Histopathology of Human Coronary Atherosclerosis by Quantifying Its Chemical Composition With Raman Spectroscopy

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Background—Lesion composition, rather than size or volume, determines whether an atherosclerotic plaque will progress, regress, or rupture, but current techniques cannot provide precise quantitative information about lesion composition. We have developed a technique to assess the pathological state of human coronary artery samples by quantifying their chemical composition with near-infrared Raman spectroscopy.

Methods and Results—Coronary artery samples (n=165) obtained from explanted recipient hearts were illuminated with 830-nm infrared light. Raman spectra were collected from the tissue and processed to quantify the relative weights of cholesterol, cholesterol esters, triglycerides and phospholipids, and calcium salts in the examined artery location. The artery locations were then classified by a pathologist and grouped as either nonatherosclerotic tissue, noncalcified plaque, or calcified plaque. Nonatherosclerotic tissue, which included normal artery and intimal fibroplasia, contained an average of 4±3% cholesterol, whereas noncalcified plaques had 26±10% and calcified plaques 19±10% cholesterol in the noncalcified regions. The average relative weight of calcium salts was 1±2% in noncalcified plaques and 41±21% in calcified plaques. To make this quantitative chemical information clinically useful, we developed a diagnostic algorithm, based on a first set of 97 samples, that demonstrated a strong correlation of the relative weights of cholesterol and calcium salts with histological diagnoses of the same locations. This algorithm was then prospectively tested on a second set of 68 samples. The algorithm correctly classified 64 of these new samples, thus demonstrating the accuracy and robustness of the method.

Conclusions—The pathological state of a given human coronary artery may be assessed by quantifying its chemical composition, which can be done rapidly with Raman spectroscopic techniques. When Raman spectra are obtained clinically via optical fibers, Raman spectroscopy may be useful in monitoring the progression and regression of atherosclerosis, predicting plaque rupture, and selecting proper therapeutic intervention. (Circulation. 1998;97:878-885.)

Key Words: spectroscopy ■ diagnosis ■ atherosclerosis

The progression and regression of atherosclerotic plaques appear to be related to the amount and type of lipids that accumulate in the intima. Recent studies have shown that plaque composition, rather than size or volume, determines whether an arterial narrowing will rupture and cause an acceleration of clinical symptoms. Although several therapies to treat atherosclerosis are available, diagnostic methods that reliably document or predict plaque development or instability have not yet been developed. Cardiac risk factors do not predict how existing disease will progress. Angiographic techniques can quantify lumen narrowing and lesion volume and recognize complications such as dense calcification or thrombosis but do not provide detailed chemical information about the lesion. Mildly calcified plaques may be missed. Although intravascular ultrasound does yield some chemical information in addition to morphological information, it cannot identify specific molecules and lacks the capability for quantitative chemical analysis. Furthermore, it is unable to accurately distinguish noncalcific atherosclerosis from nonatherosclerotic intimal thickening because of its low sensitivity in detecting lipid pools. MRI may allow the discrimination of large lipid pools, calcifications, and fibrous caps in atheromatous plaques but cannot quantify the chemical composition. Directional coronary atherectomy yields some histological information, but it is destructive and applicable only to advanced lesions. Because important chemical changes, which may predict disease progression, occur in the early, asymptomatic phases of the atherosclerotic process, athero-
tomy has limited diagnostic value. Clinical techniques capable of monitoring the chemical composition of atherosclerotic lesions in a safe and valid manner are needed to select and evaluate the effects of various interventional therapies and to advance epidemiological and clinical research relating to the pathophysiology of atherosclerosis.

Several groups have used optical spectroscopy to characterize arterial disease in situ.1–8 By delivering excitation light and collecting emitted light through flexible optical fibers, fluorescence spectra were collected from coronary artery and used to differentiate normal tissue from abnormal tissue.9,10 However, fluorescence spectra provide little quantitative chemical information, mainly because of the limited differences in the fluorescence spectra of many chemical compounds. Raman spectroscopy of tissue yields more chemical information because the Raman spectra of biological compounds are unique. For instance, Fourier-transform Raman spectroscopy has been used to study human aorta, and the spectral features of specific chemicals have been used to identify normal, intimal hyperplastic, and atherosclerotic plaques.21–25 Raman spectra were obtained from homogenized coronary artery samples and processed to calculate the relative weights (in percent) of FC, CE, TG, and PL, and CS in the examined volume of arterial tissue. These chemical weights agreed with those determined by standard lipid assays and CS assays to within a few percent.24 Furthermore, it has been demonstrated that Raman spectra from arterial wall can be collected in vivo via optical fiber probes in signal collection times of only a few seconds. These studies indicate that spectra obtained in vivo have signal-to-noise ratios sufficiently high to calculate chemical weights.25 These results suggest that similar chemical analyses can be performed on intact coronary arteries in vivo.

