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Tissue Autofluorescence as an Intermediate Endpoint in Cancer Chemoprevention Trials

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Abstract Intermediate endpoints which reflect responsiveness to chemopreventive agents are needed in clinical trials. A potential problem in the assessment of these endpoints is their requirement for invasive biopsies. Secondly, their expression within the aerodigestive tract is doubtfully uniform. Without clinically definable disease, the potential for biopsy sampling error is significant. Our approach to this problem is the analysis of intrinsic tissue fluorescence. Various cellular components exist within tissues with the innate capacity to emit a characteristic spectral signature when excited at a particular wavelength of light. It can be postulated that cells in varying stages of progression towards malignancy will differ in both the qualitative and quantitative nature of these intrinsic cellular fluorophores which include folate derivatives and cytokeratins, as well as various vitamins and coenzymes. Using current bioptical technology, we have tested the applicability of tissue autofluorescence to chemoprevention studies through various model systems. In an *N*-nitroso-*N*-methylbenzylamine (NMBA)-induced rat esophageal cancer model, alteration of the 380 nm emission pattern corresponded to disease progression from normal mucosa through dysplasia to invasive cancer. In a multicellular tumor spheroid model, *trans*-retinoic acid (RA) altered autofluorescent profiles at multiple wavelengths including intensities at 340 nm, 450 nm, and 520 nm. Such RA-induced alterations corresponded to changes in the state of spheroid differentiation. *In vivo* human studies are in progress which suggest that aerodigestive mucosal neoplasias can be discriminated from normal mucosa by their autofluorescent profile. Because aerodigestive mucosa can be scanned without the need for invasive biopsies, the assessment of tissue autofluorescence as an intermediate endpoint may prove valuable.

Key words: autofluorescence, intermediate endpoint, chemoprevention

Tissue autofluorescence has been the focus of investigation throughout the twentieth century. In the early 1900s, German scientists explored the biochemical basis for pigment in insect wings [1]. Various compounds responsible for visual characteristics, termed pteridines, were isolated and subsequently classified according to their color-emitting properties. Yellow pigment was termed xanthopterin; white pigment, leukopterin; red pigment, erythropterin; and so on. The science of spectral chemistry has grown dramatically since these pioneering efforts.

Clinical studies quickly followed these initial scientific investigations. In the 1940s and early 1950s, dermatologists and dental professionals used tissue fluorescence as a means of defining nutritional deficiency states [2–4]. Ultraviolet light emitted from a Wood-light was utilized to fluoresce the tongue. Several investigators dur-

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ing that period documented the loss of red fluorescence following ultraviolet stimulation of patients with certain deficiency states, including pernicious anemia, hypochromic anemia, sprue syndrome, and vitamin B complex deficiencies [2–4]. Schaffer *et al.* [5] subsequently demonstrated that subsequent vitamin supplementation would restore fluorescence.

Recent technological advances in fluorescence spectroscopy has opened new vistas. Such spectroscopy provides the means to characterize physical and chemical changes occurring in diseased tissues and cells, offering exciting possibilities for novel fluorescence diagnostic and therapeutic approaches. The underlying physical basis for such an approach is that fluorescence is strongly influenced by the chemical composition and cellular structure of tissues. A change in the state of a cell or tissue, such as from normal to cancerous, will change the fluorescence profile. Recent efforts investigated the use of the intrinsic fluorophores present in tissues in order to characterize their physiological state. The use of such intrinsic fluorophores enables us to gain information about the tissue microstructure and the changes therein without interfering with the native cellular environment. Tissues are made up of proteins, nucleic acids, lipids, and many other fluorescing and nonfluorescing chromophores. A number of natural fluorophores in cells and tissues fluoresce in the ultraviolet and visible regions, such as flavins, tryptophan, nicotinamide adenine dinucleotide (NADH), and collagen.

One potential benefit of measuring intrinsic fluorescence is the ability to scan the entire aerodigestive mucosa for evidence of disease. Most premalignant diseases such as bronchial metaplasia and upper aerodigestive mucosal dysplasias cannot be detected by standard endoscopic techniques. Characteristically, evidence of intraepithelial neoplasias within surgical margins is found at a distance from a primary aerodigestive cancer. This is most evident in patients with a history of chronic tobacco and alcohol abuse, leading to the term "field cancerization" [6]. All mucosa exposed to a carcinogen is at risk for malignant transformation. Patients with tobacco-induced cancers within the aerodigestive tract frequently demonstrate field cancerization by their propensity to develop multiple primary malignancies [7]. While it is clear that random sampling of mucosa for histologic evaluation may reveal areas of abnormal growth, random biopsies of the mucosa at risk for malignant transformation may lead to sampling error. In the majority of circumstances, there is no other means to clinically discriminate between mucosa expressing normal differentiation and proliferation pathways versus that with altered growth patterns [8]. Analysis of intrinsic mucosal fluorescence may obviate this problem. Mucosa in various proliferation or differentiation states may present characteristic spectral patterns. The entire mucosal surface can be scanned, spectral profiles within various sites can be mapped, and targeted biopsies can then be performed. Furthermore, changes in fluorescence profiles over time can be documented. Finally, should intrinsic mucosal fluorescence represent various stages of proliferation and differentiation, factors which modify these stages would be evidenced by tissue fluorescence analysis. In the context of chemoprevention trials, where various vitamin derivatives and other agents will impact on mucosal growth patterns, tissue autofluorescence may eventually represent a meaningful intermediate endpoint. Current evidence for this hypothesis is documented below.

