescence spectroscopy. IFS, a technique we developed in which DRS is used to remove fluorescence distortions attributable to absorption and scattering,[17] is a more accurate method for distinguishing lipoproteins from other fluorophores present in atherosclerotic plaques. Foam cells also incorporate beta carotene, a lipophilic derivative of vitamin A with antioxidant functions in the macrophage metabolism,[18] which has characteristic diffuse reflectance properties that have been used in the diagnosis of atherosclerotic plaques in animal studies.[19]

Using the fluorescence and absorption properties of oxidized lipoproteins and beta carotene, we demonstrate that our combined IFS and DRS technique can detect SFCs in atherosclerotic coronary arteries. This is a key step in our program to develop a spectroscopic technique for real-time in vivo diagnosis of vulnerable atherosclerotic plaques.

Methods

Subjects and Specimens
The study was approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects and the Cleveland Clinic Foundation's Institutional Review Board, in consultation with the Cuyahoga County Coroner's Office. Specimens were harvested from autopsy cases and hearts explanted during cardiac transplant operations.

Specimens were rinsed in PBS at pH 7.4, snap-frozen, and stored at $-80°C$ within 24 hours after collection. At the time of study, samples were randomly selected, thawed passively, and opened longitudinally. A fiber optic probe was applied in contact with the intima for spectroscopy measurements. Specimens were then ink-marked and processed for histological examination. Details regarding the consistency of spectrographic data before freezing and after thawing the specimens are presented on pages 8 and 11 of the supplemental Methods and Results, available online at http://atvb.ahajournals.org.

Histology
Coronary segments were routinely processed and cut into serial 5-μm-thick sections. Stains were performed to identify SFCs, macrophages, SMCs, proteoglycans, and apoptotic cell nuclei, as described on page 1 of the online supplemental Methods. Slides were examined by a cardiovascular pathologist who used the American Heart Association classification scheme modified by Virmani et al,[6] dividing the coronary segments into: normal, intimal thickening, intimal xanthoma, pathologic intimal thickening (PIT), fibrous cap atheroma, fibrocalcific plaque, and thin fibrous cap atheroma (cap thickness <65 μm).

Foam cells were identified by their foamy cytoplasm. The area occupied by SFCs on each slide was measured in a region of interest with the depth of 200 μm. A detailed explanation for the use of this sampling depth is offered on pages 3 and 4 of the online supplemental Methods. The method used to calculate the SFC area is described on page 1 of the online supplemental Methods.

In sudden coronary death cases, lesions causative of death were retained by the coroner and could not be used for spectroscopy.

IFS and DRS Measurements

Instrumentation
To collect fluorescence and DRS data, we used a FastEEM instrument developed in our laboratory. This unit is equipped with a fiber optic probe for light delivery and collection and has already been used in clinical studies.[17,20,21] The instrument, optical theory, and calibration methods are described on pages 1 and 2 of the online supplemental Methods.

IFS and DRS Basis Spectra Extraction and Validation
Of 11 excitation wavelengths, we chose to use IFS at 480-nm excitation (IFS_{480}) because this wavelength proved the most valuable with respect to SFC detection. IFS_{480} data were modeled by a linear combination of 3 IFS_{480} basis spectra extracted by means of multivariate curve resolution analysis (described on page 5 of the online supplemental Methods) of spectra belonging to 3 sets of coronary tissues: $FCAP_{spectrum}$ from coronary fibrous cap layers free of foam cells (n=4), $NC_{spectrum}$ from necrotic core (n=4), and $SFC_{spectrum}$ from whole coronary specimens rich in SFCs and lacking necrotic core and calcifications (n=4). The fibrous cap and necrotic core specimens were obtained through blunt dissection of coronary lesions.

To demonstrate the specificity of $SFC_{spectrum}$ and identify the main fluorescent molecules responsible for the 3 basis spectra, we compared the latter with spectra of various arterial tissue types and of high-purity chemical compounds, as described on pages 5 and 6 of the online supplemental Methods.

