SYNCHROTRON INFRARED MICROSPETROSCOPIC ANALYSIS OF COLLAGENS I, III, AND ELASTIN ON THE SHOULDERS OF HUMAN THIN-CAP FIBROATHEROMAS

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ABSTRACT
Of the many people who experience a sudden cardiac event (acute coronary syndromes and/or sudden cardiac death), a large portion have no prior symptoms. One potential in vivo spectroscopic technique for diagnosis of pathological conditions that underlie these sudden cardiac events involves the use of a near-infrared spectrometric catheter with moderate in vivo spatial resolution. To justify the time and expense of such an in vivo protocol, the putative vulnerable narrow region at the shoulder of the thin cap fibroatheroma is chemically characterized by high spatial resolution mid-infrared microspectroscopy. The sharp peaks of the mid-infrared and the previous band assignments that are readily available are useful in establishing the basis needed to support the development and validity of future in vivo NIR probing. The spatial resolution of in vivo NIR spectrometric catheters is limited by light scattering from blood and by the motion of the catheter and blood vessel wall, making it difficult to characterize a fibrous cap in the rupture zone. However, the spatial resolution of in vitro synchrotron IR microspectroscopy is high and probably sufficient to characterize chemically the actual area of disruption. A thin-cap fibroatheroma is a rupture-prone plaque. The shoulder of the cap (where the cap meets the vessel wall) is most vulnerable to rupture because mechanical stress at this point weakens the collagen and elastin fibers. It is hypothesized that the breakdown of elastin is highest in this target zone, followed by collagen III. The analysis of collagen I, collagen III, and elastin concentration in the small (ca.10 micrometer) interface zone, between the intimal wall of the artery and the fibrous cap, is of concern because it is the shoulder where the protein degradation is expected to be the highest. (A similar degradation occurs on a larger scale in the vessel wall in abdominal aortic aneurysm.) For this reason, if confirmed, testing at this location would presumably offer the highest sensitivity and provide the earliest possible warning of rupture-prone plaque. In the current study, post-mortem human tissue was used. Future experiments will be performed on animal models where in vivo NIR catheterization is followed by post-mortem mid-infrared microspectroscopy on the same animal. Subsequently it may be possible to develop in vivo near-infrared spectrometric catheter techniques suitable for use with human subjects in a clinical setting.

INTRODUCTION
Cardiovascular disease has been the primary cause of death in industrialized countries for some time, and it is rapidly becoming the number one killer in the developing countries. According to recent estimates, 61,800,000 Americans have one or more types of cardiovascular disease. Each year, more than 1 million people in the United States and more than 19 million others worldwide suffer a sudden cardiac event (acute coronary syndromes and/or sudden cardiac death). A considerable segment of this population has no preceding symptom. There is a mandate for diagnosis and treatment of the pathologic conditions that lie beneath these sudden cardiac events, and identifying vulnerable plaques and patients.

The word “vulnerable” is used to denote the probability of exhibiting an event in the future. The word vulnerable has been employed in a variety of reports in the medical literature, all of which portray conditions predisposed to injury. In this respect, the term “vulnerable plaque” is most appropriate to classify plaques susceptible to complications. In contrast, interventional cardiologists and cardiovascular pathologists retrospectively explain the plaque responsible for coronary occlusion and death as a culprit plaque, apart from its histopathologic appearance. However, for prospective evaluation, diagnosis, and treatment, clinicians require a term like culprit for identifying such plaques before an event occurs.
Plaque rupture is the most frequent type of plaque complication, accounting for in excess of 70% of fatal acute myocardial infarctions and/or sudden coronary deaths. A number of retrospective autopsy series and a handful of cross-sectional clinical studies have indicated that thrombotic coronary death and acute coronary syndromes are instigated by plaque features and associated factors. The majority of methods for detecting and treating vulnerable plaque are dedicated to rupture-prone plaque. This class of plaque is commonly called a “thin-cap fibroatheroma.”

