**In Vivo** Native Cellular Fluorescence and Histological Characteristics of Head and Neck Cancer

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ABSTRACT

Native cellular fluorescence (NCF) represents the innate capacity of tissues to absorb and emit light of a specified wavelength. The ability to define the relationship of in vivo NCF with biological characteristics of neoplastic disease may allow for an improved understanding of the clinical course of disease. Head and neck cancers from 35 patients were evaluated in vivo for NCF characteristics using a xenon lamp-based spectrometer coupled to a handheld fiberoptic probe. Spectral assessment was limited to λ 450-nm emission characteristics, in which tissues were excited at various wavelengths, ranging from λ 290 nm to λ 415 nm, and the intensity of λ 450 nm emission was recorded. Each cancer was subsequently biopsied and assessed for histological differentiation by a pathologist who was blinded to NCF analysis. Considerable variation in spectral characteristics between head and neck cancers was identified, which was determined, in part, by NCF characteristics of the normal mucosa from the same patient. Poorly differentiated tumors were more likely than well- or moderately differentiated tumors to have lower excitation maxima (P < 0.05 by ANOVA). Most significantly, the tumor differentiation status, as well as the probability of demonstrating recurrent disease, could also be related to the NCF characteristics of the patient’s normal mucosa from the same site within the upper aerodigestive tract. NCF analysis may represent an effective tool to identify biological characteristics of head and neck tumors in vivo without the need for invasive biopsies. Results suggest the need to explore the determinants of NCF characteristics expressed by clinically normal mucosa.

INTRODUCTION

NCF represents the innate capacity of tissues to absorb and emit light of specified wavelengths. The determinants of NCF are complex but principally relate to the qualitative and quantitative states of specific fluorophores, consisting of various proteins, coenzymes, nucleic acids, and other cellular components (1). Although the science of tissue fluorescence has been investigated for many years, only recent advances in optical engineering and computer technology have allowed for clinical investigations. These latter studies have consistently demonstrated that tissues in varying stages of cancer progression will differ in their NCF characteristics both in vitro and in vivo and that such differences may be useful in cancer screening (2–11).

The measurement of NCF expressed by upper aerodigestive mucosa may represent an important application of this technology (12). The oral cavity can be easily examined within the office practice setting, a factor that facilitates NCF investigations. The etiological agent for oral cancer, i.e., tobacco, has been well recognized, and its use identifies individuals at increased risk. The need for improved screening techniques in this latter population is evident due to the limited success of current screening strategies. Finally, research regarding chemopreventive agents against oral cancer suggests the need for sophisticated monitoring techniques that record the subclinical treatment effect without the need for invasive biopsies. Our previous efforts have focused on three areas of research: (a) in vitro model systems that explore the implication of cellular differentiation and proliferation on NCF characteristics of upper aerodigestive mucosa (13–15); (b) the use of animal carcinogenesis models that define the characteristics of NCF alterations induced by prolonged nitrosamine exposure (16); and (c) initial observational studies that explore differences between head and neck cancers and normal aerodigestive mucosa within an individual patient (10, 17, 18). Each of these studies has demonstrated that mucosa that exists in various biological states will differ in its NCF characteristics. Furthermore, a consistent finding in these initial studies relates to fluorescence abnormalities involving excitation wavelengths ranging from λ 300 nm to λ 340 nm and emission wavelengths at λ 450 nm. The principal fluorophore that absorbs and emits at this wavelength is NADH (1).

In this study, we performed an initial exploratory analysis of histopathological characteristics of head and neck cancer and its relationship to NCF. Given that our previous efforts suggest alterations in NCF with perturbations in cellular differentiation (13, 15), we specifically addressed this relationship in vivo.
PATIENTS, MATERIALS, AND METHODS

