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Journal

Photochemistry and Photobiology, 73(2)

ISSN

0031-8655

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Publication Date

2022-12-16

DOI

10.1562/0031-8655(2001)0730178ivfson2.0.co2

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***In vivo* Fluorescence Spectroscopy of Nonmelanoma Skin Cancer[†]**

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Received 24 July 2000; accepted 17 November 2000

ABSTRACT

In vivo and *ex vivo* tissue autofluorescence (endogenous fluorescence) have been employed to investigate the presence of markers that could be used to detect tissue abnormalities and/or malignancies. We present a study of the autofluorescence of normal skin and tumor *in vivo*, conducted on 18 patients diagnosed with nonmelanoma skin cancers (NMSC). We observed that both in basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) the endogenous fluorescence due to tryptophan residues was more intense in tumor than in normal tissue, probably due to epidermal thickening and/or hyperproliferation. Conversely, the fluorescence intensity associated with dermal collagen crosslinks was generally lower in tumors than in the surrounding normal tissue, probably because of degradation or erosion of the connective tissue due to enzymes released by the tumor. The decrease of collagen fluorescence in the connective tissue adjacent to the tumor loci was validated by fluorescence imaging on fresh-frozen tissue sections obtained from 33 NMSC excised specimens. Our results suggest that endogenous fluorescence of NMSC, excited in the UV region of the spectrum, has characteristic features that are different from normal tissue and may be exploited for noninvasive diagnostics and for the detection of tumor margins.

INTRODUCTION

Nonmelanoma skin cancer (NMSC)[†], which includes basal cell carcinomas (BCC) and squamous cell carcinomas (SCC), is the most common malignancy worldwide. About 80% of the NMSC are BCC and 20% are SCC (1). Presently there are few options for the clinician in the definitive diagnosis of NMSC. Biopsy remains the gold standard and the

accessibility of the skin makes this procedure straightforward (2,3). In this work we have initiated an investigation for the optical characterization of NMSC lesions. Tissue spectroscopy has been extensively used in the quest for the development of noninvasive diagnostic devices (4,5). Initially, the focus was on *in vitro* and *ex vivo* tissue studies aimed at determining if there were any specific distinguishing spectral features that might be used to discriminate normal from diseased tissue (6–8). Recently, research in this area has begun to emphasize *in vivo* spectroscopy. To date efforts have been devoted to the optical detection of pathologic tissue, including discrimination between cancer, precancer and inflammation in cervix, colon and esophagus, among others (5,6,9,10). Investigations have been directed for the detection of endogenous (4,11,12) as well as exogenous (13,14) fluorescent markers that could have the necessary properties to ensure a sufficient sensitivity and specificity for optical detection of malignant tissue and/or to predict the outcome of cancer therapy.

Due to the obvious accessibility of the organ, spectroscopic investigation of skin has been fundamental for the development of tissue optical spectroscopy (11,15–18). Spectroscopic methods have been applied to the investigation of NMSC using exogenous chromophores such as porphyrin whose fluorescence in the 600–650 nm region can be excited with lasers in the 400–450 nm region (13,14). Other studies have been conducted using the endogenous fluorescence of skin (19) on NMSC using He–Cd laser excitation at 442 nm. In our work we have chosen to characterize and investigate differences in the *in vivo* fluorescence of NMSC by recording the endogenous fluorescence. Data were acquired from patients *via* an optical fiber bundle coupled to a commercially available double-monochromator fluorimeter. The results show that fluorescence associated with tryptophan (12,18,20) consistently and reproducibly increases within the tumoral region of skin, whereas the collagen crosslink fluorescence (12,18) generally decreases. As such, this set of spectroscopic markers may provide the framework for the development of a method for spectroscopic discrimination of normal tissue from NMSC. In addition to the *in vivo* spectroscopic investigation we employed fluorescence microscopy to study the fluorescence emission characteristics of fresh-frozen tissue samples obtained from NMSC specimens following surgical excision. The lack of collagen fluorescence in the vicinity of the tumor loci in the tissue

[†]Posted on the website on 30 November 2000.