The objectives of the present study were to apply these Raman spectroscopy techniques to intact (nonhomogenized) coronary arteries, obtain quantitative chemical information from intact artery wall with Raman spectroscopy, and correlate this information with histological diagnoses that are based on arterial wall morphology. We developed an algorithm that uses the relative weights of arterial chemical components to classify coronary artery into three diagnostic classes: (1) nonatherosclerotic samples (class I), (2) noncalcified plaques (class II), and (3) calcified plaques (class III). This algorithm was used with the discriminant analysis technique of logistic regression. We used logistic regression to determine the diagnostic utility of each chemical component. Using the relative weights of these components, we calculated the probability that an artery location belonged to one of the three diagnostic classes. This algorithm was then tested prospectively on a second set of data.

**Methods**

**Tissue Preparation**
To minimize the effects of tissue degradation, human coronary artery samples (n=165) were obtained from 16 explanted recipient hearts within 1 hour after heart explantation. Seven patients had dilated cardiomyopathy and 9 had heart failure due to severe ischemic heart disease. In general, coronary arteries obtained from the hearts of patients with dilated cardiomyopathy did not display atherosclerotic plaque, whereas arteries from hearts of patients with ischemic heart disease exhibited advanced stages of atherosclerosis. Immediately after dissection from the explanted heart, the artery samples were rinsed with PBS solution, snap-frozen in liquid nitrogen, and stored at −85°C until use. The artery samples were collected in two sets, the first containing 97 samples and the second 68. The second set was collected after the diagnostic algorithm was developed to test the algorithm prospectively. Samples were passively warmed to room temperature, and locations on the intimal surface of each artery sample were selected for spectroscopic examination by visual inspection under ×10 magnification. After being examined spectroscopically, each artery location was marked with a spot of colloidal ink (~1 mm in diameter). The samples were then fixed in 10% formalin.

**Histology**
The artery samples were processed routinely and cut through the marked locations into 5-μm-thick sections. When necessary, samples were partially decalified by acid extraction before sectioning. The sections were stained with hematoxylin and eosin and examined with light microscopy by two experienced cardiovascular pathologists who were blinded to the outcome of the Raman spectroscopy analysis. Each set was examined by a different pathologist. The tissue sections were classified by the changes that occur within the intima and media of the artery wall according to the updated systemized nomenclature of human and veterinary medicine (SNOMed).26 The samples of the first and second sets, respectively, were classified as either (1) normal (n=12 and n=1), (2) intimal fibroplasia (n=61 and 25), (3) atherosclerotic plaque (n=3 and 0), (4) atheromatous plaque (n=6 and 16), (5) calcified atherosclerotic plaque (n=1 and 3), (6) calcified atheromatous plaque (n=7 and 13), (7) calcified fibroatheromatous plaque (n=5 and 10), or (8) calcified intimal fibroplasia (n=2 and 0). Because some of these categories had small sample numbers, three diagnostic classes were defined for the development of a diagnostic algorithm: class I, nonatherosclerotic samples (categories 1 and 2); class II, noncalcified plaques (categories 3 and 4); and class III, calcified plaques (categories 5 through 8). Three samples were histologically classified as noncalcified fibroatheromatous plaque, which is an end stage of atherosclerosis characterized by intimal sclerosis, loss of lips, and foam cells. Generally, these plaques are nonobstructive and rarely cause clinical symptoms.20 They appeared in our data set probably because many arteries were harvested from hearts with severe ischemic heart disease, which increased the likelihood of encountering these lesions. Because these plaques are rare and not clinically important, we did not consider them in the present study.

**Instrumentation and Chemical Analysis**
The experimental apparatus used to collect the Raman spectra from the tissue has been described previously.21 Coronary artery locations were illuminated in an ~100-μm-diameter location with ~350 mW of 830-nm infrared light provided by an Ar+–pumped Tisapphire laser system. A spectrograph/CCD system was used to collect 8-cm−1 resolution Raman spectra in 10 to 100 seconds over the Raman shift range of 400 to 2000 cm−1. The irradiated location did not show any signs of injury during microscopic examination. In addition, no changes to the spectra due to tissue irradiation were observed.