A thin-cap fibroatheroma is typified by a large lipid core rich in cholesterol and cholesterol esters. These plaques have a cap thickness of less than 100 micrometers and a lipid core accounting for greater than 40% of the plaque’s total volume. Potential in vivo intravascular diagnostic techniques include optical coherence tomography (OCT), intravascular ultrasonography (IVUS), elastography (palpography), MRI, angioscopy, and near-infrared spectroscopy. Increasing evidence substantiates that diverse types of vulnerable plaque with differing histopathology and biology exist. Autopsy studies have demonstrated that atherosclerotic lesions commonly exist in young and asymptomatic persons. The percentage of these lesions that represent morphologies of rupture-prone vulnerable plaques remains to be determined. Furthermore, chronic inflammation and macrophage/foam cell formation are a fundamental element of the natural history of atherosclerosis. To assess plaque vulnerability, it is apparent that a collective methodology able to appraise structural characteristics (morphology) as well as functional properties (activity) of plaque will likely be most revealing, and may offer higher prognostic value than a single method.

Among the first changes in the arterial wall in atherosclerosis is an increase in retained lipoproteins and ensuing oxidation in the subendothelial matrix. Development of lipid-laden macrophages (foam cells) is another characteristic of the early atherosclerotic progression. Proliferation and phenotypic alterations in smooth muscle cells are also observed. The highly developed atherosclerotic lesion may be distinguished by amassing of extracellular lipid, growth of a lipid-rich necrotic core, establishment of a fibrous cap, and calcification. Atherosclerosis in the arterial wall is associated with aneurysm, although it is not clear that this is an underling contributory relationship.

Abdominal aortic aneurysms (AAAs) are potentially life-threatening conditions that arise in up to 10% of the elderly populations in industrialized nations. Similar fibrous protein composition changes have been observed in both the plaque cap in atherosclerosis and in the media and adventitia of AAAs. However, an aneurysm is roughly defined as a permanent dilatation of an artery limited to a small area. AAAs occur due to considerable remodeling of the extracellular matrix and are regularly accompanied by atherosclerosis. They may be manifested by catastrophic rupture, markers of pressure on other viscera, or an embolism initiating in the aneurysm wall, but most are asymptomatic. Collagen and elastin are the main structural components of vessel walls that have been broadly implicated in aneurysm formation, progression, and rupture. These same proteins are found in the cap of fibroatheromas. The prevailing structural modification linked with human AAAs that has been reported is a loss in elastin concentration in the aortic wall. Significant correlations between lowering elastin concentration and increasing AAA diameter have been noted. One proposed mechanism for reduced elastin concentrations is degradation or loss brought about by elastolysis. Although there have been differences in the findings of many studies, it is clear that increases in the collagen-to-elastin ratio are a universal observation in AAAs. Analytical methods capable of analyzing collagen and elastin content of arteries in vivo could be valuable in the diagnosis of aneurysm and atherosclerosis, and might even permit prediction of future clinical events.

Diffuse reflection near-infrared spectroscopy has proven to be a useful technique for identifying chemical content of atherosclerotic tissues. Our laboratory has described the use of near-IR spectroscopy to categorize human aortic atherosclerotic plaques and to quantify cholesterol, HDL, and LDL in arterial wall samples. In a previously study, our laboratory also published the near-infrared spectra of collagen I, III, and elastin. The collagens and elastin possess distinctive near-IR spectra that permit identification and quantification in tissue samples. The data presented in this report show that collagen I, III, and elastin also have distinctive mid-infrared spectra. While the precise relationship between mid-IR fundamental signals and near-IR overtone and combination signals is not always clear, the fact that these target analytes are distinguishable in both spectral regions increases the likelihood that clinical observations made in the IR in vitro will translate into some form that is useful diagnostically in the near-IR in vivo.