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[†]Abbreviations: BCC, basal cell carcinoma; H&E, Hematoxylin & Eosin staining; MMS, Mohs micrographic surgery; NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma.

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sections provides additional support of our interpretation of the *in vivo* results. Furthermore the data provide compelling information that may enable the development of optical methods to detect NMSC and valuable information for the transformations that occur in the epidermis and the dermis.

MATERIALS AND METHODS

Patient recruitment

This study was performed under a protocol approved by the Subcommittee on Human Studies of the Massachusetts General Hospital (MGH). Informed consent was obtained from each patient. Subjects were recruited from a group of patients scheduled to undergo Mohs micrographic surgery within 7 days of their participation in our study. All subjects had confirmed BCC or SCC lesions, biopsies were taken at least two weeks before their participation in the present study. In all cases the wound resulting from the biopsy had been allowed to completely heal to the effect that in a few cases the lesion was difficult to locate with a typical clinical inspection. The spectroscopic results of our study were in no way used to guide subsequent treatment. A total of 18 patients agreed to participate in this investigation. Fluorescence and reflectance spectra were collected from a total of 25 NMSC lesions (20 BCC, 5 SCC). Three additional patients were chosen with clinically diagnosed BCC, but did not undergo previous biopsy of the investigated area and were not scheduled for surgery. Spectra were acquired from the tumor and from sections of normal skin located approximately 10 mm from the edge of each tumor. Histopathologic confirmation of the type of lesion was performed by the Mohs surgery staff using Hematoxylin and Eosin (H&E) staining of fresh-frozen sections of the excised tumor.

Data collection protocol. Patients were made comfortable so as to prevent movement during the data collection interval. The distal face of the fiber bundle was placed in direct contact with the lesion or normal skin. (In no case was there any exudate from the lesion of interest.) Fluorescence spectra were collected from each lesion. A reflectance spectrum was then gathered. A skin site located approximately 10 mm from the lesion was then identified as clinically normal and the spectral data collection sequence was repeated. When possible, data were collected using a large area circular fiber holder that prevents excessive pressure applied by the fiber on the investigated area. However this device was not used in areas of high curvature (such as nose and eyebrows). In spectra collected from normal subjects, however, (data not shown) we deduced that effects of probe pressure on the skin account for less than 5% of the fluorescence intensity.

Instrument description and data collection parameters

A fiber optic-based fluorimeter (Model SkinScan, Spex Industries/Jobin Yvon, Edison, NJ) was employed to gather all spectral data. Light from a 125 W Xenon arc lamp passes through an excitation monochromator. Light exiting the excitation monochromator is transmitted *via* a randomized fiber bundle to the sample of interest. The fiber bundle consists of a collection of 62 (31 delivery + 31 collection) quartz fibers, each having a core diameter of 250 μm . The distal face of the fiber bundle is placed in direct contact with the sample (without a spacer between the surface of the skin and the distal end of the fiber). Fluorescence light (or backscattered light as the case may be) emitted from the sample is transmitted by the fiber bundle to an emission monochromator and finally incident upon a photomultiplier tube having adjustable gain. The aperture of the slit, which in our case is approximately 500 μm , is determined by the rectangular arrangement of the 31 fibers at the monochromator side. Data acquisition and instrument control is governed by a laptop personal computer compatible *via* RS-232 port. Software employed for data acquisition and analysis is Grams/32 (Galactic, Salem, NH).

A series of fluorescence spectra were acquired for each lesion and adjacent normal skin. The spectral regime that was chosen for this study was selected based on the evidence that the *in vivo* autofluorescence of skin is dominated by the emission of epidermal tryptophan residues (for excitation wavelengths below 300 nm) (12) and

by the emission of dermal collagen crosslinks (for excitation in the 330–400 nm range) (11,12). Emission spectra were collected with excitation wavelengths at 295 and 350 nm. Excitation spectra were collected with 350, 380 and 470 nm emission wavelengths. Data were acquired every 2 nm. In the spectral region investigated the incident power on the skin was below 50 $\mu\text{W}/\text{cm}^2$ and in no case were the exposures of sufficient duration to induce any tissue reaction.