IN VITRO STUDIES OF FLUORESCENCE

Significant progress has been made over the past few years in using fluorescence spectroscopy to detect cancer and atherosclerosis [9–19]. After successfully applying fluorescence spectroscopy to detect tooth decay, Alfano and coworkers [9,10,20] extended their work to distinguish normal rat and human tissues from cancerous tissues. Later, Kittrel *et al.* [11] and Anderson *et al.* [15] used fluorescence spectroscopy to diagnose fibrous arterial atherosclerosis. Further studies by Deckelbaum *et al.* [14] showed that fluorescence spectra from a normal aorta and atherosclerotic tissues are different. Similar work has been done to study colon cancer by Petras and co-workers [21] and by Kapadia and co-workers [16].

Multiple excitation sources were utilized for the above studies of human tissues, including argon, helium-cadmium, and pulsed-nitrogen lasers. Our laboratory uses a fluorescence spectrometer (Mediscience Technology CD-scan,

Perkin-Elmer Spectrometer) to obtain the fluorescence and excitation spectra from tissue samples. The excitation source in this spectrometer is a special Xenon flash tube that produces intense, repetitive, short-duration pulses of radiation over the spectral range of the instrument. A small lamp close to the excitation source maintains an even triggering of the Xenon flash tube at line frequency. Energy from the source is focused by the ellipsoidal mirror and reflected by the toroidal mirror onto the entrance slit of the excitation monochromator. The monochromator consists of the entrance slit with the center wavelength determined by the setting of the grating, the angle of which is controlled by a motor. The majority of the excitation beam is transmitted to the sample area via the focusing toroidal mirror, and a small proportion is reflected by the beam splitter onto the reference photomultiplier. The fluorescence emitted by the sample is focused by the toroidal mirror onto the entrance slit of the emission monochromator. The monochromator consists of the entrance slit, a spherical mirror, grating, and exit slit. A narrow wavelength band emerges from the exit slit; the center wavelength is determined by the setting of the grating. The excitation and emission monochromators can be scanned over their ranges independently, synchronously, or driven to select points in their ranges. Synchronous scanning can be either a fixed wavelength difference or a fixed energy difference between the excitation and emission monochromators. The covered spectral ranges of the monochromators are about 250 nm to 800 nm for excitation and about 250 nm to 700 nm for emission, with suitable photomultipliers. The fluorescence and excitation spectra can be stored and processed by a personal computer, analyzed with special software (Mediscience), and displayed on a computer screen or printed. Using the above system, initial work by Alfano *et al.* [9,10] on animal and human breast and lung cancers was performed using 488 nm laser radiation and other visible (lamp) excitation wavelengths. The spectral difference between normal and cancerous breast tissues at 488 nm excitation was traced to the different amounts of blood present in the two different tissue types. The absorption of fluorescence by hemoglobin leaves a signature on the tissue emission spectra which was used to differentiate normal from cancerous tissues. Later, the inability to distinguish malignant breast tumors from benign tissues and tumors using various visible excitation wavelengths from the CD-scan system led to the excitation of NADH, tryptophan, and collagen using UV wavelengths in an effort to find a suitable diagnostic method [17, 18] for separating malignant from benign tissues.

