Alteration of Spectral Characteristics of Human Artery Wall Caused by 476-nm Laser Irradiation

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Fluorescence spectroscopy is a promising new technique for discrimination of normal and atherosclerotic arterial tissues. It has been suggested that this technique be used as a guidance system for laser angioplasty catheters; however, irradiation by 476-nm light can change the spectroscopic properties of arterial tissue. We present studies that establish intensity levels and exposure times at which alterations in tissue spectral properties are minimal. We also investigate the nature of spectral alterations following exposure of normal human aorta to high intensities of 476-nm laser light. Changes in laser-induced fluorescence (LIF) are characterized by two prominent features: the peak fluorescence intensity decreases permanently, and the fluorescence lineshape changes in a largely reversible way. We relate these changes to alterations in individual tissue chromophores; permanent changes in absolute fluorescence intensity are due to irreversible changes in tissue fluorophores, reversible changes in fluorescence lineshape are due to alterations in tissue absorbers. A simple kinetic model is used to describe the decrease in absolute fluorescence intensity.

Key words: atherosclerosis, fluorescence spectroscopy, laser angioplasty

INTRODUCTION

It has been shown that laser-induced fluorescence spectroscopy (LIF) can be used to differentiate normal and atherosclerotic human artery wall in vitro [1–7]. Fluorescence spectra of arterial tissue can be obtained using optical fiber probes [2–7]; using such devices, LIF spectra of human artery wall have been obtained in vivo [6], implying that diagnosis of atherosclerosis can be extended to in vivo applications. Thus, LIF spectroscopy provides a potential guidance system for laser angioplasty catheters [7] and may alleviate the high incidence of arterial perforation currently associated with laser angioplasty procedures [8].

However, LIF spectroscopy of arterial wall has broader applications. Because spectroscopic signals depend on the chemical structure of the irradiated sample, spectroscopy may provide a method of studying the chemical composition and thus the pathogenesis of atherosclerotic lesions in vivo [9,10].

A potential problem with LIF spectroscopy as a diagnostic for atherosclerosis is that irradiation by exciting light can change the spectroscopic properties of arterial tissue [11]. Thus, in order to apply this technique effectively, an understanding of the intensity levels and exposure times at which changes in arterial LIF become significant.

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is required. Combining the techniques of spectral diagnosis and removal of atherosclerotic lesions by laser ablation imposes an additional requirement; alterations in tissue spectral properties following exposure to the high intensities of laser light used during ablation must be understood.

In this paper, we present a study of the effects of 476-nm light on 476-nm excited tissue spectral properties. This wavelength was chosen for study as it has been shown to be useful in the differentiation of normal and atherosclerotic tissues, and furthermore, chromophores that contribute to tissue fluorescence spectra at this excitation wavelength have been determined [1,9,10]. Thus, changes in fluorescence at this excitation wavelength could be related to alterations in specific tissue chromophores.

We present studies that establish intensity levels and exposure times for 476-nm excitation light at which alterations in tissue spectral properties are minimal. As a first step toward understanding post-ablation tissue spectra, we also investigate the nature of spectral alterations following exposure of tissue samples to high intensities of 476-nm laser light. These changes are related to alterations in individual tissue chromophores. Finally, we describe these changes with a simple kinetic model, developing a technique that we use to predict features of tissue spectra following laser ablation.

METHODS AND MATERIALS

Figure 1 illustrates the experimental setup used to collect tissue fluorescence spectra. The heart of this system is a multi-fiber shielded catheter, consisting of a central fiber used to deliver excitation light to the tissue surface and six surrounding fibers used to collect returning tissue fluorescence. The six collection fibers are arranged so that they view only the illuminated portion of the tissue; thus, the collection geometry of the system is well defined [12]. 476-nm light from an argon-ion laser was focused into the distal end of the central fiber, producing a 1 mm diameter spot of light at the output face of the shield. Illumination intensities of 100 μW/mm² were used to collect tissue spectra. The distal ends of the collection fibers were arranged in a circular array and imaged onto the face of an 800-μm-diameter optical fiber. The opposite face of this optical fiber was placed at the entrance slit of a scanning monochromator-photomultiplier tube system. To block scattered excitation light, 495-nm glass cutoff filters were used.

The monochromator-PMT combination had a resolution of 6 nm full-width at half-maximum. The scanning rate of the system was 200 nm/min, and a 250-msec time constant was used to record data. Data points were taken every 0.5 nm. Fluorescence intensities in all spectra presented here are given in arbitrary units. All spectra have been corrected for the non-uniform spectral response of the detection system.