Reflectance. A reflectance spectrum was acquired from each lesion and the adjacent normal skin in the range 250–600 nm. This was done by synchronously scanning the two monochromators of the fluorimeter. Data were acquired every 2 nm. Signal was integrated for 0.2 s at each data point. A calibration spectrum was acquired by placing a reflectance standard of barium sulfate in contact with the distal tip of the fiber bundle using the same data acquisition parameters that were employed to gather patient data. Under our configuration, reabsorption by hemoglobin has little influence on the fluorescence of the skin excited between 295 and 400 nm, as the penetration of the exciting light is limited to the upper dermis and does not reach the deeper vessels of the dermis (12,21–23).

Data analysis. Emission spectra were corrected for the instrumental parameters (lamp profile, photomultiplier sensitivity, *etc.*) and divided for the apparent absorption at the excitation wavelength as retrieved from the reflectance spectra. This was an attempt to normalize the emission spectra for apparent changes in absorption between tumor and normal skin. In the overlapping spectral regions emission spectra were also multiplied by the absorption spectra (retrieved from the reflectance spectra). This was done to include effects of reabsorption of the emitted light by the tissue (11,24). Excitation spectra were corrected for the lamp emission by dividing the spectra for the apparent absorption (obtained from the reflectance spectrum) at 380 nm. The intensity of fluorescence at each excitation wavelength in tumor and normal skin was calculated as the area under the emission bands. For the fluorescence attributed to epidermal tryptophan moieties, the area was calculated between 310 and 450 nm. Similarly, for the emission of dermal collagen crosslinks, the area was integrated over the range between 380 and 600 nm. We also used the ratio I_{350}/I_{295} as an additional parameter to compare tumor vs normal fluorescence. The excitation spectra with emission at 380 nm were used to calculate I_{350}/I_{295} . These two peaks represent the maxima of the excitation spectra of tryptophan moieties and collagen crosslinks, respectively.

Acquisition of microscopic fluorescence images

Tissue collection. A total of 33 specimens (28 BCC and 5 SCC) were investigated. Our protocol included the collection of fresh-frozen tissue sections of the tumors excised during Mohs surgery from patients including those enrolled in our study. The material was collected from the Dermatological Surgery Unit at MGH. According to the standard procedures followed during Mohs micrographic surgery (25) sections (8 μm in thickness) from each specimen were mounted on glass slides, fixed and stained with H&E and preserved for further analysis. The adjacent unstained frozen sections from each specimen were mounted on a quartz slide and immediately covered with aqueous mounting medium (Aqua-Mount, Leners Lab., Pittsburgh, PA) to maintain the moisture of the tissue. A quartz coverslip was placed over the tissue section. Stained and unstained sections were kept at room temperature until used. Imaging acquisition of the tissue sample was conducted for about 3 h starting within 2 h from the excision. Imaging acquisition was conducted at room temperature.

Instrumentation and image capture. For microscopic fluorescence imaging the sample was mounted on an inverted microscope (Axioptot, Zeiss, Germany). A 150 W Mercury lamp was used for excitation. The incident light was filtered by a narrowband (20 nm full width at half maximum) filter and focused on the sample with a 5 or 10 $\times$ objective (Zeiss, Germany). Narrowband filters were selected at the following wavelengths: 350, 360, 390 and 420 nm. These wavelengths provide excitation of the collagen crosslinks. The backscattered light was collected through the same objective, the excitation wavelength was eliminated by placing a dichroic mirror (short wavelength cutoff between 380 or 450 nm depending on the excitation wavelength) in the light path, and the remaining fluorescence radiation was passed through a second narrowband filter (390, 400,

Figure 1. Schematic representation of the fluorescence microscope. The light from a Xe arc lamp is focused by means of 5 or 10 $\times$ microscope objective onto the specimen. A neutral density (ND) filter and a narrowband filter are positioned between the lamp and the objective to, respectively, decrease the amount of light incident on the filter and select the excitation wavelength. The fluorescence is collected through the same objective and the scattered excitation light is stopped by the dichroic mirror. A second narrowband filter is positioned before the detector (a charge-coupled device camera) to select the emission wavelength.

