AUTOFLUORESCENCE CHARACTERISTICS OF ORAL MUCOSA

Duncan R. Ingrams, FRCS,[1] Jagdish K. Dhingra, FRCS,[1] Krishnendu Roy, BTech,[1,3] Donald F. Perrault Jr, BS,[1] Ian D. Bottrill, FRCS,[1] Sadru Kabani, DMD,[2] Elie E. Rebeiz, MD,[1] Michail M. Pankratov, MS,[1] Stanley M. Shapshay, MD,[1] Ramasamy Manoharan, PhD,[4] Irving Itzkan, PhD,[4] Michael S. Feld, PhD[4]

[1] Otolaryngology Research Center for Advanced Endoscopic Applications, New England Medical Center, Tufts University School of Medicine, 750 Washington Street, NEMC 187, Boston, MA 02111
[2] Department of Oral Pathology, Tufts University School of Dental Medicine, Boston, Massachusetts
[3] Department of Biomedical Engineering, Boston University, Boston, Massachusetts
[4] George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Abstract: *Background.* The fluorescence characteristics of tissues depend upon their biochemical composition and histomorphological architecture, both of which undergo a change during malignant transformation. These changes are detectable as an alteration in the fluorescence spectral profile of the tissues.

Methods. Biopsy specimens from clinically suspicious lesions and normal-appearing oral mucosa were obtained from patients. Fluorescence spectroscopic measurements were obtained to study the differences between normal and dysplastic tissues and to determine the most appropriate excitation wavelength(s) for exploiting these differences.

Results. Fluorescence spectra from a total of 12 histologically normal (healthy mucosa or benign lesions) and ten abnormal (dysplastic or malignant) tissue samples were compared. Significant spectral differences were seen between the two groups. These differences were most marked at the excitation wavelength of 410 nm. Using this wavelength, fluorescence correctly diagnosed 20 of 22 samples studied.

Conclusions. This technique accurately differentiates normal from abnormal tissues in vitro and has the potential applications for in vivo use as a noninvasive diagnostic tool. © 1997 John Wiley & Sons, Inc. Head Neck **19:** 27–32, 1997.

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Correspondence to: Dr. Shapshay

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Oral cancer is expected to account for over 28,000 new cases in the United States in 1995. It is estimated that there will be more than 8000 deaths, some 1% to 2% of all cancer deaths.[1] Ninety-five percent of cases occur in people over the age of 40 years, and they are twice as common in men as in women. Alcohol and tobacco use are associated with an increased risk of developing these cancers.[2] Ninety-five percent are squamous cell carcinomas, and many arise in existing premalignant lesions of the oral cavity mucosa, such as leukoplakia or erythroplasia. The chances of surviving oral cavity carcinoma are markedly increased by early detection and treatment, with a

5-year survival rate of 80% for cancers confined to the mucosa but only 20% if lymph node metastases are present.[3] Unfortunately, early lesions can be indistinguishable from benign conditions by clinical examination alone.[4,5]

Current management of suspicious lesions includes biopsy, which may require general anesthesia, followed by definitive cancer therapy or repeated observation in clinic. Further biopsies of any areas of concern may be required. Exfoliative cytology and toluidine blue staining can be used to improve on the surgeon's ability to distinguish benign from premalignant and malignant lesions,[6,7] but both techniques rely on experience for their interpretation. A simple technique that can accurately diagnose premalignant and malignant lesions in situ might avoid unnecessary biopsy of benign lesions. If sensitive enough, it also may be able to improve prognosis, by alerting physicians to cellular changes at a stage where they are undetectable by clinical examination alone. Such a technique also may be important in screening at-risk individuals, and in assessing resection margins in excisional surgical procedures.

Fluorescence spectroscopy was first shown to be able to distinguish cancerous from normal tissue 10 years ago,[8] but to date, there have been only a few preliminary studies involving fluorescence spectroscopy of squamous epithelium from the upper aerodigestive tract.[9–11] This study seeks to determine whether fluorescence spectroscopy can differentiate normal and dysplastic tissues in vitro and choose the optimal excitation wavelength for future in vivo studies.

MATERIALS AND METHODS

Biopsy samples were collected from 11 patients with suspicious oral cavity mucosal lesions in the Departments of Otolaryngology–Head and Neck Surgery and Oral Surgery, at the New England Medical Center. Two tissue specimens measuring approximately 5 mm × 5 mm were obtained from each patient, one from the clinically suspicious lesion and the other from an anatomically corresponding site which appeared clinically normal. There was a total of 10 specimens from the buccal mucosa, 6 from lateral border of tongue, 4 from floor of mouth, and 2 from gingiva. At the time of the biopsy, the physician's clinical impression was recorded. Informed consent was obtained from each patient. This study was reviewed and approved by the Human Investigation Review Committee of Tufts University School of Medicine–New England Medical Center and the Committee on Use of Humans as Experimental Subjects of the Massachusetts Institute of Technology (MIT). The biopsy specimens were flash-frozen in liquid nitrogen–cooled isopentane and transported to George R. Harrison Spectroscopy Laboratory, MIT, for spectroscopic measurements.