Tissue samples investigated were human cadaver aorta, obtained within 24 hr postmortem. Tissue samples were washed in saline solution (8 g NaCl per liter H2O), snap-frozen in isopentane and liquid nitrogen, and stored at -40°C until use. Tissue samples were used at room temperature, within 4 hr of defrosting, and were kept moist with saline. Only samples that were morphologically normal by gross examination were used in this study. The effects of freezing on tissue susceptibility to spectral alterations following exposure to laser light were not assessed.

The following protocol was used to investigate changes in the spectral properties of arterial tissue samples following a given exposure to 476-nm light. An initial 476-nm excited LIF spectrum of tissue was recorded using a low excitation intensity, 100 μW/mm². The tissue sample was exposed to 476-nm light of a higher intensity for a given exposure time, and following this a final LIF spectrum was recorded under the same conditions as the initial LIF spectrum. Throughout this procedure, the position of the shielded spectral catheter was not altered. Comparing the initial and final LIF spectra gave a measure of the alteration in tissue spectral properties.

Spectral alterations were used to establish tissue tolerance to 476-nm excitation light. Tissue tolerance, T, is defined as the exposure time to 476-nm light at a given intensity required to produce either a 12% change in peak fluorescence intensity, I(520), or a 5% change in the peak-to-valley fluorescence intensity ratio, R = I(600) nm/I(580) nm. These quantities, I(520) and R, were studied because they have been suggested as empirical diagnostic algorithms for the presence of atherosclerosis [1–3,5,7]. Tolerances were determined at several different incident intensities including 0.32 mW/mm², 1.0 mW/mm², 1.5 mW/mm², 3.0 mW/mm², 6.3 mW/mm², and 12.8 mW/mm². At each intensity, the percentage changes in I(520) and R were determined at three different locations on the intimal surface of a sin-
Fig. 1. Experimental set-up used to collect tissue fluorescence spectra.

A single tissue sample for a given exposure time. The exposure time was increased, and the experiment was repeated at three additional locations in another sample until the average percentage change in one of these quantities exceeded the threshold values given earlier.

Threshold values for changes in R and I(520) were determined from a control experiment performed with normal cadaver aorta. Fluorescence spectra of a single sample were recorded at 10-min intervals for 1 hr with the sample kept in the dark between successive spectra. For each spectrum, I(520) and R were noted. The average value and standard deviation for each of these quantities was calculated. Threshold changes in I(520) and R were calculated as $3 \times (\text{Std. Dev.}/\text{Avg.}) \times 100\%$.

Above threshold, the nature of these spectral alterations was examined in a single normal tissue sample as a function of time following a 10-min exposure to 10 mW/mm² of 476-nm light (6 J/mm²). An initial 476-nm LIF spectrum was recorded, and successive spectra were recorded immediately following and at 10-min intervals after the 6 J/mm² exposure. Changes were gauged observationally.

To investigate the behavior of spectral alterations as a function of exposure intensity and fluence in more detail, the following study was performed. A normal tissue sample was exposed to
476-nm light of a given intensity for a specified exposure time. Immediately before and after this exposure, the fluorescence power at 600 nm was noted from a 476-nm excited LIF spectrum. This procedure was repeated three times in a given sample, and the results were averaged. At any incident intensity this procedure was followed for more than 10 exposure times using a different normal tissue sample for each different exposure time. Thus, the behavior of the post-exposure fluorescence power at 600 nm divided by the pre-exposure fluorescence power at 600 nm was obtained as a function of fluorescence for a given intensity. The experiments were performed at five incident intensities: 0.3 mW/mm², 0.5 mW/mm², 1 mW/mm², 50 mW/mm², and 100 mW/mm².

The results of these experiments were fit to a simple model of tissue spectral alteration, which is described in the following section. Parameters that describe the behavior of tissue fluorescence as a function of fluence were extracted at each intensity. The results of this fit were used to predict how tissue fluorescence spectra may be altered following laser ablation.

RESULTS AND DISCUSSION

Table 1 presents the results of our determination of tissue tolerance. Over a range of incident intensities from 0.32 to 12.8 mW/mm², the threshold for producing spectral alterations could be described in terms of a single fluence threshold of 70 mJ/mm² ± 10 mJ/mm². Tissue tolerance, T, at any incident intensity can thus be calculated as

\[ T = 70 \text{ mJ/mm}^2 I \] (1)

where T is given in seconds, and I, the incident intensity of 476-nm light, is given in mW/mm².

Figure 2 illustrates the nature of alterations of tissue spectral properties following exposures >70 mJ/mm². Here data from a single tissue sample are presented before (a) and at successive time intervals following (b–d) an exposure to 6 J/mm² (10 mW/mm² for 10 min). It was found that changes in tissue LIF could be characterized by two prominent features: a permanent decrease in the absolute fluorescence intensity and a largely reversible change in the fluorescence lineshape. Although only a single example is presented here, this behavior was found to be a general trend exhibited by many samples at many exposure fluences greater than the fluence threshold (H. Chaudhry, personal observation).

