Study of the Fluorescence Properties of Normal and Neoplastic Human Cervical Tissue

Anita Mahadevan, MS, Michelle Follen Mitchell, MD, MS, Elvio Silva, MD, Sharon Thomesen, MD, and Rebecca R. Richards-Kortum, PhD

Biomedical Engineering Program, University of Texas, Austin, Texas 78712 (A.M., R.R.R.-K.), and Departments of Gynecology (M.F.M.) and Pathology (S.T., E.S.). MD Anderson Cancer Center, Houston, Texas 77030

Fluorescence excitation–emission matrices (EEMs) were obtained in vitro for 18 cervical biopsies from 10 patients. At all excitation emission maxima, but especially at 330 nm excitation, 386 nm emission, the average normalized fluorescence intensity of histologically normal tissue is greater statistically than that of histologically abnormal tissue. A diagnostic algorithm based on the relative intensity at 330 nm excitation, 386 nm emission can differentiate histologically normal and abnormal biopsies with a higher sensitivity (89%) but a lower positive predictive value (67%) and specificity (44%) than colposcopy (78%, 88%, 88%, respectively). However, paired comparison of histologically normal and abnormal biopsies from the same patient results in a sensitivity of 76%, positive predictive value of 86% and specificity of 88% for spectroscopic identification of histologic abnormality similar to that of colposcopy. This pilot study indicates that fluorescence spectroscopy may be useful in differentiating normal and abnormal tissue; based on these results, a strategy for in vivo studies is discussed. © 1993 Wiley-Liss, Inc.

Key words: cervix, diagnosis, intraepithelial neoplasia, spectroscopy

INTRODUCTION

Early detection of cervical intraepithelial neoplasia (CIN) has played a central role in reducing the mortality associated with this disease over the last 50 years [1]. However, in recent years, the incidence of preinvasive squamous carcinoma of the cervix has risen dramatically [1], especially among young women [2]. It has been estimated that, as a result, the mortality of cervical carcinoma may rise by 20% in the years 2000–2004 unless further improvements are made in screening techniques [3]. Most commonly, cytologic screening techniques are used to detect early CIN. Estimates of the accuracy of cytologic screening vary widely, but for precancerous lesions, the false negative error rate has been shown to be between 20% and 30% [4, 5]. Once an abnormal pap smear is detected, colposcopy is used to identify the lesion and target the biopsies [6]. Thus biopsy and histologic analysis are required for accurate diagnosis of CIN [6]. Even in experienced hands, the predictive value of colposcopy is not as great as one would like. A method to increase the predictive value of this technique would allow faster, more effective patient management. In particular, if the predictive value of colposcopy by less experienced practitioners could be increased, more wide-scale implementation of colposcopy might be possible, resulting in earlier detection of CIN.

There is much evidence to indicate that spectroscopy of both exogenous [7, 8] and endogenous chromophores [9–19] can be used to identify neoplastic cells and tissues in a variety of organ sys-
As a first step toward developing a spectroscopic technique for detection of CIN, a study of the spectroscopic properties of normal and neoplastic human cervical tissue was conducted. Our goals were to characterize the optical properties of several histo-pathologic types of human cervical tissue (normal, nonspecific inflammation, HPV infection, and CIN) over the UV and visible spectral regions and to determine optimal excitation wavelength(s) for differentiating neoplastic and nonneoplastic tissue types based on their fluorescence spectra. The resulting spectra of normal and dysplastic cervix were compared to spectra of normal and dysplastic tissues from other organs to determine whether, despite the highly variable nature of the micro-anatomy, origin and environment of cancers and precancers [20–22], it would be possible to define general spectroscopic algorithms for recognizing dysplasia.