Each tissue spectrum was frequency calibrated and corrected for chromatic variations in spectrograph system detection. A fourth-order polynomial was fit to each spectrum by least-squares mini-

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**Selected Abbreviations and Acronyms**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CE</td>
<td>cholesterol esters</td>
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<tr>
<td>CS</td>
<td>calcium salts</td>
</tr>
<tr>
<td>DA</td>
<td>delipidized arterial tissue</td>
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<td>FC</td>
<td>free cholesterol</td>
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<td>NCR</td>
<td>noncalcified regions</td>
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<td>NIR</td>
<td>near infrared</td>
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<td>PL</td>
<td>phospholipids</td>
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mization and subtracted from the spectrum to leave Raman spectral features. These spectra were modeled as a linear combination of spectra from seven individual chemical components: FC, CE, CS, TG and PL, β-carotene, and DA. The DA spectra were used to model the spectral contribution of all nonlipid, nonmineral components of arterial wall. The individual DA spectra were obtained from the remnants (primarily proteins) of the delipidized artery samples, one that appeared nonatherosclerotic and another that appeared calcified by visual inspection. All spectra in our two data sets could be modeled accurately with the set of spectra from the seven individual chemical components. The Raman spectral model was used to calculate the relative weights (in percent) of six of the chemical components at each selected artery location. The seventh component, β-carotene, is an intense Raman scatterer and sometimes contributes spectral features to coronary artery spectra, although it is present in minuscule quantities. Therefore, its spectrum was included in the spectral model but not used for calculating its relative weight. Consequently, its spectral contribution is not expressed as relative weight but as arbitrary units. Details of the model and its validation with standard chemical assays have been described previously.

In calcified plaques, CS occupied a large part of the tissue volume examined by Raman spectroscopy. To obtain information about the remaining noncalcified tissue, we renormalized the chemical fractions in the NCR, eg,

\[ FC_{NCR} = \frac{FC}{FC+CE+TG&PL+DA} \]

Similarly, the fractions of CE, DA, and TG and PL were renormalized to exclude the relative weight of CS. The TC in the NCR is

\[ TC_{NCR} = FC_{NCR} + CE_{NCR} \]

Algorithm Development and Statistical Analysis
The relative weights of the chemical components calculated with Raman spectra obtained from the first sample set were used to develop an algorithm to classify the tissue into nonatherosclerotic tissue (class I), noncalcified plaque (class II), or calcified plaque (class III). Because the relative weights of the measured chemical components do not appear to be normally distributed, logistic regression was used to analyze the data. Ternary logistic regression was used to generate discriminant scores, \( R_i \), based on a linear combination of weights (\( C_j \)) of chemical component \( j \) as

\[ R_i = \alpha + \beta_1 C_1 + \beta_2 C_2 + \ldots \]

where \( \alpha \) is a constant and \( \beta_j \) is an adjustable coefficient for each component. The probability \( P_i \) that an artery location belongs to one of the three diagnostic classes, \( k \), can be estimated as:

\[ P_i = \frac{e^{R_i}}{1 + e^{R_i}} \]

which sum to one. The variance of a score, \( R_i \), can be calculated as:

\[ \text{var}(R_i) = \sigma_\alpha^2 + \sum_j (C_j \sigma_{\beta_j})^2 + \sum_j (2 \sigma_\alpha \beta_j C_j) + \ldots \]

with \( \sigma_\alpha \) and \( \sigma_{\beta_j} \) the standard deviation of \( \alpha \) and \( \beta_j \) and \( \sigma_{\beta_j} \) the covariance between \( \alpha \) and \( \beta_j \). The algorithm was then used to classify prospectively the artery locations of the second sample set into the three diagnostic classes.

Results
Comparison With Histopathology
Typical NIR Raman spectra from coronary arteries representing each of the diagnostic classes (I, II, and III) are shown in Figs 1 through 3. The spectra provide distinct features for the determination of the chemical composition and the diagnostic classification of arterial wall.

Figure 1A shows a spectrum from an arterial wall location exhibiting intimal fibroplasia (class I) and the result of the
least-squares fit of the chemical component spectra. The bottom curve displays the residual, obtained by subtracting the model fit from the artery spectrum. This spectrum is dominated by TG and protein features visible at ≈1650, 1250, and 1450 cm\(^{-1}\) Raman shifts. The TGs located in the adventitial layer are stronger Raman scatterers than the proteins in the intima and media. Therefore, the TG features are more prominent than the protein features, although in the spectroscopically examined volume, the relative weight of TG is lower than that of proteins. The relative weights of proteins, lipids, and CS were calculated from this spectrum (Fig 1A), and the results were compared with the morphology at the location marked with colloidal ink. The microscopic section of this artery wall location (Fig 1B) shows that the intima and media are primarily composed of protein fibers (collagen and elastin) and smooth muscle cells (actin and myosin), indicating consistency between the morphological constituents and the calculated chemical composition.