Using this altered fluorescence spectrometer design, we conducted studies to determine our ability to detect carcinogen-induced premalignant changes in the upper aerodigestive tract using an animal model (Glasgold MR, Savage HE, Pinto J, Alfano R, Schantz SP, submitted for publication). The well-defined *N*-nitroso-*N*-methylbenzylamine (NMBA) model was used to induce esophageal carcinoma in Sprague-Dawley rats. Following varying intervals of exposure to intraperitoneal-injected NMBA (4 mg/kg), gross and histological analyses of esophageal mucosa were performed. Concomitantly, esophageal tissue was isolated and analyzed for spectral characteristics. Following five weeks of exposure, mucosal changes consisted primarily of diffuse severe hyperplasia with areas of dysplasia. No gross abnormalities could be identified. By 13 weeks, nearly all rats exposed to NMBA developed esophageal tumors. Concomitant with histologic changes identified at five weeks were characteristic spectral abnormalities. Specifically, evaluation of the excitation emission spectra at 380 nm identified two peak intensities (290 nm and 330 nm). Normal esophagus expressed a 290 nm/330 nm ratio of approximately $1.12 \pm .04$ (S.E.). After five weeks of NMBA exposure, when premalignant histologic changes were noted but before gross disease became apparent, the 290 nm/340 nm ratio significantly increased to 1.69 $\pm$ .11 (S.E.). Results demonstrated the capacity of intrinsic fluorescence changes within mucosa to characterize progression from a normal to a premalignant state. The physiologic mechanism responsible for observed spectral changes is currently under investigation.

Along with the above animal model studies, our laboratories are simultaneously investigating the capacity of chemopreventive agents to modify intrinsic tissue fluorescence *in vitro* [22]. Experiments were conducted on a multicellular tumor spheroid (MTS) derived from a metastat-

ic squamous cell carcinoma of the larynx. Initial fluorescence analysis was performed on a MTS treated for ten days in either control or β-all-*trans*-retinoic acid (RA)-containing medium. Following ten days of exposure, RA consistently inhibited growth. Accompanying alterations in the MTS growth were alterations in the intrinsic MTS fluorescence. Excitation of the MTS by 340 nm light showed several fluorescent changes, including a slight shift from the 450 nm fluorescence maxima of the untreated MTS (Fig. 1). Likewise, an increase in bandwidth of approximately 20 nm at 50% fluorescence intensity of the 450 nm maxima was observed. These data support the original hypothesis underlying the investigation, *i.e.*, chemopreventive agents have the capacity to modify the fluorescence of aerodigestive mucosa. Tissue fluorescence may in future years represent a valuable endpoint in chemoprevention trials involving patients with neoplasias of the upper aerodigestive tract.

IN VIVO STUDIES OF TISSUE FLUORESCENCE: AERODIGESTIVE MUCOSA

Extrapolating from the initial *in vitro* studies demonstrating the capacity of fluorescence analysis to discriminate between various diseased tissues, investigators have applied this technology to *in vivo* circumstances. Colonic mucosa has been the most extensively evaluated [23–25].

Cothren *et al.* [23] performed one of the initial *in vivo* studies on colonic mucosa. The authors used a nitrogen-pumped dye laser (370 nm wavelength) with an average power of 270 μW at the distal tip delivered in 3 ns pulses. Fluorescence emission spectra were collected from 350 nm to 700 nm. Colonoscopy was performed in twenty patients. Spectral analysis was performed on 31 adenomas, 4 hyperplastic polyps, and 32 histologically normal areas. These investigators noted that fluorescence intensity at 460 nm was approximately fourfold greater in normal mucosa as compared with adenoma. In addition, the fluorescence intensity at wavelengths greater than 650 nm was consistently greater in adenomas when compared with normal mucosa. The 480 nm intensity emitted by hyperplastic polyps was intermediate to adenomas and normal mucosa. Using an algorithm involving the 460 nm and 650 nm intensity readings, Cothren *et al.* [23] were able to diagnose colonic adenomas with a sensitivity of 100% and a specificity of 97%. The predictive value of a positive test for adenoma was 97%.