Interpreting these changes in terms of alterations in individual tissue chromophores is complicated, because composite arterial LIF spectra are generated through an interplay of intrinsic fluorescence, scattering, and reabsorption [12,6,7,12,13]. Figure 3 is a schematic diagram that illustrates how the broadband intrinsic fluorescence of normal aorta is modulated by reabsorption to produce the composite tissue LIF spectrum. Observations with the fluorescence microscope indicate that the intrinsic fluorescence is due to chromophores associated with the structural proteins collagen and elastin [12,14]. The absorption peaks at 540 and 580 nm, which produce the valleys in the composite tissue fluorescence spectrum, are consistent with the absorption lineshape of oxy-hemoglobin [12,6,12,13,15,16]. Although oxy-hemoglobin is present in normal and atherosclerotic human endartery aorta, preliminary experiments indicate that it is present only in calcified atherosclerotic plaques in vivo [6].

It is known that oxy-hemoglobin can be deoxygenated by exposure to laser light [17], resulting in a change in absorption properties that is consistent with the changes in tissue fluorescence lineshape presented in Figure 2. However, the absorption of oxy-hemoglobin, as well as changes in absorption upon de-oxygenation, are close to a minimum at 600 nm [15]. Thus, by studying changes in fluorescence power at 600 nm, P(600),
changes in tissue fluorophores can be monitored separately from changes in tissue absorbers.

Our preliminary investigations indicate that, with 476-nm excitation, the fluorescence lineshapes of powdered bovine collagen and elastin (Sigma Chemical Co.) are unaltered following exposure to 476-nm light in the range of intensities and exposure times studied with tissue, although the fluorescence intensity decreases (H. Chaudhry, personal observation at $1 - 50$ mW/mm$^2$, up to fluences of 8 J/mm$^2$). Thus, to monitor alterations in tissue fluorophores, as a function of fluence over the range 500--600 nm, data need only be collected at a single wavelength in this region, since their fluorescence lineshape is not altered. By choosing 600 nm, a point where the contributions of tissue absorbers is minimal and minimally altered upon exposure, we can obtain complete information about the nature of alterations in tissue fluorophores over the range 500--650 nm as a function of fluence.

Figure 4a--e illustrates the behavior of absolute fluorescence power at 600 nm as a function of fluence for several incident intensities. This change is characterized as $P(600)/P(600)$, the ratio of absolute fluorescence power at 600 nm in the final LIF tissue spectrum to that in the initial LIF spectrum. As the similarity of these figures indicates, over the range of intensities studied, alterations in tissue fluorophores were found to depend only upon fluence.

This behavior suggests that alterations in tissue fluorophores may be mediated by a photochemical mechanism. We have developed a simple
photochemical model for the changes in absolute fluorescence power at 600 nm, which is consistent with the dependence of these changes on fluence. Although we have no direct experimental evidence to support this photochemical model, it suggests an interesting possible mechanism that is consistent with our data. In our simple model, we have considered tissue to be composed of two types of fluorophores (Fig. 5). Type 1 fluorophores can exist in three states, a ground state with population N_1, and two excited states. The highest energy state, ΔE above the ground state, has population N^*; fluorophores can make transitions to this state from the ground state by absorbing light. This process is governed by the rate constant α/ΔE, where α is the absorption cross section of excitation light for the fluorophore, and I is the incident intensity of 476-nm light. Fluorophores in this highest energy state can either make transitions to the ground state, emitting fluorescence with rate constant k_f, or branch with rate constant β to form a non-fluorescing species, population N_2. Type 2 fluorophores are similar to type 1 fluorophores in all respects but one; they
In the initial LIF spectrum this will be given by

\[ P_i = \sigma I \left( N_{IT} \left( \frac{k_f}{k_f + \beta} \right) + N_{2T} \right). \]  

(3)

However, following exposure of the sample to a given fluence, the proportion of type 1 fluorophores in the ground state will decrease. If we assume that steady state is achieved in the exposure, we can calculate \( N_i \) as a function of fluence as

\[ N_i = N_{IT} e^{-\alpha F} \]  

(4)

where \( \alpha \) is given by

\[ \alpha = \frac{\sigma \beta}{\Delta E (k_f + \beta)} \]  

(5)

Thus, the ratio of the absolute fluorescence power post-exposure to that pre-exposure can be written as

\[ \frac{P_f}{P_i} = \frac{N_{IT} \left( \frac{k_f}{k_f + \beta} \right)}{N_{IT} \left( \frac{k_f}{k_f + \beta} \right) + N_{2T}} e^{-\alpha F} + \frac{N_{2T}}{N_{IT} \left( \frac{k_f}{k_f + \beta} \right) + N_{2T}} \]  

(6)

or

\[ \frac{P_f}{P_i} = B e^{-\alpha F} + (1 - B). \]  

(7)

A least-squares fit of our data to this equation generated the results shown in Table 2. For each intensity, \( \alpha \) and \( B \) were not significantly different from the average values \( \alpha = 2.5 \times 10^{-5} \text{mm}^2/\text{mJ} \) and \( B = 0.46 \), indicating that fluence is the only parameter needed to describe the LIF changes owing to tissue fluorophores.