Specimens were thawed and brought to room temperature and immersed in phosphate-buffered saline (pH 7.4); spectroscopic measurements were performed using a commercial fluorimeter (Fluorolog, Instruments SA Inc, NJ).[11] The system consists of a continuous white light source (Xe lamp), an excitation monochromator (Spex 6810), a sample chamber, an emission monochromator, a photomultiplier tube detector cooled with a peltier cooling system, and a personal computer with the Spex DM3HOST data acquisition, control, and analysis software.

Tissue specimens were placed in a quartz cuvette in phosphate buffer inside the sample chamber. The samples were orientated so that the mucosal surface was facing the excitation beam. The excitation beam measures 3 mm × 5 mm in size and strikes the tissue specimen at normal incidence. A small fraction of the excitation beam is diverted by means of a beam splitter into a reference cell containing a standard fluorescent solution (Rhodamine B) whose fluorescence is monitored with a photomultiplier tube. The measured reference intensity is used to correct time-to-time intensity variations of the arc lamp during one spectral measurement and normalize out the intensity variations at different excitation wavelengths. The sample fluorescence is divided by this standard fluorescence of Rhodamine B to correct for such variations. Excitation wavelengths in the range of 250 nm to 500 nm in 10-nm steps were used to induce autofluorescence, and emission spectra were measured in the wavelength range of 350 nm to 700 nm in 10-nm steps. Each spectrum was corrected for source-intensity variations and spectral response of the system. These spectra were then processed to generate a single excitation-emission matrix (EEM) for each sample. Surface and contour plots were created for each sample.

After the spectral measurement, the mucosal surface of the specimens was marked with India ink, fixed in 10% formalin and submitted for histopathology. The fluorescence spectral results were compared, with histology as the gold standard.

RESULTS

A total of 22 clinically normal and abnormal samples were collected from the oral cavity and analyzed as described. On histological examination, 10 specimens were classified as abnormal (dysplastic or malignant), and 12 samples were classified as normal (healthy mucosa or benign changes).

The fluorescence spectra from all the samples exhibited characteristic contours centered at 350 nm (300 nm excitation), 480 nm (350 nm excitation), 515 nm (460 nm excitation), and 630 nm/670 nm (410 nm excitation). Important differences were observed between normal and abnormal oral tissues. To better analyze the spectral differences, ratio contours were calculated, by dividing the EEMs of the abnormal tissue by that of the normal site from the same patient. The ratio maps summarized all the spectroscopic bands where the fluorescence properties of normal and dysplastic tissue differed. Figure 1 shows one such ratio EEM contour map.

Fluorescence spectra were then plotted for individual excitation wavelengths, and most marked differences between normal and dysplastic tissues were observed with excitation wavelength of 410 nm. The most important finding in dysplastic and malignant samples were increased fluorescence above 600 nm, as compared with normal with prominent emission peaks at 635 nm and 690 nm, indicating that dysplastic lesions exhibited a fluorescence feature which is not present in normal mucosa. Figure 2 shows the typical fluorescence spectrum of normal and dysplastic tissue using 410 nm excitation wavelength.

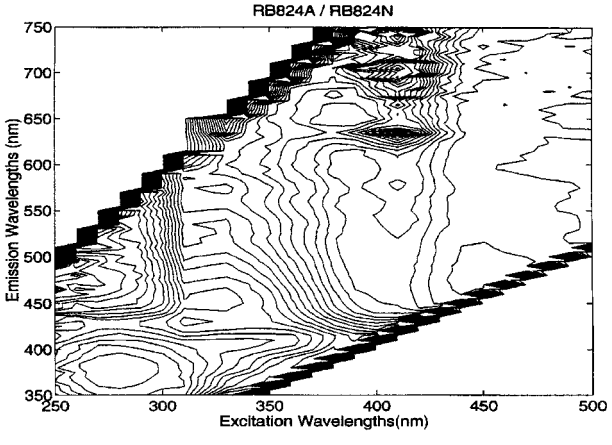

FIGURE 1. Ratio of excitation to emission matrix (EEM) contour map between dysplastic and normal oral mucosal tissues highlighting the intense emission peaks at 635 nm and 690 nm in dysplastic tissue, with excitation wavelength of 410 nm.

