Intravascular Ultrasound Combined With Raman Spectroscopy to Localize and Quantify Cholesterol and Calcium Salts in Atherosclerotic Coronary Arteries


Abstract—Coronary intravascular ultrasound (IVUS) can assess arterial wall architecture and localize large intravascular deposits, but it does not provide quantitative chemical information, which is essential in the evaluation of atherosclerotic lesions. Previously, it has been shown that Raman spectroscopy can be used to accurately quantify the relative weights of cholesterol, calcium salts, triglycerides, and phospholipids in homogenized arterial tissue. In the present study, we explore some benefits of combining IVUS and Raman spectroscopy to evaluate the intact arterial wall. IVUS images were collected in vitro from human coronary arterial segments in various stages of disease (n=7). The images were divided into radial segments (11 to 28 per image, 332 in total), each of which was classified visually as calcified or noncalcified tissue. The arteries were opened longitudinally, and Raman spectra were collected from locations at 0.5-mm intervals across the arterial luminal circumference. The spectra were used to calculate the chemical composition of the arterial wall at the examined locations. Generally, locations containing large amounts of calcium salts, as determined with Raman spectroscopy, were classified as calcified with IVUS. However, small calcific deposits (<6% of weight) were not readily detected with IVUS. The amounts and location of cholesterol determined with Raman spectroscopy were correlated closely with the presence of cholesterol observed by histochemistry, but these deposits could not be located accurately by IVUS. The combination of Raman spectroscopy and IVUS applied in vitro provides detailed information about the amount and location of calcific deposits and lipid pools in atherosclerotic plaques. Future advances in optical fiber technology may allow simultaneous collection of Raman spectra and IVUS images through the same catheter in vivo. (Arterioscler Thromb Vasc Biol. 2000;20:478-483.)

Key Words: coronary arteries ■ atherosclerosis ■ intravascular ultrasound ■ Raman spectroscopy

Previous studies have demonstrated that intravascular ultrasound (IVUS) can detect the 3 layers of vascular wall and the presence of intimal thickening, lipid deposits, and calcifications.1–4 Although IVUS adds valuable information to that obtained by coronary angiography about the degree of luminal narrowing, it cannot detect calcific deposits measuring <0.25 mm in diameter, which are visible by histological examination under high-power magnification.5 Also, the sensitivity of IVUS in detecting lipid pools is low (46%).5–7 Recent studies have suggested that plaque composition and morphology, rather than size or volume, are indicators of plaque rupture, which may lead to an acceleration of clinical symptoms.8–10 Specifically, the accumulation of cholesterol in the arterial intima has been shown to play an important role in the progression and regression of atherosclerotic plaques.8

Raman spectroscopy is a promising technique that can be used to characterize the chemical composition of biological tissue. A Raman spectrum of a given molecule is unique,11–14 which makes Raman spectroscopy ideal for detecting, identifying, and diagnosing diseases that involve gross chemical changes in tissue, such as atherosclerosis. Raman spectra can be obtained by processing the collected light that is scattered from an artery as it is illuminated with a laser beam. With sensitive laboratory equipment, quality spectra can be collected in less than a second, and most spectral features are visible in spectra collected in only a few seconds via optical fiber catheters.15 Because Raman spectroscopy is nondestructive, one can collect spectra of the tissue in situ, which can be processed to provide quantitative information about the chemical composition of the arterial wall.16 Previously, it has been demonstrated that the relative weights of the major lipid classes and calcium salts (CS) in homogenized human coronary arterial wall can be calculated with Raman spectra and that estimates of these amounts are correlated closely with...
those determined by standard lipid assays (±3%) and CS assays (±5%).17 This quantitative chemical information obtained with Raman spectroscopy has been used to identify histopathologically atherosclerotic lesions in vitro.18 In vivo measurements have been hindered by the high background noise generated by light scattering within the optical fibers used to construct intravascular Raman probes. We anticipate that recent technical advances in optical fiber technology will allow adequate reduction of the background signal and the collection of high-quality Raman spectra of the arterial wall in vivo.19–21 Once in vivo Raman measurements are made possible during catheterization, one will need a means to identify from where the spectra are collected. IVUS can provide morphological information from the vascular wall. A combination of Raman spectroscopy diagnosis and IVUS technologies, which may be possible with current catheter technology, may produce a powerful diagnostic tool.

In the present in vitro study, we explore the diagnostic capabilities created by combining these techniques. We investigate the value of adding the quantitative chemical information provided by Raman spectroscopy to the information given by IVUS. Future applications of the Raman/IVUS combination may allow the possibility of multidimensional chemical mappings of an arterial wall. This information may be useful in a variety of ways. For instance, the clinician could monitor the effects of lipid-lowering therapies22 and identify lesions that are prone to rupture.