In contrast to the mid-IR, near-IR spectrometry is characterized by low molar absorptivities and broad overlapping bands. In vivo vibrational spectrometry has been plagued historically by problems with high water absorbance in tissue, light scattering, peak overlap, and peak shifting with temperature and sample-matrix composition. The intense absorbance of water more than any other factor prevents the use of mid-IR spectrometry as a catheter-based diagnostic tool in atherosclerosis. Instead, near-IR spectrometry has been driven into increasingly complex biological and medical problems as more intense and more stable light sources as well as more efficient detectors (and in many cases, more efficient imaging detectors) have been developed. Improved methods of obtaining rapid wavelength selectivity using tunable lasers and integrated sensing and processing using molecular factor filters are also playing an important role in advancing near-IR spectrometry in a clinical setting.
Synchrotron infrared light is approximately 1000 times more intense than a conventional infrared source. In addition, synchrotron infrared light is highly collimated like a laser, making it more easily focused onto a small spot. However, unlike a laser, the synchrotron emits a wide range of infrared wavelengths, enabling FTIR microspectroscopy. Consequently, with synchrotron infrared light, samples can be studied that are smaller and/or more dilute in concentration. In addition, the 1000-fold increase in brightness translates to data collection times that are about 30 times faster with the synchrotron source in comparison to a globar source. For this study, use of intense synchrotron radiation permits small microscopic apertures near the diffraction limit of the light to be used in microspectrometry, increasing the spatial resolution of collagens and elastin attainable at the site of plaque rupture. Measuring IR fundamentals provides stronger signals with less peak overlap than available with NIR overtone and combination bands.

Infrared spectrometry has been employed a number of times in the analysis of arterial collagens and elastin. Human arterial tissue has been characterized using FT-IR microspectroscopy and chemometrics\(^{12, 13}\). Comparative studies of plaques and proteins using FTIR and other methods have been performed\(^{14}\). Collagen modifications and surface properties of arteries have been studied in a bovine model\(^{15}\). The effect of elastin on the calcification of collagen-elastin matrix systems has been studied with infrared spectrometry\(^{16}\). Finally, fundamental vibrational information on collagens and elastin has been derived from studies using FT-Raman spectrometry\(^{13, 17}\).

Similar composition changes have been observed in both the fibrous cap of lesions in atherosclerosis and in the media and adventitia of AAAs, albeit on different physical scales\(^{15, 18, 19}\). Near-infrared spectrometry, immunohistochemistry, and scanning electron microscopy with morphometry have been employed in these studies of collagen and elastin composition changes. The spectrometric correlation methodology employed in the following study relies on collagen and elastin content changing within the samples as suggested by previous research. This preliminary study tests the hypothesis that the changes observed previously in collagen I, III, and elastin in aneurysm on a millimeter scale are similar to the changes that occur in the fibrous cap of vulnerable atherosclerotic plaque on a scale of micrometers or tens of micrometers. If successfully demonstrated, the similarities could be used as \textit{in vivo} markers of the vulnerable plaques most in need of treatment, and could be used in monitoring therapies in atherosclerotic plaques treated by drugs.

**EXPERIMENTAL**

**Tissue Samples.** Twenty human coronary tissue sections were obtained post-mortem without identifiers from a single patient through the University of Kentucky Medical Center, Clinical Pathology Services. The study was approved by the University of Kentucky IRB and HIPAA compliance office. If components other than collagen and elastin were in the tissue and were varying in concentration, then the correlation maps produced by IR imaging would be biased and would not likely reflect the individual contributions from collagen I, III and elastin in the sample. The potential for bias was eliminated by removing all other components of the tissue visible on IR spectra by a washing, solvent-extraction and formalin-fixing process prior to mounting the tissue sections on the slides\(^7\). The tissue sections were mounted in paraffin onto two low-e glass slides (SensIR) for infrared microspectroscopy. A visible light image of a stained section of human coronary atherosclerotic plaque appears in Figure 1. This section was adjacent to the section used for IR microspectrometric imaging (see Figure 2). An arrow marks the location on the fibrous cap from which infrared spectra were obtained. Figure 3 depicts representative spectra from a small area of the fibrous cap. A total of 80,600 spectra were obtained from all plaque sections.
control image section was at the bottom of the vessel, away from the fibrous cap.

**Figure 3.** Representative infrared spectra from the region near the arrow in Fig. 2. The sharp peak at 1469 cm\(^{-1}\) arises from paraffin.

**Instrumentation.** The infrared microspectrometer used at beamline U2b of the vacuum ultraviolet (VUV) storage ring of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL), Upton NY consisted of a Nicolet PLAN® infrared microscope interfaced to a Nicolet Magna® 860 infrared spectrometer (Thermo Electron, Madison WI). A liquid nitrogen cooled 250 cm MCT detector that had maximum signal intensity at 1250 µm was used.