Using the criteria of increased red fluorescence and an emission peak at 635 nm, we developed an empirical algorithm to quantify the slope of the spectral curve between 615 nm and 640 nm, and the area under the curve between 600 nm and 650 nm. The resulting values were plotted on a scatter chart. The ratio of emission intensity at 640 nm and 615 nm was plotted on the X axis, and the area under the curve between 600 nm and 650 nm was plotted on the Y axis. A decision line was drawn to minimize the number of misclassified samples (Figure 3). Twenty of the 22 specimens fell within the correct-decision plane. There was one false-positive result in a tissue sample with inflammatory ulceration, and one false-negative result in a cancerous tissue where the peak intensity in the red region was at 600 nm and was therefore not documented by the statistical algorithm. The sensitivity, specificity, and overall accuracy of fluorescence to differentiate histologically normal from dysplastic and cancerous tissues were 90%, 91%, and 91%, respectively, histology being the gold standard.

DISCUSSION

Fluorescence spectroscopic techniques to detect malignant tumors have been under investigation for many years, but most of the early studies were based on the use of external fluorescent agents such as hematoporphyrins, which are preferentially concentrated in malignant tissues.[12] Autofluorescence from malignant tumor was observed by Policard as early as 1924. Alfano et al[8] in 1984 conclusively showed that autofluorescence can effectively differentiate between normal and cancerous tissues. Several researchers have studied the use of such techniques in diagnosis of malignant and premalignant tissues in different parts of the human body.[13] Alfano et al reported studies in gynecologic and colon tissues as well as breast tumors and lung cancers.[14–17] Prospective studies on colon cancer and colonic adenomatous polyps have shown a high degree of diagnostic sensitivity and specificity.[18–24] Similar results from cervical lesions,[25–28] bladder cancer,[29] lung cancer,[30–34] and esophageal cancer[35] have been reported recently. All these groups use different approaches (in terms of wavelengths of excitation, diagnostic algorithms, and data analysis) for differentiating between normal and diseased tissues.

Oral cavity carcinomas occur at a rate of 6.8 per 100,000 in the population of the United States.[36] Approximately 90% of all malignant tumors of the oral cavity are squamous cell carcino-

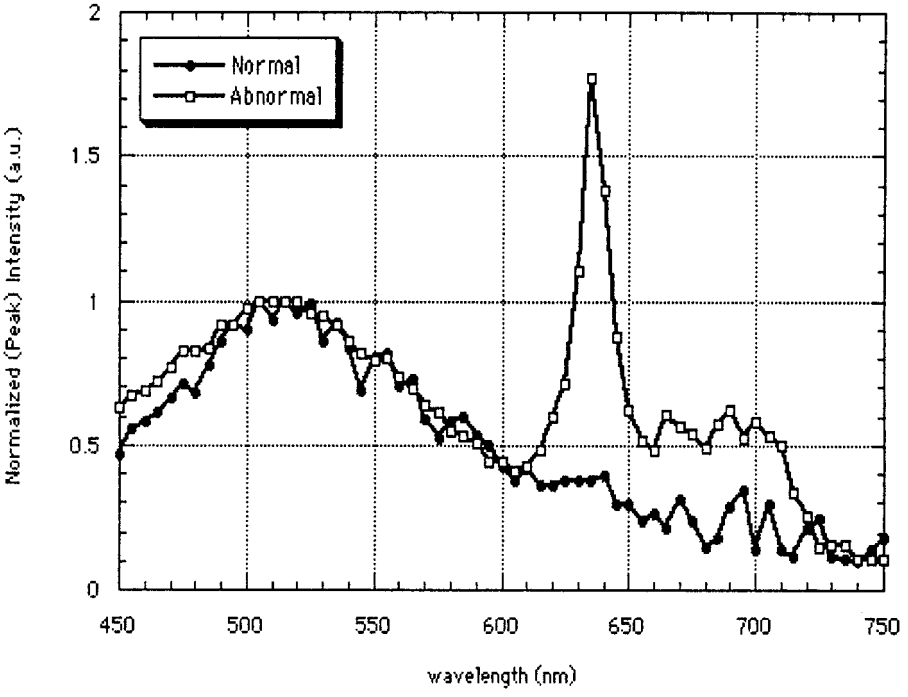

FIGURE 2. Fluorescence spectrum of normal and cancerous oral mucosal tissues with 410 nm excitation wavelength, showing increased red fluorescence (>600 nm), with a prominent peak at 635 nm.

mas, which arise from the mucosa and which, in most cases, probably are preceded by premalignant lesions. Histological examination suggests that invasive squamous cell carcinoma may be the end point of a sequence of increasing cellular atypia known as dysplasia. This typically progresses from mild to moderate to severe dysplasia, and to carcinoma in situ, before developing into invasive cancer. These premalignant conditions, and carcinoma in situ, are especially diffi-