MATERIALS AND METHODS

Twenty cervical biopsies were obtained from 10 patients at the time of colposcopy; 10 biopsies were colposcopically normal and 10 colposcopically abnormal in appearance. The patients entered in the study were selected from a group of patients referred for follow-up colposcopy for suspected CIN on the basis of abnormal cervical cytology. Informed consent was obtained from each patient and the project was reviewed and approved by the Institutional Review Boards of the University of Texas MD Anderson Cancer Center and the University of Texas at Austin. The biopsies measured 1–4 mm in diameter and ~1 mm in thickness. They were snap frozen and stored initially in liquid nitrogen and later at −70°C in an ultralow temperature freezer until the time of spectroscopic study. Preliminary experiments at excitation wavelengths from 360–500 nm with a single colposcopically normal biopsy indicate <12% change in the fluorescence intensity and line shape of fresh and snap frozen tissue. At the time of spectroscopic study, a sample was brought to room temperature and moistened with buffered isotonic saline solution (TRIS, pH 7.4). Control experiments with TRIS showed its fluorescence was at least a factor of 1,000 times less than that of cervical tissue at all wavelengths investigated. The epithelial surface of the sample was mounted on the inner surface of a fused silica cuvette for spectroscopic measurements. The samples were later fixed in 4% formaldehyde and sent for his-
Fluorescence Properties of Cervical Tissue

TABLE 1. Histologic Analysis

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Patient number</th>
<th>Colposcopic appearance</th>
<th>Histologic appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>1</td>
<td>Abnormal</td>
<td>Inflammation</td>
</tr>
<tr>
<td>002</td>
<td>1</td>
<td>Normal</td>
<td>Inflammation</td>
</tr>
<tr>
<td>003</td>
<td>2</td>
<td>Abnormal</td>
<td>HPV</td>
</tr>
<tr>
<td>004</td>
<td>2</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>005</td>
<td>3</td>
<td>Abnormal</td>
<td>Inflammation</td>
</tr>
<tr>
<td>006</td>
<td>3</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>007</td>
<td>4</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
<tr>
<td>008</td>
<td>4</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>010</td>
<td>5</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>011</td>
<td>6</td>
<td>Abnormal</td>
<td>HPV</td>
</tr>
<tr>
<td>012</td>
<td>6</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>013</td>
<td>7</td>
<td>Abnormal</td>
<td>CIN III</td>
</tr>
<tr>
<td>014</td>
<td>7</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>020</td>
<td>8</td>
<td>Normal</td>
<td>Inflammation</td>
</tr>
<tr>
<td>027</td>
<td>9</td>
<td>Abnormal</td>
<td>CIN II</td>
</tr>
<tr>
<td>028</td>
<td>9</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>035</td>
<td>10</td>
<td>Abnormal</td>
<td>CIN II-III</td>
</tr>
<tr>
<td>036</td>
<td>10</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

RESULTS

Two cervical biopsies were too small for fluorescence measurements. The results of the histologic sample analysis of the 18 samples studied are shown in Table 1. Nine samples were classified as normal, 4 showed nonspecific inflammation, 2 showed evidence of HPV infection, and 3 exhibited CIN. The positive predictive value for colposcopic detection of histologic abnormality was 88%, with a specificity and sensitivity of 89% and 78%, respectively (26). An average decrease of 14.8% (±11.5%) was observed in the intensities of the initial and final 260 nm emission spectrum; no changes were observed in lineshape. No data were excluded due to photobleaching.

Figure 1a,b shows the average normalized EEMs of histologically normal and abnormal biopsies, respectively. EEMs are presented as contour plots with emission wavelength on the abscissa and excitation wavelength along the ordinate. Contour lines connect points of equal fluorescence intensity. In each plot, 3 sets of linearly spaced contours are shown: 10 from 0.001 to 0.01 (-), 10 from 0.01 to 0.1 (-) and 10 from 0.1 to 1.0 (+) where fluorescence intensities are given in units relative to the intensity at 260 nm excitation, 330 nm emission. An additional contour is shown at 0.0285 (+) in Figure 1b.