Patient Population. The patient population analyzed here consisted of 35 patients with squamous cell carcinoma of the head and neck. The patients consisted of 16 women and 19 men. As will be described below, NCF analyses in each instance were performed in vivo on the patient’s cancer and on the clinically normal tissue from the contralateral site of the cancer. The median age of the population was 64 years (range, 27–87 years). Biopsies of neoplastic and contralateral normal mucosa were performed after fluorescence analysis for routine histopathology. All samples were examined by a single pathologist who was blinded as to the fluorescence profile. Tumors were characterized routinely by members of the pathology staff as well-differentiated, moderately differentiated, or poorly differentiated, depending on the degree of keratinization. All pathological assessments were performed blinded as to fluorescence results. Sites of cancer within the upper aerodigestive tract included 18 tongue cancers, 8 floor-of-mouth cancers, 5 gingival cancers, 1 hard palate cancer, 1 buccal mucosa cancer, and 2 pharyngeal cancers. These 35 patients were chosen at random from the head and neck cancer population who presented for treatment at Memorial Sloan-Kettering Cancer Center. These patients were chosen without regard to previous treatment. Twenty-nine patients had previously untreated disease, whereas 6 patients had disease recurrence or a second primary malignancy at the time of NCF analysis. This study was approved by the institutional review board of Memorial Sloan-Kettering Cancer Center, and each patient’s written consent was obtained prior to study entry.

Fluorescence Instrumentation and in Vivo Analyses.
NCF analyses were performed in vivo in all circumstances using a handheld fiberoptic probe attached to a fluorescent spectrometer (CD Scanner; Mediscience Technology Corporation, Cherry Hill, NJ), as described previously in detail (18). The probe was 180 cm long and had an inner diameter of 3 mm and an outer diameter of 6 mm. The fibers were randomly bifurcated, with half to the emission side of the instrument. The optical fibers were recessed 3 mm in a metal sleeve at the tip of the probe, which prevented the fibers from coming into contact with the mucosal surface and also prevented extrinsic white light from interfering with the fluorescence analysis (18). The characteristics of the optical instrumentation have, likewise, been described in detail and consisted of a special xenon flash tube excitation source that produced an intense, repetitive (50–60 Hz), short-duration (8 μs) discharge of radiation over a spectral range (18). Because this measure NCF characteristics of tissue, no extrinsic dyes or supravital stains were used.

Emission scans were performed by exciting the tissues at one particular wavelength while the emission was measured over a variable wavelength. By custom, these scans were identified first by the chosen excitation wavelength, followed by the specific emission interval. Conversely, the excitation scans were performed by exciting tissues with a variable wavelength while the emission was measured at a fixed wavelength. These scans were also identified first by the range of tissue excitation wavelength used, followed by the specific emission wavelength. Although multiple emission and excitation analyses have been performed, we confined our analyses to one excitation scan (excitation, λ 290–430 nm; emission, λ 450 nm) for the purposes of this study. Our previous studies indicated that this scan may distinguish neoplastic from nonneoplastic mucosae within specific oral cavity sites (10, 17, 18). It is of note that results from this scan can be related to the same fluorophore, NADH, which excites at λ 340 nm and emits at λ 450 nm (1, 19, 20). Previous in vitro studies have demonstrated abnormalities involving this coenzyme with transformation of tissue (21, 22).

RESULTS

For the purposes of scan analysis, we calculated the excitation maximum, i.e., the wavelength that induces maximal λ 450-nm emission, the λ 335 nm:λ 375 nm ratio, and the area under the curve, as well as Fourier analysis, as we reported previously (10, 17, 18). The only factor that we could identify that reflected the tumor biological characteristics under consideration was the excitation maximum (Fig. 1). Only the results of this measure will be presented.

We noted that the excitation maximum of the 35 tumor samples analyzed varied from λ 291 nm to λ 363 nm. In 33 patients, the corresponding normal mucosa from the opposite site of the cancer was assessed. The range of excitation in these latter samples was λ 320–353 nm. Overall, the excitation maxima were significantly higher in the normal mucosae than they were in the cancers from the same patients (λ 334 ± 8 nm versus λ 327 nm ± 14 nm, respectively; P < 0.01 by paired t test). This pattern of decreased excitation maximum was seen in 25 of the 33 patients. In eight individuals, the excitation maximum was higher than in the corresponding cancer.
We have shown previously that there is considerable variance in the NCF fluorescence profiles of normal mucosae from head and neck cancer patients, as well as healthy volunteers, and that this may have significance in the assessment of disease states (18, 23). Although neoplastic tissue also differed from normal tissue in its fluorescence characteristics, we noted that a correlation between the two could be identified. Ramanujam et al. (9) described a similar phenomenon in the NCF analyses of patients with diseases of the uterine cervix (9). Fig. 2 demonstrates that the excitation maximum of a particular tumor corresponded with the excitation maximum of the normal contralateral mucosa from the same patient. To perform this analysis, we divided the total population into tertiles based on the $X$ maxima of the normal mucosa, i.e., the lowest, middle, and upper tertiles (11 patients in each group). We then calculated the mean $X$ maximum and SD for the corresponding tumors from the 11 patients in each group. Differences between the three groups are significant by ANOVA ($P < 0.05$). NL, normal mucosa; TU, head and neck tumors.