The 2 known absorbers in coronary arteries are oxyhemoglobin and beta carotene,[19] and their spectra were obtained respectively from http://omlc.ogi.edu/spectra/hemoglobin/index.html (Dr Scott Prahl, Oregon Medical Laser Center, Portland, Ore) and from breast tissue measurements. Breast tissue was selected for measurement of the beta carotene spectrum because beta carotene has an environment-sensitive spectrum and is insoluble in aqueous media, and the breast tissue is rich in fat and nearly lacks hemoglobin.

IFS and DRS Modeling
Modeling IFS_{480} and DRS allowed us to extract the contributions of the 3 basis spectra to IFS_{480} ($SFC_{contribution}$, $FCAP_{contribution}$, and $NC_{contribution}$) and those of beta carotene and oxyhemoglobin to DRS. Details regarding the IFS and DRS modeling and its goodness of fit are offered on pages 6 and 11 of the online supplemental Methods and Results.

Analysis Methods and Diagnostic Algorithms
We used 3 diagnostic algorithms with the purpose of identifying thick fibrous cap atheromas and PIT lesions with SFCs. The 3 intimal xanthomas with SFCs are not vulnerable plaques, and for this reason, we included them in the group of normal specimens and intimal thickening lesions. One algorithm used as diagnostic variable the $SFC_{contribution}/FCAP_{contribution}$ ratio, the second used beta carotene contribution, whereas a third used the combination of these parameters. We chose the ratio $SFC_{contribution}/FCAP_{contribution}$ because it reflects the balance between the 2 pathological features that confer instability and respectively stability to the atherosclerotic plaque: SFCs and fibrous cap. To analyze the data set in an unbiased manner, leave-one-out cross-validation and logistic regression were used as described on page 7 of the online supplemental Methods.[20]

Results
Coronary specimens were randomly selected after being harvested from 17 human hearts obtained during postmortem autopsy (9 sudden cardiac death and 3 nonsudden cardiac death cases) or heart transplant surgery (5 cases of ischemic cardiomyopathy).

Histology
There were a total of 132 specimens: 40 normal and intimal thickening lesions, 4 intimal xanthomas, 24 PIT, 49 fibrous cap atheromas, 0 thin fibrous cap atheromas, and 15 fibrocalcific plaques. We detected SFCs in a total of 21 thick segments (13 fibrous cap atheromas and 8 PIT) and 3 intimal xanthomas. Histological characteristics of the 21 thick coronary specimens with SFCs and remaining segments are presented in Table 1. As illustrated by the PIT specimen presented in Figure 1, combined accumulations of superficial proteoglycans, SMCs, and extracellular lipids in the region of

TABLE 1.   Histological Characteristics of Coronary Segments

Tissue Structure Inside Region of Interest	Thick Fibrous Cap Atheroma and PIT Lesions With SFCs (n=21)	Remaining Segments (n=111)	P
Increased proteoglycans, n (%)	20 (95%)	26 (23%)	<0.0001
Increased SMCs, n (%)	16 (76%)	27 (24%)	<0.001
Extracellular lipids, n (%)	21(100%)	39 (35%)	<0.0001
Combined structures, n (%)*	15 (71%)	12 (11%)	<0.0001
Deep fibrous intima, n (%)	21 (100%)	63 (57%)	0.01
Increased hyaluronan, n (%)	13/16† (81%)	...	...
Macrophages, n (%)	21 (100%)	...	...
Apoptotic SFCs, n (%)	16 (76%)	...	...
Apoptotic SMCs, n (%)	12 (57%)	...	...

*Proteoglycans, SMCs, and extracellular lipids; †cuts with SFCs available for hyaluronic acid binding protein staining in only 16 specimens.

interest were present in 15 of 21 (71%) thick specimens with SFCs and only 12 of 111 (11%) of the remaining segments ($P<0.0001$). SFCs were macrophages as demonstrated by the anti–HAM-56 immunostaining (supplemental Figure Ia and Id, available online at http://atvb.ahajournals.org). Staining with hayluronic acid binding protein was performed in 16 of

the 21 thick specimens with SFCs. Sections with SFCs were not available for this stain in 5 specimens because of numerous previous cuts. Hyaluronan colocalized with SFCs in 13 of 16 segments (81%; supplemental Figure Ic and If).