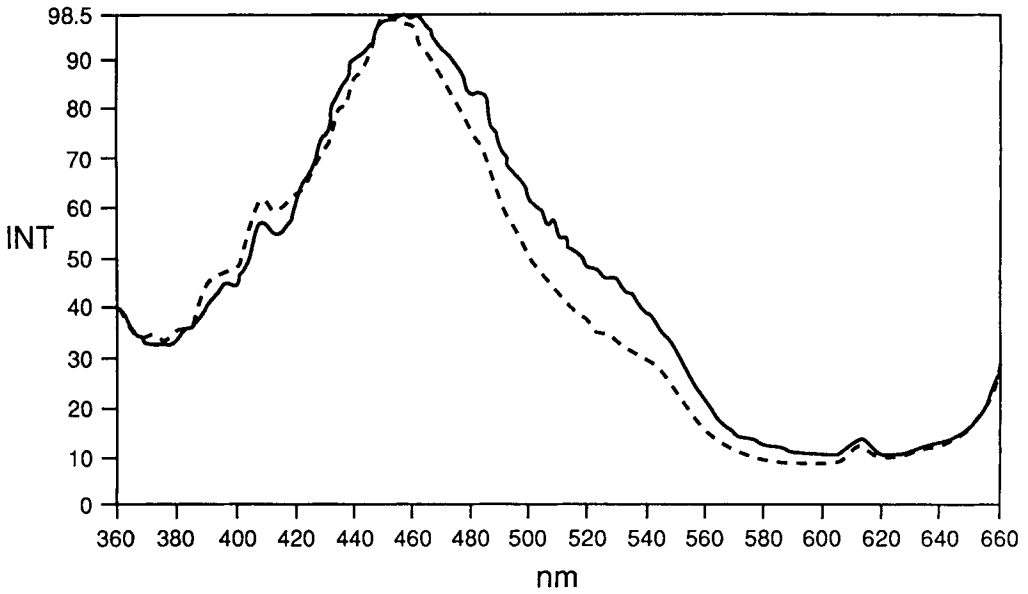

Fig. 1. Retinoic acid (RA) induces changes in intrinsic tissue fluorescence. Following 10 days of treatment with 10^{-6} M RA, an increase in intensity at 520 nm was identified. Solid line represents RA-treated spheroid. Dotted line represents untreated control.

The authors suggest that the diagnostic accuracy of autofluorescent examination exceeded normal endoscopy by 20%.

Subsequent studies by Schomacker *et al.* [24] confirmed the ability of laser-induced fluorescence analysis to discriminate between neoplastic and non-neoplastic mucosa *in vivo*. In contrast to the study of Cothren *et al.* [23], Schomacker and colleagues [24] used a pulsed-nitrogen laser emitting a 337 nm light for tissue excitation. All *in vivo* samples were observed to fluoresce at two maxima, *i.e.*, 390 nm and 460 nm, which were believed to arise from collagen and NADH, respectively. The principal difference between normal mucosa and neoplastic mucosa was related to a decrease in the 390 nm emission from the latter tissue. A multivariate linear regression analysis was used to determine the predictive value of *in vivo* autofluorescence analysis. Results showed a sensitivity, specificity, positive predictive value, and negative predictive value toward neoplastic tissue of 80%, 92%, 82%, and 91%, respectively. The authors concluded that fluorescence differences reflected polyp morphology rather than changes in fluorophores specific to polyps.

Studies on lung and upper aerodigestive mucosa have just begun. Hung and colleagues [26] have used an argon source (wavelength 488 nm), a krypton ion laser (wavelength 405 nm at 15 mW/cm^2 radiance), and a helium-cadmium laser (wavelength 442 nm). Laser light was transmitted through a 600 nm core diameter quartz fiber via a standard bronchoscope. Light emitted from the excited tissue was measured from 420 nm to 750 nm. The intent of this study was to distinguish premalignant and malignant conditions of bronchi from normal tissue by spectral characteristics. The authors point to the significance of calculating the ratio of, *i.e.*, the relative intensity of, light at one wavelength to the intensity of light at another. The use of this ratio facilitates spectral analysis through several mechanisms. For example, it minimizes variables regarding light intensity such as angle and distance, which are difficult to control during endoscopic procedures. The authors noted differences in various lung tissues at the red:green ratio, *i.e.*, 690 nm versus 560 nm. They suggested that the 560 nm/690 nm ratio would be diminished in intraepithelial neoplasias.

Subsequent work by this group compared fluorescence bronchoscopy versus standard white light bronchoscopy [27]. They used a helium-cadmium (442 nm) laser on 55 patients with known lung cancer and 41 asymptomatic non-cancer volunteers. The laser-induced fluorescence was found to improve the detection of dysplasia and carcinoma *in situ* by 50% as compared to white light bronchoscopy with a specificity of 94% for both.

DISCUSSION

Studies involving upper aerodigestive mucosa have just begun. However, it is apparent that normal spectral signatures within various sites within the aerodigestive tract will differ. Each site must therefore represent its own control. This is reasonable given the differing characteristics of the mucosa within the oral cavity. Buccal mucosa, for instance, may differ dramatically from the mucosa on the dorsal tongue or the floor of the mouth, both in terms of thickness and keratinization patterns. Initial studies must clearly define these normal site-specific characteristics. Additionally, the normal mucosal profile population must be clearly defined. Spectral "normality" in populations predisposed to carcinogen damage, such as heavy smokers, would be suspect. As has been shown in initial studies utilizing the Wood-light, dietary deficiencies may confound analysis. Additional confounding factors may include infectious processes, *e.g.*, Candidial or bacterial. These issues will undoubtedly be addressed in the coming years. Also to be addressed are additional spectral analyses that can be performed, such as infrared and Raman analyses, as well as more sophisticated means of analyzing light transmission through tissues. Future years will determine the relevance of autofluorescence as an intermediate endpoint in cancer prevention strategies.

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