Methods

Tissue Preparation

Human coronary arterial segments were dissected from hearts during autopsy within 24 hours after death. Subjects (n=12) ranged in age from 44 to 83 years. The adventitial fat surrounding the arterial segments was kept intact to mimic the in vivo situation during the collection of IVUS images. Arterial segments (n=17) of a few centimeters in length were flushed with PBS (pH 7.4), frozen in liquid nitrogen, and stored at −80°C until use. After they were thawed, the arterial segments were submerged in PBS during IVUS measurements. No perfusion pressure was applied to the arterial segments.

IVUS Images

An IVUS imaging catheter (Visions FX plus, EndoSonics) was used to collect images at various locations along the segments. This imaging system is composed of a catheter with acoustic elements mounted circumferentially around its distal tip and a digital imaging system. The image, generated perpendicular to the axis of the catheter, has a slice thickness of ~0.5 mm. Digital reconstruction of the acoustic vectors produces an accurate 2D image of a 360° circumferential slice of the coronary arterial wall at an axial resolution of 80 µm. During IVUS measurements, calcifications were identified by the shadows behind echo-dense areas on the real-time IVUS images. These arterial planes were marked with a curved surgical needle positioned opposite the calcification, which was clearly visible in the IVUS image. The needle mark served to ensure that the Raman spectroscopic measurements would be obtained from the same axial plane. Digital images of nonatherosclerotic arterial sites and atherosclerotic plaques were stored and analyzed. The arterial segments were then snap-frozen in liquid nitrogen and stored at −80°C until Raman spectroscopic examination. IVUS images were analyzed by 2 experienced investigators, and calcifications were graded after agreement had been reached by these 2 investigators. The Raman spectroscopic measurements were performed off-line on separate occasions to prohibit bias.

Raman Spectroscopic Examination

The arterial segments were thawed, opened longitudinally, flattened, and positioned on an aluminum sample holder. Raman spectra were obtained from the luminal side at 0.5-mm intervals over the entire circumference; intervals were marked by a needle (Figure 1).

A Raman microspectroscopic system was used for these studies. This system coupled infrared laser light of 850-nm wavelength from a titanium/sapphire laser (model 375 B, Spectra Physics) pumped by an argon-ion laser (model 2020, Spectra Physics) into a microscope by means of a holographic notch filter (Kaiser Optical Systems). In the microscope, a 20× objective (PL-FL 20 Nachet) of numerical aperture 0.35 concentrated the laser light onto the sample (Figure 2). The samples were irradiated with ~300-mW laser light in a 25-µm-diameter spot. Scattered light was collected and collimated by the same objective. Inelastically scattered light was transmitted by the holographic filter and focused onto the 100-µm core of an optical fiber. This optical fiber led the collected light to a laboratory-built single-stage F/2 Littrow spectrometer. For signal detection, a charge-coupled device (CCD) camera (Princeton Instruments) equipped with a back-illuminated deep-depletion CCD chip was used. Spectral resolution was ~8 cm⁻¹, and signal collection times were 8 seconds. Laser irradiation did not cause visually or spectroscopically noticeable degradation of the samples. Repeated measurements at the same location did not result in spectral changes.

Raman Spectrum Processing

After background correction,15 the spectra were modeled as a linear combination of spectra of 17 arterial components.17 These components were free cholesterol, cholesterol esters, CS, triglycerides and phospholipids, 2 delipidized arterial segments, and β-carotene. The contribution of some components in the coronary arterial spectrum, such as triglycerides and proteins, is difficult to model with spectra obtained from commercially available chemicals because these components in the artery contain mixtures of related molecules. Therefore, these components were extracted from the arterial wall.
of H2O2 from cholesterol by cholesterol oxidase.23 With this enzymatic method based on the production of cholesterol esters, peroxide production is visualized by a brown reaction product formed after peroxidation of diaminobenzidine by H2O2. In a number of cases, the sections were counterstained with hematoxylin to enhance contrast.

Comparison Between IVUS and Raman Spectroscopy in Detecting Calcifications

The detection of calcification by IVUS was compared with the relative amounts of CS as determined by Raman spectroscopy. Each IVUS image was divided in radial segments, like the pieces of a pie. For each of the 17 arterial samples, the number of radial segments was made equal to the number of Raman spectra obtained from the entire circumference of the arterial lumen. Because Raman spectra were obtained in 0.5-mm steps, this number of spectra varied (from 11 to 30; this variation was dependent on the luminal diameter of the arterial wall. The radial segments of the IVUS images were analyzed for the presence of a calcification and separated into a group of radial arterial segments with calcification (IVUS calcification-positive segments) and a group without calcification (IVUS calcification-negative segments). The Raman-determined CS amount for each segment was then compared with the IVUS classification. In total, 332 radial segments were analyzed for CS.