SFC apoptosis was detected by TUNEL stain in 16 of 21 specimens (76%), and apoptosis of SMCs was identified in 12
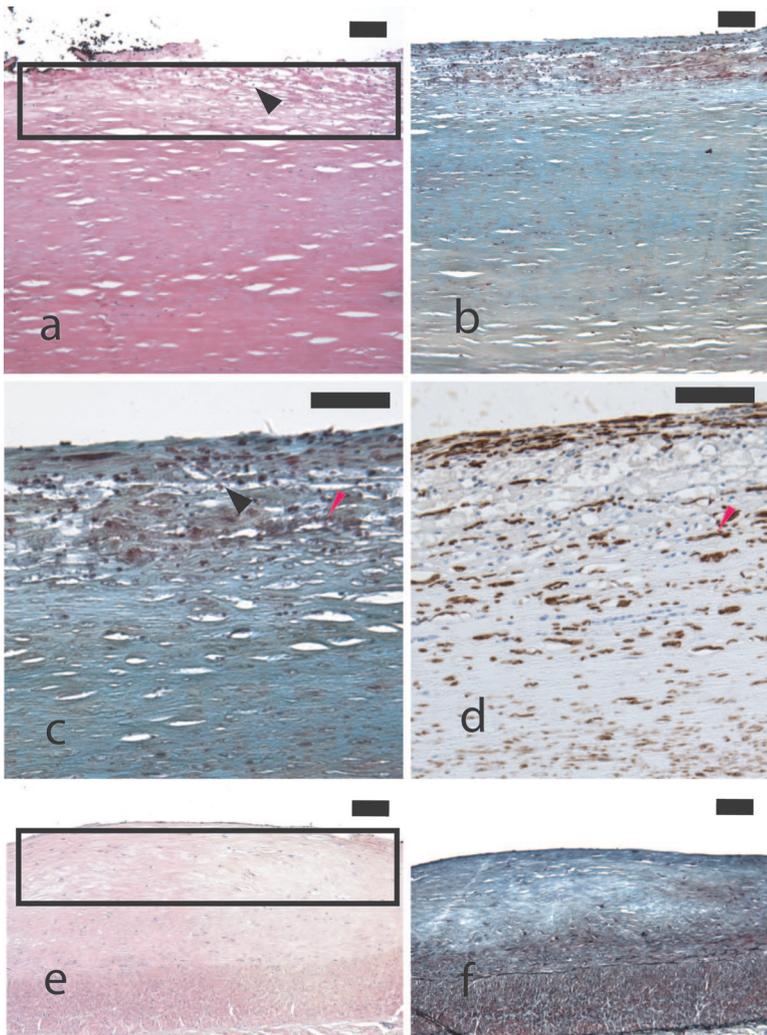