In each tissue type, the most intensely fluorescent peak occurred at 290 nm excitation, 330 nm emission (320, 330 nm). In addition to this peak, several other fluorescence excitation emission maxima were observed in the EEMs of all tissue types. A shoulder was noted at (330, 385 nm) that was most well defined in the EEMs of normal tissue. A shoulder was also observed at (290, 420 nm), which was least well defined in the EEM of normal samples. In the average EEM of normal tissue, a single maximum was observed at (350, 450 nm); however, two maxima at (350, 450 nm) and (370, 470 nm) were present in the aver-
Fig. 1. Average normalized fluorescence EEMs of (a) 9 histologically normal cervical biopsies and (b) 9 histologically abnormal cervical biopsies. The EEMs are presented as contour plots with emission wavelength on the abscissa and excitation wavelength along the ordinate. Contour lines connect points of equal fluorescence intensity. In each plot, 3 sets of linearly spaced contours are shown: 10 from 0.001 to 0.01 ( ), 10 from 0.01 to 0.1 ( ), and 10 from 0.1 to 1.0 ( ) where fluorescence intensities are given in units relative to the intensity at (290 nm, 350 nm). An additional contour is shown at 0.025 ( ) in (b) to highlight the excitation emission maximum at (370, 470 nm).

Figures a) and b) show that the average EEM of abnormal biopsies. Finally, an emission maximum was observed within 10 nm of (470, 545 nm) in the EEMs of all tissues. It should be noted that all valleys between peaks correspond to the hemoglobin Soret absorption maximum at 415 nm [26].

Table 2 summarizes the average relative fluorescence intensities ±95% confidence intervals at the excitation emission maxima for the histologically normal and abnormal tissues. The average relative peak intensities of the normal samples are greater than those of the abnormals; in addition, the associated confidence intervals for the normals are greater than those for the abnormals. These data were used to determine whether relative fluorescence intensities of the histologically normal tissues were statistically different than those of the abnormal tissues at each of these maxima. A standard one-sided t-test was employed to test the hypothesis that the average relative intensities of the normal tissues were greater than that of the abnormal [27]. This hypothesis was found to be true below the 0.1 level of significance for all excitation emission maxima. A set of histologically normal and abnormal biopsies was obtained from each of 9 patients (Table 1). Paired and unpaired t-tests were performed on these data to test the hypothesis that the fluorescence intensities of a normal sample were greater than that of the abnormal from the same patient. At all excitation emission maxima other than (290, 420 nm), the level of significance for the paired test was lower than that of the unpaired test.

Although Table 2 highlights differences in the relative fluorescence intensities, it fails to indicate those present in the spectral line shapes of the different tissue types. Figure 2 illustrates the average relative fluorescence emission spectra at the excitation wavelengths corresponding to excitation emission maxima observed in all tissue types. Although these data are represented in the EEMs of Figure 1a,b, (an emission spectrum can be represented by a line parallel to the emission axis, an excitation spectrum by a line parallel to the excitation axis), it is difficult to appreciate subtle differences in the line shapes of broad emission peaks in these contour plots.

The average relative emission spectra at 260 nm excitation (Fig. 2a), shows that the 330 nm emission peak is broadened in the spectra of normal tissues and narrowed in the spectra of tissues with CIN. At 330 nm excitation, the average relative emission spectra (Fig. 2b) clearly demonstrates that the shoulder at (330, 385 nm) is most well defined in normal tissue. However, examination of these spectra also reveals that the emission peak near 440 nm is shifted to longer wavelengths in tissues with nonspecific inflammation. This shift is also seen to a lesser degree in the emission spectrum obtained at 350 nm (Fig. 2a).
and 450 nm excitation (Fig. 2d) in tissues with nonspecific inflammation relative to other tissue types. The average relative excitation spectra at 470 nm emission (Fig. 2e) illustrates a single excitation maxima for normal tissues at 350 nm excitation; a double excitation maximum is present for tissues with nonspecific inflammation at 350 and 370 nm. The average relative excitation spectra of tissues exhibiting HPV infection and CIN show some evidence of this double peak structure as well. The double peak structure was present above the noise level in excitation spectra of 4 of the 9 abnormal samples—2 with nonspecific inflammation, one with HPV infection, and one with CIN. None of the excitation spectra of normal samples exhibited this structure. Spectroscopic differences between histologically abnormal tissues are greatest at 350 nm excitation, in particular, differences between tissues with HPV infection and CIN are most pronounced here.