Results

In Figure 3, a representative illustration is given of an IVUS image and the corresponding Raman spectroscopic analysis. Figure 3A shows an IVUS image obtained from a cross section of an artery that contained a calcified area of a coronary artery. The needle mark can be recognized by the radial echo-dense (bright) area at the top of the IVUS image. The presence of a calcification opposite the needle is indicated by the characteristic combination of a shadow behind a strong echo-dense area. The IVUS image indicates that the calcification is present over an arch of ~170°. The artery was cut open, and a series of 16 Raman spectra was collected from the luminal side along the entire circumference of the same cross section. Each of the 16 Raman spectra was processed and fit with the spectra from the individual chemical components. Figure 3B shows one of these spectra (dots) and the result of the model fit (line). The Raman spectral model calculated that this particular arterial site contained 19% total cholesterol and 26% CS (signal attenuation within the tissue was not taken into account).24 Figure 3C-1 shows the relative amounts of CS for all 16 spectra of this arterial cross section. The location of the high amounts of CS as determined with Raman spectroscopy corresponds with the presence of a calcification as indicated by IVUS. Total cholesterol, determined with Raman spectroscopy (Figure 3C-2; note the difference in scale), is primarily found in the noncalcified areas but cannot be clearly visualized with IVUS.

Cholesterol present in histological sections of the marked arterial plane was visualized by an enzymatic method and diaminobenzidine staining. No other stains were used for the section shown in Figure 3D. High contrast can be observed in the center, at the bottom, and at the 2 shoulders of the core of the plaque. The thickened intima next to the core of the plaque had also accumulated cholesterol. The high concentrations of cholesterol at these locations correspond with the high relative amounts of total cholesterol found with Raman spectroscopy (in Figure 3C-2, solid arrowhead). A fine line can be observed around the atheromatous core, indicating the border of calcific deposits before decalcification (Figure 3D, open arrowheads). This line suggests that calcific deposits were located throughout the core of the plaque. These observations are in agreement with the size of the calcification detected with IVUS and with the high amounts of CS found with Raman spectroscopy.

Figure 4 illustrates for 4 arterial samples how the calcification-positive areas over 360° that were detected by IVUS compare with the parts of the arterial wall containing >6% CS that were determined by Raman spectroscopy.18 In general, the arch that contains >6% CS is slightly larger than the arch that is found positive for calcification with IVUS. In addition, Raman spectroscopic techniques can detect and quantify the presence of cholesterol in the arterial wall. As shown in Figure 4, the cholesterol in these arterial samples is found to be located at the border of calcified areas extending into the noncalcified areas.

The radial segments that were IVUS calcification positive (n=2) were found to have high amounts of CS by Raman spectroscopy (Figure 5). In the radial segments that were IVUS calcification negative (n=80), the relative amounts of CS were found to be low. With IVUS considered to be the standard in this comparison, sensitivity and specificity values for detecting calcifications can be determined with Raman spectroscopy by calculating the CS values. A receiver operating characteristic curve analysis accounting for the association between segments of the same sample25 shows that the sensitivity for detecting IVUS calcification-positive areas with Raman spectroscopy equals the specificity (83%) at a CS value of 7%. Thus, it appears that the CS deposits must have a relative weight of >7% to be detected accurately with IVUS.

Discussion

The present study suggests that IVUS, capable of providing tomographic information, in combination with Raman spectroscopy, which can quantify the chemical composition of an artery, may be valuable in the diagnosis of atherosclerosis. A validation of IVUS as an imaging technique has been demonstrated extensively in the past by other researchers. Raman spectroscopic measurements of coronary arteries have recently been validated.17,18 The goal of the present study is to show where the 2 techniques complement each other, not to validate Raman spectroscopy with IVUS.

The arterial segments that were found to be calcified by IVUS correlated closely with the location of high amounts of CS detected by Raman spectroscopy. In addition, cholesterol
deposits could be detected and quantified by Raman spectroscopy, but they could not be located accurately by IVUS. IVUS can accurately detect the presence of CS deposits >0.25 mm but lacks the ability to detect the presence of lipid pools. Not only can Raman spectroscopy identify the major chemical components of the coronary arterial wall, such as total cholesterol, CS, triglycerides + phospholipids, and β-carotene, but it can also quantify these amounts. The
detection and quantification of cholesterol may be important in clinical practice, in view of the fact that a number of recent studies have demonstrated that plaque rupture occurs primarily in lipid-rich plaques covered with a fragile fibrous cap.\textsuperscript{10,27–29} An important goal in our future Raman spectroscopy–assisted IVUS studies is to detect these rupture-prone atherosclerotic plaques in coronary arteries.