Figure 1. PIT lesion "A" features numerous SFCs: hematoxilin and eosin (a), Movat's pentachrome (b and c), and anti–smooth muscle α-actin (d). b, the differential accumulation of proteoglycans (blue) around SFCs and a deep collagenous intima (yellow). Proteoglycans (blue) and SMCs (dark red) are spatially associated with SFCs (c). d confirms numerous and large SMCs (brown), adjacent to SFCs. Specimen "B" with no SFCs shows no superficial accumulation of proteoglycans or SMCs: hematoxilin and eosin (e) and Movat's pentachrome (f). Magnifications: ×10 (a, b, e, and f) and ×20 (c and d). Black and red arrows point to SFCs and SMCs, respectively. Black rectangles indicate region of interest. Bars=100 μm.
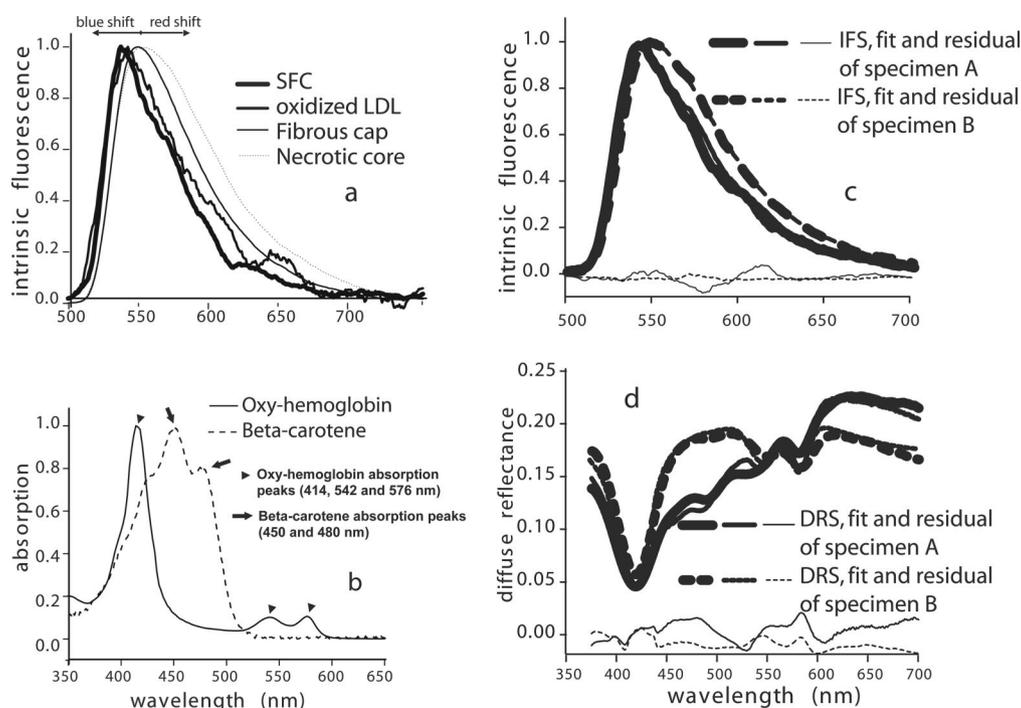
Figure 2. IFS basis spectra at 480 nm excitation of SFCs, fibrous cap, and necrotic core compared with that of LDL oxidized with the myeloperoxidase/H_2O_2/NO_2^- system for 22 hours (a). Extinction spectra of beta carotene and oxyhemoglobin (b). IFS_{480} (c) and DRS spectra (d), plotted with fits and residuals, of coronary plaques illustrated in Figure 1.

of 16 (75%) of the coronary specimens with SFCs that featured accumulations of SMCs (supplemental Figure Ib and Ie). A deep fibrous intima was identified in all thick fibrous cap atheromas and PIT specimens with SFCs (Figure 1b).

IFS and DRS Measurements

IFS and DRS Basis Spectra Extraction and Validation

The IFS_{480} basis spectra ($FCAP_{spectrum}$, $NC_{spectrum}$, and $SFC_{spectrum}$) are illustrated in Figure 2a. $SFC_{spectrum}$ was shifted toward the blue region of the spectrum when compared with $FCAP_{spectrum}$. In contrast, $NC_{spectrum}$ was red-shifted when compared with $FCAP_{spectrum}$, in agreement with our previous studies of necrotic core fluorescence.[1]

The spectrum of LDL oxidized for 22 hours with myeloperoxidase was nearly equivalent to $SFC_{spectrum}$ (Figure 2a). Spectra of all other chemical compounds possessed lineshape features distinct from $SFC_{spectrum}$. More results regarding the chemical etiology of the basis spectra and specificity of the $SFC_{spectrum}$ are offered on pages 9 and 10 of the online supplemental Results.

To fit the DRS data, we used the extinction spectra of beta carotene and oxyhemoglobin (Figure 2b). To prove that the contribution of beta carotene to DRS is generated mainly by SFCs, we compared this parameter in the 3 sets of coronary tissue structures used to extract the IFS_{480} basis spectra. The beta carotene contribution ranged between 16.2 and 62.3 a.u. in segments rich in SFCs, greater than in necrotic core (4.6 to 14.7 a.u.) or fibrous cap (0 to 7.8 a.u.; Figure 3b). Figure 3a illustrates the average DRS spectra of the 3 sets of tissues.