Note that these data are consistent with our earlier results for tissue tolerance, where it was found that a fluence threshold of 70 mJ/mm² was required to produce spectral alterations. Our model predicts that the fluence necessary to produce a 12% change in absolute fluorescence intensity, \( F_{th} \), is

\[ \frac{P_f}{P_i} = (1 - B) + B e^{-\alpha F_{th}} = 0.88. \]  

(8)

Using average values for \( \alpha \) and \( B \), \( F_{th} \) is found to
TABLE 2. Results of Least Squares Fit to Data in Figure Four

<table>
<thead>
<tr>
<th>Intensity (mW/mm²)</th>
<th>α (×10⁻³ ± νₐ (×10⁻⁶)</th>
<th>B ± νₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>3.95 ± 1.14</td>
<td>0.469 ± 0.041</td>
</tr>
<tr>
<td>0.5</td>
<td>1.90 ± 0.49</td>
<td>0.368 ± 0.003</td>
</tr>
<tr>
<td>1.0</td>
<td>1.26 ± 0.86</td>
<td>0.467 ± 0.104</td>
</tr>
<tr>
<td>10.0</td>
<td>3.13 ± 1.33</td>
<td>0.416 ± 0.046</td>
</tr>
<tr>
<td>100.0</td>
<td>1.19 ± 0.92</td>
<td>0.443 ± 0.072</td>
</tr>
<tr>
<td>Average</td>
<td>2.50 ± 0.28</td>
<td>0.469 ± 0.066</td>
</tr>
</tbody>
</table>

be 120 mJ/mm², close to our threshold value of 70 mJ/mm².

This simple model can be used to predict features of tissue spectra following laser ablation. If we model ablation as a thermal process and assume that the properties of tissue are those of water, we must have an energy density of 2.5 J/mm² at the bottom of the ablation crater, regardless of the incident intensity [18]. This energy will be distributed over a depth of unablated tissue corresponding to one penetration depth of the incident radiation. For argon ion laser light in arterial tissue, this is approximately 300 µm [18]. Thus, the floor of the ablation crater is exposed to a fluence of

\[ F = (2.5 \text{ J/mm}^3)(3 \text{ mm}) = 7.5 \text{ J/mm}^2. \] (9)

Using our expression for the change in absolute fluorescence intensity following exposure to 476-nm light with average values of \( \alpha \) and \( B \) for normal tissue, we see that this study predicts a decrease of approximately 40% in the absolute fluorescence power following ablation. This estimation is strictly valid only for tissues in which fluorophores are distributed homogeneously and neglects effects that are due to thermal damage and charring, which may be produced during ablation. In future studies, we will assess the effects of these changes on our ability to discriminate normal and atherosclerotic tissues based on 476-nm excited LIF spectroscopy.

CONCLUSIONS

We have demonstrated that arterial LIF can be altered by excessive exposure to laser irradiation. This has important consequences for the application of diagnosis of atherosclerosis using LIF spectroscopy. We have established that tissue tolerance to 476-nm laser light can be given by a fluence threshold of 70 mJ/mm², over a wide range of incident intensities. Although our results describe in vitro behavior of normal aorta only, preliminary experiments suggest that threshold exposure times for atherosclerotic tissue are not greatly different from those of normal aorta in vitro, and that in vitro tolerances approximate in vivo tolerances reasonably well.

At fluences above threshold, detectable changes in tissue LIF spectra can be divided into two types irreversible changes in absolute fluorescence intensity and reversible changes in fluorescence lineshape. Reversible changes in the fluorescence lineshape are due to reversible dissociation of oxyhemoglobin.

Changes in absolute fluorescence intensity are due to changes in tissue fluorophores found in collagen and elastin. These changes are likely mediated by a photochemical mechanism and can be described in terms of the fluence delivered to
the tissue. Our experimental data could be fit to a kinetic equation generated from a simple photocchemical model. This model was used to predict changes in tissue LIP following laser ablation.

Due to the ubiquitous nature of collagen and elastin, the study of laser-induced alterations in these fluorophores has significant relevance to other types of human tissue.

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