When IVUS images and quantitative chemical information obtained with Raman spectroscopy of the same specimen are compared, possible sampling errors must be taken into account. IVUS images collected in the present study were obtained from arterial slices with a thickness of 350 to 500 \(\mu\)m. The IVUS information from this arterial volume is depicted 2-dimensionally and compared with the quantitative information about the chemical composition at a number of spots in this volume. The measuring volume of the Raman spectroscopic method was \(\approx 10\) times smaller than that of the IVUS measurements, in view of the fact that the diameter of the laser spot at the arterial surface was \(\approx 25\ \mu\)m. (Deeper in the tissue, the diameter of the cone of laser light traveling through the tissue will become larger because of the diffuse light scattering but not substantially larger than the illumination spot size.) Areas that appear calcification positive on IVUS images may have contained small areas free of CS, which may have been examined with Raman spectroscopy. Areas that appear calcification negative on IVUS images may have contained small calcifications that are detected with Raman spectroscopy. Some of the mismatches between the IVUS and Raman spectroscopy data may have originated from this disparity in examined volume. Another source for mismatches between the IVUS and Raman spectroscopy data may be caused by the attenuation of the Raman signal from calcified deposits located deeply within the tissue. Recently, we completed a study involving the attenuation of Raman scattering from cholesterol at various depths in atherosclerotic plaques in vitro.\textsuperscript{24} A Raman signal attenuation curve was made with human intima/media coronary tissue that showed that \(\approx 300\ \mu\)m of tissue attenuates cholesterol signals by 50%. We assume that this type of tissue attenuates scattering light from calcified tissue in approximately the same manner as from cholesterol deposits. This could have resulted in a measured CS content <7%, whereas IVUS detected these segments as calcification positive.

Other researchers who conducted in vitro IVUS studies used arterial samples that were submerged in saline or filled with agar-agar solution,\textsuperscript{30} or the arterial samples were perfused with saline at 100 mm Hg\textsuperscript{31} to mimic the physiological shape and size of the artery. In the present study, arterial samples were submerged in saline and were not perfused at 100 mm Hg during IVUS measurements because this could have increased the diameter of the artery asymmetrically. Comparison with Raman spectroscopic examination and histology, conducted on flattened nonperfused arterial tissue, would then have become difficult.

The histological section that was stained specifically for cholesterol (see Figure 3D) shows that the thickened intima contains cholesterol. However, these sections did not contain cholesterol clefts, which are indicative of the presence of cholesterol crystals. This clearly demonstrates the need for specific cholesterol-staining techniques\textsuperscript{23} to visualize the cholesterol content of an artery. Before it was sectioned, the arterial sample in Figure 3 was decalcified to avoid cutting artifacts, but the CS deposit residue is still visible in the atheromatous core of the plaque after hematoxylin and eosin staining.

Clinical intravascular use of Raman spectroscopy requires the use of optical fibers. With sensitive laboratory spectroscopic equipment, high-quality spectra can be collected in less than a second, and with the use of optical fiber catheters, most spectral features are visible in spectra collected in only a few seconds.\textsuperscript{15,20} Optical fibers are currently being optimized for the collection of high-quality intravascular Raman spectra in vivo.\textsuperscript{32} Current efforts are aimed at the simultaneous acquisition of IVUS images and Raman spectra through an integrated IVUS/
Raman spectroscopic sideways-looking catheter. To create a blood-free environment during Raman measurements, such a catheter could be equipped with a proximal port for saline flush or an inflated balloon mounted at the catheter tip. IVUS may then serve as a guiding tool for the intravascular use of Raman spectroscopy in vivo, enabling in vivo histopathology and monitoring the chemical composition of an artery after medical therapeutic intervention, percutaneous transluminal coronary angioplasty, or coronary atherectomy.

Our recent study involving the attenuation of Raman scattering from cholesterol at various depths in real plaques in vitro (≈300 μm of tissue attenuates cholesterol signals by 50%) implies that Raman spectroscopy can detect subsurface structures that are >1 mm beneath an arterial surface. Therefore, Raman spectroscopy should be capable of detecting atherosclerotic deposits under thick fibrous caps.16,24,33

This information, combined with tomographic information about the architecture of the artery provided by IVUS, may be used to calculate the concentrations of the major chemical components at various depths. If serial measurements with a motorized pull-back system are collected, detailed chemical mappings of lesions at interest could be made. Such information may provide improved risk stratification, a means to guide therapeutic intervention (for instance, assessment of the need for stent implantation even at moderately stenosed coronary sites), and an instrument to monitor the effects of medical therapy in the individual patient. Future studies will aim at exploiting the tomographic capabilities of IVUS to create detailed chemical mappings over the entire depth of the arterial wall.

In conclusion, the present study suggests that information about the chemical composition of an arterial wall obtained with Raman spectroscopic techniques and morphological information generated with IVUS may be a very useful combination. When catheter technology allows intravascular Raman spectroscopy in vivo, the combination with IVUS may allow the clinician to monitor the effects of lipid-lowering therapies and to identify lesions that are prone to rupture.

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References

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