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.

Key Words: spectroscopy □ diagnosis □ atherosclerosis
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. 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. 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 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 decalcified 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). The samples of the first and second sets, respectively, were diagnosed 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 fibroscerotic 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 fibroscerotic plaque, which is an end stage of atherosclerosis characterized by intimal scarring, loss of lipids, and foam cells. Generally, these plaques are nonobstructive and rarely cause clinical symptoms. 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. 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 spectrometer system detection. A fourth-order polynomial was fit to each spectrum by least-squares mini-
Histopathology by Raman Spectroscopy

Figure 1A shows a spectrum from an arterial wall location exhibiting intimal fibroplasia (class I) and the result of the determination of the chemical composition and the diagnostic classification of arterial wall.18–20

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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 thickens, 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 atheromatous 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
Probability Values of Scores $R_1$ and $R_2$

The regression coefficients, along with their standard errors and statistical significance, are given in Table 2. Discriminant scores were found to be $R_1 = -13.1 + 1.1 \times TC_{NCR}$ and $R_2 = -20.0 + 2.5 \times CS + 0.7 \times TC_{NCR}$. The $TC_{NCR}$ and the relative weight of $CS$ of each artery sample can be plotted in a two-dimensional space, where $TC_{NCR}$ 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 $P_3 = P_{III}$, which is a vertical line at $TC_{NCR} = 12 \pm 1\%$. 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 $P_1 = P_{III}$, which is a line described by the equation $CS = 12.0 - 0.44 \times TC_{NCR}$, and the border separating noncalcified plaques from calcified plaque is determined by $P_3 = P_{III}$, which yields the equation for a line $CS = 4.1 + 0.2 \times TC_{NCR}$.

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

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### TABLE 1. Mean ($\pm$SD) of Each Chemical Component, Calculated With Raman Spectroscopic Techniques

<table>
<thead>
<tr>
<th>Diagnostic Category</th>
<th>$TC_{NCR}$</th>
<th>$FC_{NCR}$</th>
<th>$CE_{NCR}$</th>
<th>$CS$</th>
<th>$DA_{NCR}$</th>
<th>$TG &amp; PL_{NCR}$</th>
<th>$\beta$-Carotene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonatherosclerotic (1, 2)</td>
<td>3.8$\pm$2.5</td>
<td>3.0$\pm$2.5</td>
<td>0.8$\pm$1.3</td>
<td>1.2$\pm$1.4</td>
<td>75.2$\pm$22.6</td>
<td>210$\pm$21.8</td>
<td>13.4$\pm$18.6</td>
</tr>
<tr>
<td>Noncalcified plaques (3, 4)</td>
<td>25.8$\pm$9.5</td>
<td>16.7$\pm$8.2</td>
<td>9.1$\pm$4.8</td>
<td>1.1$\pm$1.6</td>
<td>66.3$\pm$13.5</td>
<td>7.8$\pm$4.5</td>
<td>152.6$\pm$61.1</td>
</tr>
<tr>
<td>Calcified tissue (5-8)</td>
<td>19.0$\pm$9.5</td>
<td>4.4$\pm$3.6</td>
<td>14.5$\pm$7.7</td>
<td>40.9$\pm$20.7</td>
<td>78.5$\pm$10.2</td>
<td>3.2$\pm$3.3</td>
<td>23.1$\pm$55.3</td>
</tr>
</tbody>
</table>

*Expressed as arbitrary units. All other values are percents.

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### Table 2. Regression Coefficients, Standard Errors, and Probability Values of Scores $R_1$ and $R_2$

<table>
<thead>
<tr>
<th>Estimated Coefficient*</th>
<th>Standard Error</th>
<th>$P$</th>
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<tr>
<td>$R_1$</td>
<td>$\alpha_1 = -13.1$</td>
<td>6.8</td>
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<tr>
<td></td>
<td>$\beta_1 = 1.1$</td>
<td>0.6</td>
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<tr>
<td>$R_2$</td>
<td>$\alpha_2 = -20.0$</td>
<td>8.9</td>
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<tr>
<td></td>
<td>$\beta_2 = 0.7$</td>
<td>0.4</td>
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<tr>
<td></td>
<td>$\beta_2 = 2.5$</td>
<td>1.1</td>
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</tbody>
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*The estimated covariances between pairs of coefficients ($P = .05$) are as follows: $(\alpha_1, \beta_1) = -3.8; (\alpha_1, \beta_2) = -6.7.$

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### Figure 6. Diagnostic algorithm based on the relative weights of $TC_{NCR}$ and $CS$. This decision plot is divided by ternary logistic regression into regions of nonatherosclerotic tissue (class I), noncalcified plaque (class II), and calcified tissue (class III). The algorithm, developed with the first set (A), separated a second set of artery locations prospectively into the same regions (B).
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useful parameters in classifying the arteries as nonatheroscle-
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the correlation between the relative weights of various chem-
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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, 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. Because further advances in optical fiber catheters are needed, improved techniques to reduce fiber background noise are currently being developed. 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 TC_NCR 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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