Diagnostic Algorithms

We used 3 diagnostic algorithms to identify fibrous cap atheromas and PIT lesions with SFCs. Each algorithm used either the ratio $SFC_{contribution}$/$FCAP_{contribution}$ or beta carotene contribution as diagnostic variables or a combination of both parameters. These algorithms were designed to identify subgroups of specimens defined by SFC area greater than 6 cutoff thresholds, representing fractions of the region of interest set at intervals increasing in a doubling fashion: 0%, 2.5%, 5%, 10%, 20%, and 40%. Table 2 lists the sensitivity and specificity of the combined 2-parameter algorithm and compares the accuracy values obtained by each of the 3 algorithms. Figure 4 illustrates the decision areas for identifying fibrous cap atheromas and PIT lesions with SFC area >40%, 10%, and 0% of the region of interest.

Discussion

In this study, we report for the first time the development of a bimodal spectroscopy technique based on a combination of IFS and DRS for identifying human coronary segments with features of vulnerable plaques. Preliminary data regarding the spectroscopic identification of SFCs were published by our group in 2003.[22]

Fibrous cap rupture and plaque erosion are the 2 main types of vulnerable plaques. Along with SMCs and proteoglycans, and especially hyaluronan,[2,6,7] macrophages in the superficial region of eroded plaques play a critical role in their vulnerability.[3–5]

Foam cells are activated macrophages that have stored oxidized LDL particles. They represent the landmark cellular feature of the coronary plaque, possessing important degrading and inflammatory functions.[9–11] We found SFCs, defined as those foam cells situated in the top 200 μm of the plaque, in 24 of the specimens studied, of which 21 were either fibrous cap atheromas or PIT. All 21 specimens were coronary segments in
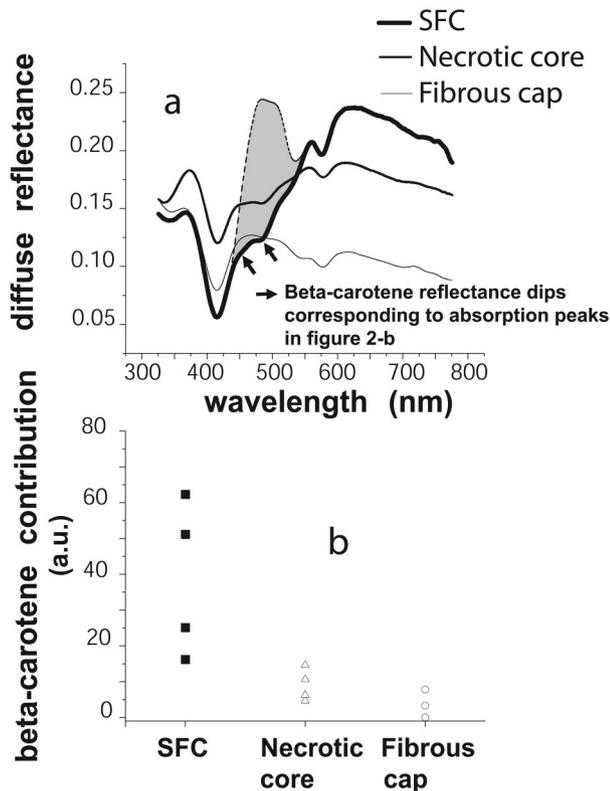
Figure 3. Average diffuse reflectance spectra of 3 sets of coronary layers (a). Note the absorption signature of beta carotene in the SFCs and necrotic core spectra. The grayed area indicates the imprint of beta carotene absorption, obtained by comparison with a hypothetical diffuse reflectance spectrum where the beta carotene absorption has been removed (dashed line). Contribution of beta carotene to DRS in the individual specimens used to generate the average spectra (b).

advanced stages of atherosclerosis, with a thickness of up to 1300 μm and a deep fibrous intima (Figure 1b).