450 $\pm$ 10 nm). The fluorescence image was captured with a charge-coupled device CH250/A camera, 512 $\times$ 512 pixels, (Photometrics Inc., Tucson, AZ) (Fig. 1). Image manipulation and analysis were conducted with a commercial image processing software (IP Lab Spectrum, Signal Analytics, Vienna, VA). To obtain images of the same location in stained and unstained sections, the original Mohs micrographic surgery map (MMS) (25) served as a rough guide to the location of the tumor. The H&E image was first carefully inspected to locate landmarks (such as hair follicles, sebaceous glands and MMS ink marks) that were then used to locate the corresponding region in the unstained sections. A bright field image of the H&E image was stored before recording the fluorescence image of the corresponding unstained tissue.

RESULTS

Emission and excitation spectra of normal skin at the wavelength used in our investigation are in agreement with what was reported previously (11,12,15,18,21,26). We will limit the presentation of our results to the differences between normal and tumor fluorescence.

In vivo emission spectra (tumor vs normal)

The *in vivo* emission spectra of both BCC and SCC lesions upon excitation at 295 nm consistently showed a larger fluorescence signal from the lesion compared to the surrounding "normal" skin (Fig. 2a). The fluorescence of BCC lesions was 2.9 ± 1.4 larger than in normal surrounding skin, whereas that from SCC lesions was 2 ± 0.9 times larger. At each excitation wavelength we estimated the increase (or decrease) of the fluorescence in the tumor by calculating the ratio between the fluorescence intensity (as the area under the emission peak) of the tumor and of the surrounding normal tissue. The ratios are reported $\pm$ standard deviation. The increase in fluorescence was also detected in those patients whose lesions were not previously biopsied. No spectral shift was observed between tumor and normal skin. Upon excitation at 350 nm the measured fluorescence intensity was

Figure 2. Representative *in vivo* emission spectra of normal skin and NMSC. The spectra shown are for a single subject and are representative of the data obtained in NMSC. The variability of the intensity (given as a ratio between the tumor intensity and the surrounding normal tissue intensity) is indicated in the "Results" section: (a) excitation at 295 nm. The emission of the tumor (dotted line) is more intense than the one of the surrounding normal skin (solid line); and (b) excitation at 350 nm. The emission of the tumor (dotted line) is much lower than the one of the surrounding normal skin (solid line).

reduced in the tumor area relative to normal skin surrounding it (Fig. 2b). On average the fluorescence of BCC was 0.72 ± 0.14 that of the surrounding normal skin and that in SCC was 0.85 ± 0.11 .

In vivo excitation spectra (tumor vs normal)

The *in vivo* excitation spectra with emission at 350 nm confirmed a larger fluorescence intensity of the tumors. Excitation spectra with the emission at 380 nm show the opposite trend in the intensity of the peaks near 295 and 350 nm between tumor and normal (Fig. 3). In cancerous tissue the peak at 295 nm was higher than in normal tissues, whereas in normal skin the intensity at 350 nm was higher than in the tumor. The ratio between the peak at 350 nm and the peak at 295 nm (I_{335}/I_{295}) was higher in normal skin (0.51 ± 0.3) than in the tumor (0.33 ± 0.21 for BCC and 0.19 ± 0.08 for SCC).

Autofluorescence of fresh-frozen tissue sections

The combination of excitation and emission narrowband filters used in our experiments allowed for the detection of the microscopic fluorescence of collagen and elastin. In the presence of the tumor, the comparison between the autofluorescence

Figure 3. Representative *in vivo* excitation spectrum of normal skin and NMSC. The spectra shown are for a single subject and are representative of the data obtained in NMSC. The variability of the ratio of the fluorescence intensity at 335 and 295 nm between the tumor and the surrounding normal tissue is indicated in the "Results" section. The emission wavelength was set at 380 nm. The concomitant increase of the peak at 295 nm and decrease of the peak near 350 nm in the tumor can be appreciated. Tumor (dotted line), normal skin (solid line).