Given that differentiation status could be associated with a tumor’s excitation maximum and that the same tumor’s excitation maximum correlated with the maximum of the normal mucosa from which it was derived, we assessed whether the excitation maximum of the normal mucosa could be related to the histopathological differentiation of the same patients’ tumors (Fig. 3b). As in the case of direct assessment of the tumor, the overall excitation maxima of the normal mucosa tended to differ, depending on the differentiation status of the cancer ($P = 0.17$). In paired analyses, these differences reached statistical difference when patients with moderately differentiated disease were compared with those with well-differentiated disease ($P < 0.05$). This suggests the possibility that the biological characteristics of the “normal mucosa” within an individual patient could govern the differentiation status of the same patient’s tumor and that this capacity may be reflected in NCF characteristics of the normal aerodigestive epithelium.

Multiple considerations exist when biological traits of head and neck cancer are defined. We considered histopathologically...
defined tumor differentiation, as described above. In an initial exploratory analysis, we also considered whether the excitation maxima of these tissues could be related to disease progression. A comparison was, therefore, made between those patients who remained free of disease with longitudinal follow-up versus those who either presented with recurrence at the time of NCF analysis and/or developed recurrence of the index cancer following NCF assessment. Such recurrence consisted of local, regional, or distant disease. Follow-up (median follow-up = 29 months) information was available on 29 patients to make this assessment. Ten patients were noted to either present with recurrence or develop recurrent disease in the follow-up period. We noted that patients whose tumors or whose normal mucosa expressed low excitation maxima were more likely to demonstrate disease recurrence. This was significant in the analysis of the excitation of normal mucosae of those without recurrence versus those with recurrence (337 ± 8 nm versus 330 ± 7 nm, respectively; \( P < 0.05 \) by \( t \) test). The excitation values of the tumors were 328 ± 14 nm in those patients without recurrence versus 320 ± 12 nm for those with recurrence. This latter difference showed a trend but was not, however, statistically significant (\( P = 0.11 \)). Fig. 4 demonstrates the percentages of recurrence in patients categorized into three equal groups based on the excitation maxima of both the tumors and the normal mucosa. In each incidence, the probability of demonstrating recurrence decreased in those with the highest excitation maxima. Again, this relationship was most evident in the NCF analysis of clinically normal mucosa.

DISCUSSION

Multiple studies, including those involving head and neck cancer, have demonstrated that cancers can be discriminated from normal corresponding mucosae by their NCF characteristics (4–11, 17, 18). This study, however, represents the first to address whether spectral characteristics of a particular tumor reflect biological properties. The biological properties we addressed were histological differentiation and disease progression. Results demonstrate that, when a single excitation scan (excitation, \( \lambda \) 290–430 nm; emission, \( \lambda \) 450 nm) is used, differences can be observed between tumors that are poorly, moderately, or well-differentiated. Differences were related to the excitation wavelength that contributed to maximum \( \lambda \) 450-nm emission; the more poorly differentiated the tumor, the lower the excitation maximum. Such analysis helps to explain part of the considerable variance seen in the NCF characteristics expressed by head and neck cancers in vivo. Results are also consistent with our previous in vitro analyses, which demonstrate that modulating the differentiation status of normal mucosa or head and neck tumor spheroids could similarly alter \( \lambda \) 450-nm emission characteristics (13, 15). It should be emphasized, however, that considerable overlap in spectral characteristics between the various categorical groupings existed. Thus, it is doubtful that factors that account for the differentiation status of the tumor are solely responsible for determining the excitation maximum. Indeed, NCF may not reflect differentiation status but, rather, may reflect some other tumor property associated with tumor differentiation, such as the metabolic activity of the tumor mass, the number and density of neoplastic cells, cellular proliferation, or the tendency of cells within the tumor mass to undergo terminal differentiation and apoptosis. We also noted that certain cancers in this study expressed heterogeneity in differentiation status, depending on from where within the tumor mass samples were taken. This heterogeneity was not accounted for in NCF assessments and may have influenced the results.