Association Between SFCs and Erosion-Prone Plaques

Macrophages are an important characteristic of the erosion-prone plaques, as demonstrated by several important studies.[3–5] We hypothesized that SFCs are associated with this type of plaques as well. Using anti–HAM-56 immunostaining, we demonstrated that SFCs were all macrophage derived (supplemental Figure Ia and Id), suggesting that SFCs are important players in the process of erosion. Well known mechanisms that may directly involve SFCs are matrix degradation

and endothelial denudation.[10,12] Also, recently emerged data point to the fact that apoptotic foam cells promote platelet aggregation through the release of tissue factor,[23] which could represent an important step in the thrombus-formation stage of the erosion process. Seventy-six percent of the thick specimens with SFCs in our study showed SFC apoptosis (Table 1; supplemental Figure Ib and Ie).

In addition to direct mechanisms, we suspect that SFCs participate in the erosion process via indirect pathways that involve interactions with surrounding proteoglycans and SMCs. We were able to demonstrate that SFCs colocalized in thick fibrous cap atheromas and PIT specimens with SMCs and proteoglycans, including hyaluronan (Figure 1; Table 1; supplemental Figure Ic and If). These are histological features that have been shown by Virmani et al to be pathognomonic for erosions.[2,6,7] The spatial association between SFCs and accumulations of proteoglycans and SMCs is in agreement with reports that demonstrate close interactions between these structures.[24–28] Importantly, Wight et al demonstrated that hyaluronan is closely associated with macrophages in atherosclerotic lesions.[25] It is also known that SMCs can proliferate and undergo apoptosis while coming in contact with macrophages and foam cells, as illustrated in our thick coronary specimens containing SFCs (Table 1; supplemental Figure Ib and Ie).[24,28] It is believed that hyaluronan promotes erosion by ways of receptor-mediated platelet adhesion and inflammatory cell recruitment,[7] and that SMC apoptosis causes the release of tissue factor, a key element of the extrinsic coagulation cascade.[29] We therefore suspect that SFCs, in addition to their direct actions, may promote plaque erosion through indirect mechanisms that involve the accumulation of proteoglycans, especially hyaluronan,[2,7,26,27] and SMC apoptosis.[28,29]

Detection of SFCs in Fibrous Cap Atheromas and PIT Lesions

Having demonstrated an association between SFCs and erosion-prone plaques, we assessed the capability of a bi-modal method of IFS$_{480}$ and DRS as a tool for SFC detection. We showed an exclusive similarity between SFC$_{spectrum}$ and the spectrum of LDL oxidized for 22 hours with myeloperoxidase, as illustrated in Figure 2a and detailed on pages 9 and 10 of the online supplemental Results. We also demonstrated that SFCs, along with necrotic core layers in lesser degree, were the structures responsible for the beta carotene absorption effects seen in DRS (Figure 3). We suspect that beta carotene contribution was higher in SFCs than in

TABLE 2.   Sensitivity, Specificity and Accuracy for Identifying Thick Fibrous Cap Atheromas and PIT Lesions With SFCs Using Two Discriminatory Variables, Combined and Alone

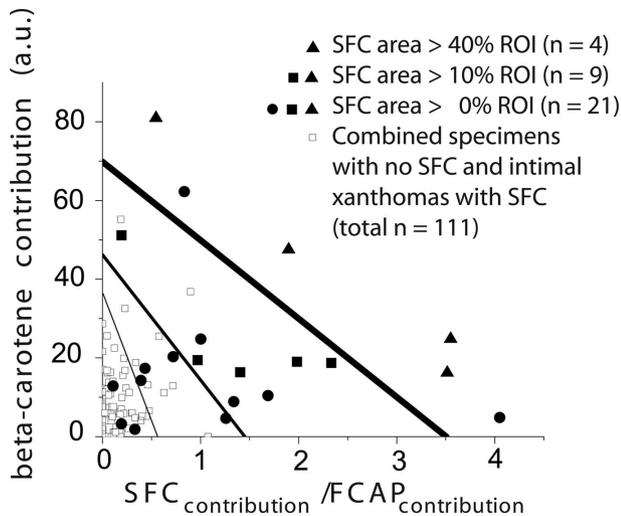| Discriminatory Variables	Diagnostic Characteristics	SFC Area Threshold (% of region of interest)					
		40%	20%	10%	5%	2.5%	0%
SFC$_{contribution}$/FCAP$_{contribution}$	Specificity	98%	98%	93%	93%	93%	91%
combined with	Sensitivity	100%	80%	100%	100%	100%	86%
beta carotene contribution	Accuracy	98%	98%	93%	94%	93%	90%
SFC$_{contribution}$/FCAP$_{contribution}$	Accuracy	96%	95%	87%	89%	91%	89%
beta carotene contribution	Accuracy	89%	88%	83%	85%	86%	70%