In the spectrum of an atheromatous plaque (class II) shown in Fig 2A, spectral features from the sterol rings of FC and CE can be recognized. Significant relative weights of FC and CE were calculated from this spectrum, which is again consistent
with the morphology of the microscopic section shown in Fig 2B and 2C. In these sections, one can see many lipid-laden foam cells and FC crystals.

Raman spectra obtained from calcified plaque are distinguishable by the symmetrical stretch vibration of phosphate (960 cm\(^{-1}\)) found in CS, mainly calcium hydroxyapatite. A large relative weight of CS was calculated from the spectrum of a highly calcified atheromatous plaque (class III) shown in Fig 3. The microscopic section of this location showed traces of large mineral deposits, even after acid decalcification.

**Chemical Analysis**

For each artery wall location of samples in the first set, the chemical components are plotted versus the eight histological categories (Fig 4). Fig 4A shows that TC NCR in nonatherosclerotic tissue (categories 1 and 2) is lower than that in noncalcified plaques (categories 3 and 4) and calcified plaques (categories 5 through 7). Noncalcified plaques have the highest FC NCR (Fig 4B), and Fig 4C shows that CE NCR increases as atherosclerosis progresses. Fig 4D shows that the relative weight of CS in calcified plaques is elevated. On average, DA NCR (Fig 4E) is not much different among the eight histological categories. TG and PL NCR (Fig 4F) decreases as atherosclerosis progresses because as the intima and media thicken, the adventitial layer is further away from the luminal surface and thus contributes less to a spectroscopically examined volume of tissue than in nonatherosclerotic tissue. Fig 5 shows that the spectral contribution of carotenoids, expressed in arbitrary units, is increased in atherosclerotic and atheroma-tous tissue (categories 3 and 4).

The mean ± SD of the relative weight of each chemical component in nonatherosclerotic tissue (I), noncalcified plaque (II) and calcified plaque (III) is listed in Table 1. A Wilcoxon test showed that the difference in TC NCR between nonatherosclerotic tissue and noncalcified plaque was highly statistically significant (\(P < .001\)). The difference in the relative weight of CS between noncalcified and calcified plaque was also found to be highly statistically significant (\(P < .001\)).

**Diagnostic Algorithms**

Ternary logistic regression determined that TC NCR and the relative weight of CS of artery locations in the first sample set...
could be used to classify an artery location as either nonatherosclerotic tissue (class I), noncalcified plaque (class II), or calcified plaque (class III). The regression coefficients, along with their standard errors and statistical significance, are given in Table 2. Discriminant scores were found to be $R_1 = -13.1 + 1.1 \times TCNCR$ and $R_2 = -20.0 + 2.5 \times CS + 0.7 \times TCNCR$. The $TCNCR$ and the relative weight of $CS$ of each artery can be plotted in a two-dimensional space, where $TCNCR$ is the horizontal axis and $CS$ is the vertical axis (Fig 6A). Each point in this space has a corresponding value of $R_1$ and $R_2$ from which the probability that a sample belongs to one of the three diagnostic categories can be assigned. The border separating the regions of nonatherosclerotic tissue and noncalcified plaque in Fig 6 is given by $P_1 = P_{11}$, which is a vertical line at $TCNCR = 12 \pm 1\%$. The error of 1% was calculated from the standard deviation of $R_1$. The border separating the regions of nonatherosclerotic tissue and calcified plaque is given by $P_1 = P_{1II}$, which is a line described by the equation $CS = 12.0 - 0.44 \times TCNCR$, and the border separating noncalcified plaques from calcified plaque is determined by $P_2 = P_{1II}$, which yields the equation for a line $CS = 4.1 + 0.2 \times TCNCR$.

The algorithm, developed with the first sample set, was then used prospectively to classify the artery locations of the second sample set into one of the three diagnostic classes (Fig 6B). Prospectively, the decision of the algorithm agreed with that of the pathologist for 64 of 68 samples.

In this study, we have shown that the pathological state of human coronary artery can be assessed by quantifying its chemical composition with Raman spectroscopy techniques. The relative weights of the chemical components of the artery are first calculated and then used in a diagnostic algorithm to estimate the probability that the artery belongs to a morphologically defined diagnostic class. For 95 of 97 artery locations studied in the first sample set and for 64 of 68 artery locations studied in the second sample set, the decision of our diagnostic algorithm correlated with that of the pathologist. This high level of agreement indicates that

**Discussion**

In this study, we have shown that the pathological state of human coronary artery can be assessed by quantifying its chemical composition with Raman spectroscopy techniques. The relative weights of the chemical components of the artery are first calculated and then used in a diagnostic algorithm to estimate the probability that the artery belongs to a morphologically defined diagnostic class. For 95 of 97 artery locations studied in the first sample set and for 64 of 68 artery locations studied in the second sample set, the decision of our diagnostic algorithm correlated with that of the pathologist. This high level of agreement indicates that
alterations that might result from autolysis, we used coronary artery tissue. For noncalcified plaques, the presence of carotenoids was also present, and although it did not represent a significant weight fraction, its spectrum was used in modeling the artery sample.