ence image of fresh-frozen tissue sections and the H&E image shows that a dark area with a marked loss of collagen autofluorescence includes the tumor loci and a large part of the surrounding dermal tissue (Fig. 4). The areas characterized by the loss of fluorescence are delimited by the autofluorescence of intact collagen and elastin fibrils. The elongated shape typical of collagen and elastin fibers can be observed in the fluorescent peritumoral area of Fig. 4. The marked loss of fluorescence of the tumor and peritumoral areas was observed in 26 of the 33 (78%) NMSC (Table 1). The areas characterized by the loss of autofluorescence were two- to three-fold larger than the actual tumor size estimated from the H&E images. In a limited number of cases (7 BCC) the autofluorescence pattern showed sharply demarcated "dark" areas amidst brightly fluorescent dermal collagen fibrils. Control cases without tumor showed diffuse autofluorescence of dermal collagen and elastin fibrils. The autofluorescence of dermal appendages (*e.g.* pilosebaceous units) is also larger than the autofluorescence of tumor and peritumoral areas.

DISCUSSION

Spectral differences *in vivo* between tumor and normal

In vivo fluorescence of both normal skin and tumor with excitation at 295 nm produces the emission expected from the tryptophan moieties located in the epidermis (12,18,22). The fairly large standard deviation of the fluorescence intensity ratios and of the I_{335}/I_{295} reflects the high subject-to-subject and site-to-site variability of skin fluorescence. However, the statistical significance of the data presented here is due to the very low within-subject, within-site variability of the fluorescence signal in the area surrounding the tumor. The within-subject, within-site variation of the fluorescence intensity recorded in normal volunteers is below 5%.

The most striking observation among those reported here is the increase in the fluorescence of tryptophan moieties (emission band near 345 nm) in the tumor relative to the surrounding normal skin. This increase is probably due to

Figure 4. Fluorescence microscopy image: (a) white light H&E stained image of a NMSC section. The tumor nests can be distinguished by the lighter appearance delimited by a dark purple rim; (b) white light image of the same area in a section adjacent to the one in (a); and (c) fluorescent image of (b). Excitation at 360 ± 5 nm; emission at 428 ± 5 nm. The area where the tumor nests are located result in a dark, nonfluorescent region surrounded by a brighter area where fibrous structures (from collagen fibrils) can be observed. The nonfluorescent area is larger than the area occupied by the tumor nests in (a).

hyperactivity or epidermal hyperproliferation (18). One hypothesis to explain this observation is that increased epidermal activity results in higher transient concentration of proteins in cells (therefore larger fluorescence signals) and/or increased thickness of the epidermis.

Upon excitation at 350 nm the *in vivo* fluorescence spectra of normal skin and tumor is believed to result from crosslinks of collagen and elastin in the dermis (11,12). In fact, radiation with wavelength longer than 330 nm travels into the upper dermis (22,27) where collagen is predominantly laid. The emission and excitation properties of collagen and elastin are very similar resulting in spectra that could not be distinguished. The fluorescence of the collagen crosslinks in the tumor decreases drastically in comparison with the surrounding normal skin. This decrease is likely due to tumor-induced changes in the connective tissue. The activity of NMSC results in the partial or total destruction of the collagen and elastin crosslinks surrounding the tumor nests. Several mechanisms have been introduced to explain the erosion of the connective tissue (28–31). In summary NMSC is associated with a higher synthesis of metalloproteinases in fibroblasts. The activity of the metalloproteinases decreases

Table 1. Summary of *in vivo* fluorescence findings in NMSC

Tumor type	No. of lesions analyzed with autofluorescence microscopy	% of less-fluorescent tumor and peritumoral areas*
BCC	28	78 (21 of 28)
SCC	5	60 (3 of 5)

*This table represents the percentage of lesions in which the fluorescence of the tumoral area is smaller than in the peritumor. The data are obtained from the fluorescence of the fresh-frozen tissue sections of BCCs and SCCs. Data were analyzed first by visual inspection of the fluorescence image then by calculating the fluorescence in a 400 pixel² area of the tumor and the peritumor. Average reduction in fluorescence intensity greater than 20% between tumor and peritumor were classified as "less-fluorescent". The fluorescence images used for the analysis were collected under 360 nm excitation and 450 nm emission.