Schwartzchild 32x and 10x all reflecting mirror lenses were used for the objective and condenser respectively. A remote projected image plane mask before the objective produced the apertures used for single point spectra or raster scan mapping via a digitally controlled motorized microscope stage. Spectra were recorded in a reflection absorption mode. A clear location on the infrared reflecting microscope slide ReflectIR® (SensIR, Danbury CT) was used to obtain a reflection background spectrum.

Mapping was also accomplished from a globar source focal plane array instrument. The Perkin-Elmer Spotlight model 300 was used to obtain rectangular maps of select regions of the sections being examined. For focal plane array images, the 6.25µm x 6.25µm pixel size was used.

Preliminary examination of each map was done from a locally baseline corrected peak area for the triplet at 1236 cm\(^{-1}\) and the doublet associated with the 1082 cm\(^{-1}\) band. A map of the ratio of the area of the 1236 cm\(^{-1}\) band to that of the 1082 cm\(^{-1}\) was used to locate the region with the highest relative amount of the collagen I.

Reference FTIR spectra of collagens I, III, and elastin (Sigma) were obtained (see Figure 4). While such reference compounds are sometimes contaminated by small amounts of lipid, potential contamination posed no problem for this research because the lipid regions of the spectrum were not used in the analysis to avoid the paraffin. The spectral data were scatter-corrected prior to data analysis (see Figure 5). Representative spectra from a human coronary tissue section are presented in Figure 3 for comparison with Figure 5. The sharp peak at 1469 cm\(^{-1}\) in Figure 3 arises from paraffin. The chemical composition of the tissue samples between adjacent areas of tissue is typically similar, and as a result, the gross appearances of the spectra are similar.

**Figure 4.** Spectra of lyophilized standards of collagen I, III, and elastin.

**Figure 5.** Spectra of standard mixtures of collagen I, III, and elastin used for mean-centered correlation analysis.

SEM morphometry provided the reference method for collagens and elastin\(^ {20} \). A contract laboratory (Industrial Analytical Services, Leominster, MA) was
used to blind the spectroscopists and electron microscopists examining the tissue sections. A similar methodology was employed by the authors in a previous near-IR study of aneurysms.

Data Analysis. Analytical software was written in Matlab 6.5 (The Math Works, Inc., Natick, Mass.). In an effort to determine which spectral changes in the coronary sections were associated with collagens I and III and elastin composition changes, a set of sample mixtures of collagens I and III and elastin was prepared using pure lyophilized standards (see Fig. 5). A triangular array of compositions was constructed for this study similar to the one used in reference 6. The composition of each of the prepared sample standards was represented by a vertex in the array, with the pure collagen I (C1) standard in one corner of the triangle, the pure collagen III (C3) in another corner of the triangle, and finally the pure elastin in the remaining corner of the triangle. The concentrations of each constituent in the standard mixtures were set at 0, 25, 50, 75, or 100 wt % of each lyophilized protein. The vertexes represent all possible combinations of mixtures in the percentages given (a total of 15 mixtures including the pure corner standards). The center (i.e., group mean) would represent a mixture of one-third of each protein, but this sample was not actually prepared in the set. The reflection spectra of the 15 mixtures were compared to the reflection spectra of the coronary sections by mean-centering the spectra of the mixtures and the spectra of the coronary artery sections. The difference spectra between each standard sample spectrum and the mean spectrum of the standard samples were calculated. Likewise, the difference spectra between each coronary section spectrum and the mean spectrum of the coronary sections were also calculated. Finally, the difference spectra of the standards and the coronary sections were then correlated using the product-moment correlation coefficient. The values of these correlation coefficients were contour plotted to produce images corresponding to collagen I, III, and elastin in Figures 6-9.

Figure 6. Collagen I distribution in targeted region of fibrous cap (red=high, blue=low concentration).

Figure 7. Collagen III distribution in targeted region of fibrous cap (yellow=high, blue=low concentration).

Figure 8. Elastin distribution in targeted region of fibrous cap (red=high, blue=low concentration).