Carotenoids have been used by others to detect atherosclerotic plaque. In the present study, carotenoids were found to occupy this area under the ink dot. Fig 7 illustrates how a CS deposit could have been missed. This section was classified as calcified fibroproliferative plaque because of the CS deposits next to the ink dot. However, if the tissue had been sectioned at another angle, this deposit might have been missed and the sample would have been diagnosed as noncalcified. Raman spectroscopy examines a volume of tissue and can detect structures that are beneath the luminal surface. A Raman spectrum of human aorta media. These results indicate that the sections were obtained from the same spectroscopically examined area under the ink dot. The present study did show certain advantages of Raman spectroscopy over microscopic examination. The ability to observe lipids, such as cholesterol crystals, and calcification in tissue sections is essential to recognizing and classifying atherosclerotic tissues by microscopic examination. Representative sections must be made of the tissue to encounter these structures with light microscopy. The high level of agreement between the pathologists’ diagnostic decisions and those of our algorithm indicates that the sections were obtained from the same tissue.

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Figure 7. Microscopic section of calcified fibroproliferative plaque. If the tissue was sectioned at another angle, the CS deposit may have been missed (bar indicates 100 μm). Processing of the Raman spectrum measured from the location marked with ink revealed a 7% relative weight of CS.

In the present study, to minimize any artifactual chemical alterations that might result from autolysis, we used coronary arteries dissected from surgically explanted hearts that were removed during heart transplantation. The majority of these coronary arteries showed either severe end-stage atherosclerotic plaques or only mild intimal thickening. As a result, in our dataset the total number of plaques is small compared with the number of nonatherosclerotic arteries. In addition, the number of noncalcified plaques is disproportionately small, the number of heavily calcified plaque is disproportionately large, and some histological categories have small sample numbers. Therefore, it was not possible to devise diagnostic algorithms that could subclassify arteries to the same degree that they could be subclassified by histological examination. However, statistically significant differences were seen in relative weights of chemical components, such as TCNCR and CECNCR, that may correlate with the presence of particular morphological structures, such as cholesterol crystals or foam cells, used in histological classification. Future studies will seek to quantify the correlation between the relative weights of various chemical components and the volume of tissue occupied by specific morphological structures. We expect that these studies will lead to the development of improved diagnostic algorithms based on Raman spectroscopy.

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The presence of small quantities of lipids or CS is difficult to detect on sections stained with hematoxylin and eosin. Additional histochemical stains for neutral lipids and von Kossa stains for calcium may be needed to visualize fine lipid droplets in fibroblasts, smooth muscle cells, or punctate calcification in the fibrous cap. These stains do not identify individual chemical components, and their interpretation is subjective and nonquantitative. Raman spectroscopic techniques can detect and objectively quantify even small amounts of individual lipids and CS, regardless of tissue handling.

The major chemical components and morphological structures that make up of coronary artery tissue can be expected to remain stable under the freeze preservation and storage conditions used in the present study. Therefore, the Raman spectroscopy techniques, which we have verified in vitro,32 should be applicable in vivo. To verify this, we are in the process of collecting Raman spectra in vivo via an optical fiber catheter in the operating room at the time of heart transplantation, allowing in vivo “Raman histopathology” to be compared with microscopic histopathology of the same location.32 Because further advances in optical fiber catheters are needed, improved techniques to reduce fiber background noise are currently being developed.32 We will report on our work in this field separately.

In conclusion, we have shown that quantitative chemical information of coronary arteries, determined with NIR Raman spectroscopy techniques, can be correlated with arterial wall histology. We have developed diagnostic algorithms that accurately discriminate between nonatherosclerotic tissue, noncalcified plaques, and calcified plaques on the basis of the relative weights of CS and TCNCB, and can be used prospectively. Future investigations may show the value of Raman spectroscopy for basic studies of the pathogenesis of atherosclerosis and when obtained clinically, for assessing the effects of medications on progression and regression of atherosclerosis, predicting acute events like plaque rupture, and selecting proper interventions.

Acknowledgments

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