To investigate the ability of a simple spectroscopic algorithm to differentiate histologically normal and abnormal samples, the relative fluorescence intensities at (330, 385 nm) were plotted as a function of sample type (Fig. 3a). The average relative intensity of normal tissues is higher than that of abnormal. However, the diagnostic algorithm represented by the horizontal line in Figure 3a has a positive predictive value for detection of histologic abnormality of 67%, lower than that of colposcopy in this study. The sensitivity (83%) is higher than that of colposcopy, whereas the specificity (44%) is significantly lower than that of colposcopy.

The increased variability of normal tissue fluorescence and the better results of the paired t-test suggest a paired comparison may yield better discrimination. Figure 3b shows the same relative fluorescence intensities at (330, 385 nm) for the paired colposcopically normal and abnormal biopsies from 8 patients. Histologic diagnoses are also indicated. Here, the relative fluorescence intensity of colposcopically normal tissue is greater than that of colposcopically abnormal in all but one case. A combined spectroscopic and colposcopic algorithm using paired data was defined for histologic abnormality. Using colposcopy to identify normal samples, and spectroscopy to identify those samples as abnormal with a fluorescence intensity lower than 0.67 times that of the normal, resulted in a positive predictive value of 86%, a specificity of 85% and a sensitivity of 75%, comparable to that of colposcopy in experienced hands.

**DISCUSSION AND CONCLUSIONS**

This study indicates the potential of fluorescence spectroscopy for improving colposcopic detection of CIN. A simple, paired algorithm using colposcopic identification of normal tissue and spectroscopic identification of abnormal tissue based only on the relative fluorescence intensity at (330, 385 nm) approaches the performance of colposcopy in experienced hands. This combined technique has the advantage of being simple to automate, and future algorithms based on spectroscopic information at a multiplicity of wavelengths have the potential to differentiate various categories of histologically abnormal tissue. The small number of samples investigated and possible spectral differences between tissue
Fig. 2. Average relative fluorescence emission spectra of cervical tissue obtained at (a) 230 nm excitation, (b) 330 nm excitation, (c) 350 nm excitation, (d) 450 nm excitation. (e) Average relative fluorescence excitation spectra of cervical tissue obtained at 470 nm emission.

in vitro and in vivo [14, 28] point to the need for further work. This pilot study has important implications for pursuing these studies. Although Figure 2 indicates distinct spectral differences in the fluorescence of abnormal tissues, data from more samples are needed to establish the predictive value for differentiation of CIN, HPV infection, and nonspecific inflammation. Emission spectra at 330 nm excitation show the greatest differences among tissues with HPV infection and CIN and should be pursued further.
Fig. 3.  a: Relative fluorescence intensity at (330, 385 nm) for all 18 biopsies.  b: Relative fluorescence intensity at (330, 385 nm) for 16 paired colposcopically normal and abnormal biopsies from 8 patients.  Histologic diagnoses are also indicated.
Implementing a paired algorithm as in Figure 3b requires better understanding of the intriguing variability in the spectra of histologically normal samples. In particular, it is important to determine whether inter or intrapatient variability is more significant. In an attempt to understand this variability in future studies, correlations between the spectroscopy of normal cervix and the physiologic state of the cervix, such as stage in menstrual cycle, gestational history, and previous history of vaginal examinations, will be noted. In addition, correlations between histologic features such as the intensity and cellular components of any inflammation, the relative percentage of glandular mucosa and the thickness of the squamous mucosa will be noted.