Figure 9. Localized distribution of collagen I, collagen III, and elastin at individual pixel resolution.
RESULTS AND DISCUSSION

The correlations between the coronary tissue section spectra and the set of standard sample spectra ranged between ±0.99. In consequence, the contours in Figures 6-8 covered a wide range of correlation values (as did the pixels in Figure 9). Color was used to represent correlation between the spectra of the coronary sections and reference standards. Violet represents the lowest correlation, while red represents the highest. The region at the far left of Figures 6-8, off the edge of the thin cap, served as a sort of internal standard or control for the images as the scanning progressed toward the right and the thinnest portion of the cap.

Figure 6 shows the correlation between the tissue spectra and amount of collagen I in the standards in the region marked by the arrow in Figure 2. Pixel values were interpolated between the concentrations of the standards to reach the maximum correlation, as performed in reference 6. While the amount of collagen I does not increase monotonically from left to right in the image, there is a gradient in concentration over the 80 micrometer distance across the fibrous cap. The right side of the image in Figure 6 is the side closest to the thinnest area of the fibrous cap, as shown in Figure 1.

Figure 7 depicts the distribution of collagen III predicted in a similar manner. Collagen III also shows an overall gradient, but instead generally decreases nonmonotonically from left to right in the image. In contrast, the amount of elastin did not appear to change substantially across the field of the image (see Figure 8). However, most of the loss of elastin could have occurred long before the coronary tissue was collected. The control section imaged at the bottom of the vessel was similar to Figure 8 in that it showed no obvious concentration trends for either the collagens or elastin. Overall, the trends in collagen I, III, and elastin observed in human coronary plaque are similar to those observed in AAAs. At single pixel resolution on the right side of the sample zone nearest the thinnest region of the fibrous cap (see Figure 9), gradients are not as clear.

Rupture-prone plaques are not the lone vulnerable plaques. All categories of atherosclerotic plaques with high probability of thrombotic complications and swift progression should be regarded as vulnerable plaques. Furthermore, vulnerable plaques are not the only culprit features leading to the occurrence of acute coronary syndromes, myocardial infarction, and sudden cardiac death. Vulnerable blood (i.e., blood inclined toward thrombosis) and vulnerable myocardium (inclined toward lethal arrhythmia) perform an essential role in the clinical outcome. The phrase “vulnerable patient” has even been proposed for the classification of persons with high probability of emergent cardiac events in the near future. A quantitative means of cumulative risk assessment of vulnerable patients should be created that includes variables describing plaque, blood, and myocardial vulnerability. Newly developed assays (e.g., for C-reactive protein), imaging techniques (e.g., CT and MRI), noninvasive electrophysiological tests (for vulnerable myocardium), and promising catheters (to localize and characterize vulnerable plaque), together with prospective genomic and proteomic methods, will lead researchers in the hunt for vulnerable patients. These analytical methods will also lead to the expansion and exploitation of new therapies, and eventually to reduction in the incidence of acute coronary syndromes and sudden cardiac death.

This preliminary study has some important limitations. A single patient served as the source of the 80,600 spectra collected from 24 coronary sections, limiting the observable variation in the data set. The lack of detailed histological data for the samples and lack of clinical history from the patient prevents the association of the spectra with specific tissue pathologies and comparison of pathology. Most importantly, the exact location of any rupture (the culprit lesion) was not uncovered in the tissue sections. For this reason, the exact nature of the gradients within 10 micrometers of any tear in the fibrous cap cannot be determined. However, the fact that gradients in collagen and elastin similar to those observed in AAAs do exist in the vicinity of a plaque rupture suggests that similar mechanisms of protein degradation may be responsible in both disease states. Thus, an increase in collagen I at the expense of collagen III (and possibly of elastin) might serve as a marker of plaques needing an immediate intervention.

CONCLUSION

Like near-IR spectra, mid-IR spectra are distinctive for proteins in the blood vessel wall (specifically collagens and elastin). The results of this preliminary study suggest that synchrotron IR microspectroscopy is a potentially useful technique for investigating vascular changes and protein composition associated with cardiovascular disease. In particular, synchrotron IR microspectroscopy has the light intensity, and thus spatial resolution using small mask apertures, needed to quantify collagens and elastin within 10 micrometers of the site of plaque rupture. These early results support an expanded study in the future employing IR spectra to characterize completely chemical compositions within 10 micrometers of the location of plaque rupture. If the compositions of many of these small regions prove similar in many different patients, a useful marker will have been demonstrated that can be targeted by near-IR catheters in vivo.

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