es the amount of collagen and elastin crosslinks and consequently also reduces the fluorescence from the connective tissue. It is likely that the destruction of collagen and elastin occurs primarily in the extracellular matrix surrounding the tumor nests (Fig. 4). The slight difference in the fluorescence changes between BCC and SCC that we recorded could be due to the more extensive invasion of the dermis by BCC compared to SCC. It is expected that variations to the data recorded may occur in specific types of tumor such as SCC *in situ* and in morpheaform BCC. In SCC *in situ* there is no invasion into the dermis, and therefore we would not expect a suppression of collagen fluorescence; in the case of morpheaform BCC the distribution of tumor cells is to surround the collagen fibers resulting in a sclerosed lesion which might lead to an increase of the collagen fluorescence. We have anecdotal data to this effect which is not sufficient, however, to draw statistical conclusions.

Autofluorescence of fresh-frozen tissue sections

The investigations we have performed indicate a correlation between the *in vivo* fluorescence signals and events occurring in the dermis and epidermis by investigating the autofluorescence of fresh-frozen tissue sections. Based on the *in vivo* fluorimetric results we hypothesized that autofluorescence could be extended to the spatial microscopic imaging of NMSC. As shown in Fig. 4, the loss of fluorescence is not limited to the tumor nests but includes also a large area of extracellular matrix surrounding the tumor. This is consistent with the decrease of the collagen fluorescence recorded *in vivo*. These results seem to confirm our hypothesis that the erosion of the dermal matrix surrounding the tumor is associated with the presence of NMSC and is due to an increased level of metalloproteinases (32–34) that modify the collagen crosslinks (35).

The decrease of the *in vivo* endogenous fluorescence of the tumor in the visible region upon UVA or visible excitation is common to many cancerous tissues (4). Our microscopic results show that in addition to the loss of autofluorescence due to changes in epidermal tumor cells (4), there is an additional loss of fluorescence due to modification in the extracellular matrix as reported in some lung cancers (36). On the other hand, the increase of the *in vivo* autofluorescence of the tumor in the 300–380 upon excitation in the UVB is a novel finding in the field of tumor autofluorescence and should be explored further. In fact it may hold the possibility of enabling a method for the use of *in vivo* fluorescence spectroscopy in the diagnosis of NMSC.

CONCLUSIONS

In summary our investigation shows that *in vivo* autofluorescence of NMSC is characterized by an increase in the emission of tryptophan moieties and a decrease in the emission of collagen and elastin crosslinks. The two effects can be explained with an increase in epidermal proliferation (37) and the erosion of the dermal matrix (32,34), respectively.

The latter interpretation is confirmed by our microscopic autofluorescence imaging of fresh-frozen tissue section in which the areas of connective tissue surrounding the tumor loci show a marked loss of fluorescence from collagen and elastin crosslinks. The limitation of the optics used in our

fluorescence microscope prevented autofluorescence microscopy in the spectral region of the tryptophan moieties. Future work will hopefully overcome this limitation and allow investigating tryptophan fluorescence in NMSC fresh-frozen tissue sections.

To our knowledge this increase of *in vivo* autofluorescence of tryptophan moieties related to skin cancer is the first reported in the literature. This unique finding could be due to the particular epidermal hyperproliferation (18) or due to the fact that this is in fact the first *in vivo* investigation of cancer that uses excitation at 295 nm. This result certainly opens the possibility of retrieving diagnostic information through the combination of the increase of tryptophan fluorescence and the decrease of the collagen. Although this increase requires investigations on skin lesions different from NMSC, in future it may provide the most important piece of information for the diagnostic potential of this technique, especially if considered in combination with the parallel decrease of dermal fluorescence.

Acknowledgements—The project was in part funded by the DOD Medical Free Electron Laser Grant N00014-94-1-0927. The authors would also like to thank Erin Bowler for the preparation of stained and unstained tissue sections.

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