It is interesting to note that fluorescence EEMs of normal and neoplastic cervix are similar to those of normal and neoplastic epithelial tissues reported previously. The 4 principle fluorescence excitation emission maxima identified in this study were also observed in the EEMs of normal and adenomatous colon [12] and normal and neoplastic urinary bladder [29], indicating that similar fluorophores may contribute to the signals of these tissues. This is not surprising; the tentatively assigned fluorophores (tryptophan, vitamin B3, derivatives, collagen, NADH, elastin) and modulating absorbers (oxy-hemoglobin) contributing to the EEMs of normal and neoplastic colon [12] and urinary bladder [29] are common to many biologic tissues. In addition to the common excitation emission maxima, the fluorescence EEM of normal colon exhibited an additional peak at (315, 430 nm) not observed in cervical tissue, illustrating potential differences in the fluorescence of these tissues.

Although similar fluorophores may contribute to the fluorescence spectra of a variety of neoplastic tissues, the significant variability in the biology of neoplasia [20–22] implies that the spectroscopic diagnostic algorithms for dysplasia may not be general, but, rather, organ specific. In an attempt to answer this question, we compared the EEMs and algorithm presented here at 330 nm excitation to fluorescence spectra and algorithms published for identifying dysplasia in other epithelial tissues. At 330 nm excitation, 385 nm emission, EEMs of neoplastic colon, bladder, and cervix all exhibit a decreased fluorescence intensity relative to that of normal tissue. Algorithms for differentiating adenomatous polyps from normal colon and hyperplastic polyps at 325 nm excitation [13] and 337 nm excitation [14] have utilized, in part, the relative drop in the intensity of fluorescence at this emission wavelength. It has been suggested that this emission peak can be ascribed to fluorophores associated with the structural protein collagen [12, 14, 18, 29] and that the reduction of this peak may be due to thickening of the epithelium, which contains less collagen than the supporting stroma. However, attempts to correlate fluorescence intensity to epithelial thickness have thus far been unsuccessful [14] and further, detailed histologic studies are required to confirm this hypothesis. It must be pointed out that, although epithelial thickening occurs in some dysplasias, it is not a universal finding [20–22] and that thickening of the epithelium may be associated with a variety of chronic irritative conditions as well [30].

A reduction in the peak fluorescence intensity of dysplastic colon relative to normal colon has also been observed at 370 nm excitation [12, 18]. At this excitation wavelength, a diagnostic algorithm was developed to differentiate normal colon from adenomatous polyps based primarily on changes in the peak fluorescence intensity. Specificity, sensitivity and positive predictive value exceeded 90% in a study of 65 samples from 32 patients using an unpaired comparison algorithm. This is in contrast to the fluorescence data from normal cervix reported here, where significant variability necessitated the use of paired diagnostic algorithms for optimal identification of dysplastic tissue.

This contrast illustrates the need for a better morphologic and biochemical understanding of the origin of fluorescence data from normal and dysplastic tissues. Particular emphasis must be given to the relation between architectural and cellular changes that accompany dysplasia and the fluorescence spectrum before a more general understanding of algorithm transportability can be developed.

In conclusion, the availability of systems for in vivo data acquisition [11, 14, 18, 19] and the implications of this study suggests a strategy for future in vivo work. Fluorescence emission spectra at 337 nm excitation (closest to 330 nm) will be obtained from multiple normal and abnormal sites from each participating patient. Data pairs will be compared with colposcopic and detailed histologic analysis to assess the predictive value of spectroscopic algorithms for identification of CIN, HPV infection, and nonspecific inflammation.
ACKNOWLEDGMENTS

Financial support from the Whitaker Foundation is gratefully acknowledged. The authors acknowledge the assistance of Shalini Jain, Barbara Tommasovic, and Nirmala Ramanujam in freezing tissue biopsies.

REFERENCES