Figure 4. A binary diagnostic plot identifies thick coronary specimens with SFC area greater than 3 percentages of the region of interest (ROI). The thick, intermediate, and thin lines represent diagnostic borders that make the separation between each of the 3 groups of thick lesions and the remaining specimens (for example, the intermediate line makes the separation between 9 thick specimens with SFC area >10% and the remaining 123 segments).

necrotic core layers because beta carotene, a moiety with known antioxidative properties, while being initially stored in foam cells,[18] is subsequently consumed in the highly oxidative milieu of the necrotic core.

Excellent accuracy values were obtained for the detection of SFCs in thick fibrous cap atheromas and PIT lesions using a combination of 2 variable parameters (Table 2; Figure 4). The accuracy was better when using the 2 spectroscopic parameters combined ($SFC_{contribution}/FCAP_{contribution}$ and beta carotene contribution) than separately (Table 2). At the same time, improved accuracy was obtained for increasing threshold values of SFC area (Table 2). This could be of particular importance because the magnitude of the SFC content might be an important factor in plaque rupture and erosion. Since a study to investigate the critical threshold of the SFC content in these processes has not yet been published, we can only speculate that improved accuracy in identifying plaques with higher SFC content will reflect into improved accuracy of recognizing plaques with a higher degree of vulnerability.

Limitations

We selected the segments investigated at random, which gave us the opportunity of comparing segments with SFCs with a wide variety of coronary specimens. However, we did not find thin fibrous cap atheromas among the specimens studied. Thin fibrous cap atheromas feature SFCs, a thin fibrous cap, and necrotic core.[2] Although our study did not investigate this particular type of lesion, $SFC_{spectrum}$ and $NC_{spectrum}$ have distinct features (Figure 2a), which creates the potential for simultaneous detection of SFCs and necrotic core in thin fibrous cap atheromas.

In this study, we focused on identifying 21 thick fibrous cap atheromas and PIT lesions featuring SFCs, proteoglycans, and SMCs. However, in the group of specimens with no

SFCs, there were 26 segments that harbored increased proteoglycans (Table 1). Our method could not detect these specimens. It is conceivable that specimens with proteoglycans could erode even in the absence of macrophages and SFCs.[2,6,7] Our laboratory is studying an alternative excitation wavelength that would detect this type of lesions. However, it is possible that the combination of SFCs, proteoglycans, and SMCs poses a higher risk of erosion than any of these features taken alone. The method investigated in this article provides a basis for identifying this combination.

Conclusion and Plans for Future Studies

The FastEEM instrument is equipped with a thin fiber optic probe suitable for coronary artery studies. This instrument has already been used in in vivo clinical studies in other organ systems.[17,20,21] We have now used the bimodal spectroscopy technique combining IFS_{480} and DRS to detect SFCs in coronary artery plaques with features associated with plaque vulnerability in vitro. The IFS technique combines fluorescence and diffuse reflectance data to eliminate the spectral distortion produced by blood absorption.[20] Data acquisition is almost instantaneous, which makes the method clinically robust. For these reasons, we believe that IFS will be particularly useful in vivo, in which blood absorption, flow of blood, and organ motion are serious impediments for other spectroscopy and imaging methods. We are optimistic that our bimodal spectroscopy technique can be successfully developed for real-time in vivo diagnosis of vulnerable atherosclerotic plaques in the future.

Disclosures

None.

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