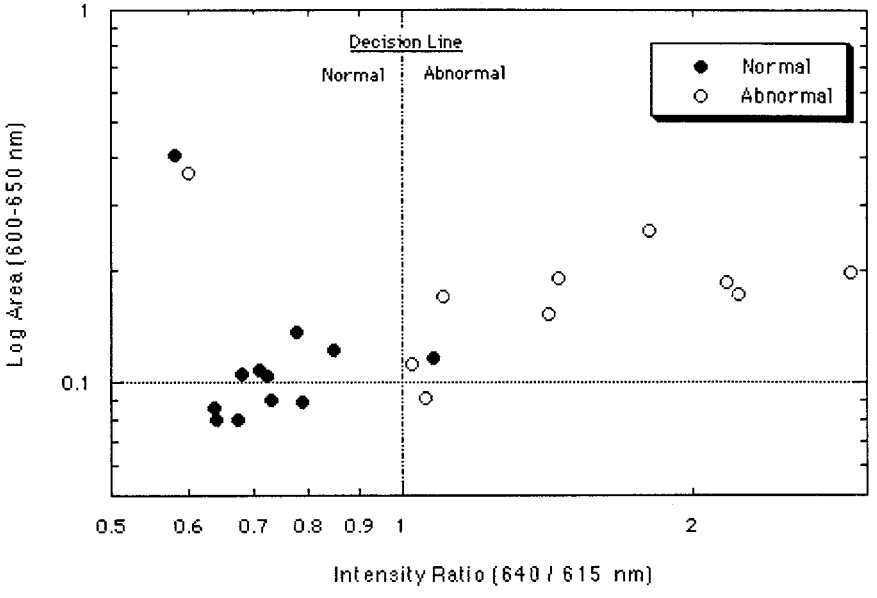

FIGURE 3. Scatter plot showing the distribution of normal and abnormal (dysplastic/cancerous) tissue samples after application of a diagnostic statistical algorithm to the fluorescence measurements with 410 nm excitation light.

cult to distinguish from normal mucosa or nonmalignant conditions. Patches of mucosa in the mouth that appear white are sometimes called by the descriptive term leukoplakia, but this appearance can be due to a variety of pathologies. Most are due to benign epithelial hyperplasia, but patches also may contain areas of dysplasia, carcinoma in situ, and frankly invasive carcinoma.[37] These conditions all may appear identical on visual examination, and, unless an area of leukoplakia regresses quickly, the area must be biopsied for histological examination. Diagnostically, the areas of mucosa that form red, raised areas called erythroplasia present less of a challenge, as they carry a much greater risk of representing malignancy, and therefore all should be biopsied.

A few studies have been reported involving fluorescence spectroscopy of oral cavity carcinomas using a single excitation wavelength.[13] Preliminary work has recently been presented on autofluorescence studies of squamous epithelium from the upper aerodigestive tract,[9–11] but the field of spectroscopic diagnosis of oral lesions is still largely underexplored.

Our in vitro study was performed to achieve two main objectives: first, to test our hypothesis that autofluorescence spectroscopy can differentiate normal from diseased oral tissue; second, to determine the most appropriate excitation wavelength(s) for exploiting these differences. We based our diagnoses of dysplasia or cancer on an increase in fluorescence in the red region at 600–710 nm and the presence of a fluorescence peak at 635 nm when the specimen was excited by 410 nm light. Emissions in this area have not been fully characterized but may be due to porphyrins in general and protoporphyrin IX (PP IX) in particular. There is some evidence that PP IX accumulates in malignant cells.[38,39] This seems to occur because of a relative lack of ferrochelatase, an enzyme required to incorporate chelated iron into PP IX to form the heme molecule. The rapid growth of malignant cells exhausts the available iron supply, leading to an increase in intracellular concentration of PP IX.[38,39] If that is the case, then we are in effect carrying out an in situ biochemical assay for PP IX and assigning the diagnosis of "abnormal" to cells that contain greater than a certain concentration. However, some researchers attribute the red region peak and the increased red fluorescence to porphyrins produced by certain bacteria and have cautioned that because other cells also may concentrate porphyrins, diagnosis based on this technique may be inaccurate.[12] A detailed biochemical study needs to be performed to assign exactly the spectral signatures to individual chromophores.

Our study is significant because it conclusively demonstrates the ability of autofluorescence spectroscopy to differentiate normal and dysplastic mucosa in vitro with great degree of accuracy. It also provides important guidance for the future in vivo studies, as the results clearly show that of the whole spectrum of light between 250 nm and 500 nm, excitation wavelength of 410 nm is the most significant for differentiating normal and dysplastic or cancerous oral mucosa.

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