Our analysis was restricted principally to emission of a single wavelength, \( \lambda \) 450 nm. The fluorophore that is classically considered to emit at this wavelength is the reduced pyridine nucleotide, NADH. Pyridine nucleotides play key roles in multiple cellular metabolic activities, including energy transfer to the respiratory chain within mitochondria and DNA synthesis (19–22). Schwartz and Passonneau (21) previously evaluated the effect of growth conditions on NADH and NADH concentrations in normal and virally transformed fibroblasts. They noted that the transformed cells contained lower levels of each substance. Furthermore, nucleotide levels within the virally altered fibroblasts failed to modulate with growth confluence in culture, in a manner similar to that of nontransformed cells. It is possible that such changes within oral mucosa may precede later genetic events that contribute to clinically apparent disease. Likewise, factors that would induce changes in the shape of the curve could relate to qualitative changes in particular molecules, such as that which occurs with binding of molecules to proteins. This may influence how emission energy may be transmitted to adjacent molecules and, thus, modify the intensity of detectable emission. Other factors that may confound measurements include the shift of beam penetration and mucosal differentiation and proliferation characteristics (7, 14, 17). Finally, differences in emission characteristics at a single wavelength may simply reflect the influence of not one but multiple fluorophores. Other molecules that emit at that interval include flavins and folic acid derivatives. Critical biochemical analyses that would address these considerations have yet to be performed.

One of the more intriguing observations in this exploratory study relates to the relationship between spectral characteristics...
of the normal mucosa and the head and neck cancer of the same patient. Although considerable variance could be identified in the tumors, part of that variance could be explained by the NCF characteristics of the normal mucosa. An increased excitation maximum of the normal mucosa was associated with an increased maximum of the tumor derived from the same patient. Furthermore, a relationship could be identified between the excitation maxima of the normal mucosa and the differentiation status of the tumor obtained from the same patient. Thus, patients whose normal mucosa expressed a low excitation maxima were more likely to have a poorly differentiated tumor. Conversely, an increased excitation maxima or the normal mucosa was associated with the probability of increasing tumor differentiation. This relationship could also be seen when tumor behavior was explored. Patients whose normal mucosa expressed a low excitation maximum were more likely to demonstrate recurrent disease. This latter analysis was, however, limited due to the small number of patients analyzed. Supportive evidence is required.

These latter results raise the hypothesis that the condition of the normal mucosa of an individual patient at the time that his/her cancer is initiated will influence the biology of the disease. It is well known that certain disease states are prone to head and neck cancer development (24–26). For instance, individuals with syphilitic glositis, chronic oral candidiasis, or iron deficiency disorders have been characterized by both abnormal histological characteristics of noncancerous mucosa and an increased risk of oral cancer. Certain congenital disorders that are characterized by abnormal upper aerodigestive epithelial differentiation, such as dyskeratosis congenita, are similarly prone to head and neck cancer development (26). The nontransformed mucosa in most of these disorders is atrophic, which may predispose the basal cell layers to increased carcinogen exposure. Whether tumors from such individuals are more likely to present a certain differentiation status has not, to our knowledge, been addressed. Likewise, it has been shown that certain carcinogens will lead to specific mutational events and that these events may govern tumor differentiation within head and neck cancers (27). Loss of heterozygosity at 3p21, for instance, is frequently identified in tobacco-induced cancers and occurs early in the transformation process before frankly invasive disease becomes clinically apparent (28–30). El-Naggar et al. (30) have reported that loss of heterozygosity at 3p21 is more frequently associated with poorly differentiated cancers. Additionally, constitutive genetic makeup of an individual, which would include the genetic characteristics of aerodigestive mucosa, may influence the differentiation status of the tumor. Saranath et al. (31), for instance, have shown that polymorphisms involving the L-myc gene within the normal cells of a cancer patient can be associated with the tumor differentiation status of the patient’s cancer. It is conceivable that these conditions, whether environmentally induced or inherited, will lead to a physiological state within noncancerous mucosa that could be reflected in measurable NCF characteristics. These physiological properties of normal mucosa at the time that the tumor is initiated may set the stage for the phenotypic and behavioral characteristics of subsequent clinically apparent disease. Future studies relating NCF analysis to mucosal biology may answer these questions.

REFERENCES


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