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Detection and Diagnosis of Oral Cancer by Light-Induced Fluorescence

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Background and Objective: New techniques for non-invasive early detection and diagnosis of oral dysplasia and carcinoma are required. Our objective was to determine in the hamster cheek pouch model whether differentiation between the healthy tissue and the different stages of oral premalignancy and malignancy is possible using laser-induced fluorescence after tissue exposure to 5-Aminolevulinic acid (ALA).

Study Design/Materials and Methods: DMBA carcinogenesis was applied to one cheek pouch in 18 hamsters for 0–20 weeks. Prior to sacrifice, 20% ALA was applied to the cheek tissues. Excised cheek tissues were cryosectioned and imaged using fluorescence microscopy with excitation at 405 nm, detection at 635 nm. After fluorescence measurement, H&E staining and histopathological evaluation were performed.

Results: Fluorescence intensity was significantly lower in healthy tissue than in pathological tissues. Significantly higher intensities and more “fluorescence hot spots” occurred in severe dysplasia and carcinoma than in healthy tissue, hyperkeratosis, mild and moderate dysplasia.

Conclusions: Light-induced fluorescence after ALA exposure can differentiate between the different stages of premalignancy and malignancy. Its ability to differentiate between healthy tissue and early pathology is particularly interesting *Lasers Surg. Med.* 32:17–24, 2003.

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Key words: 5-aminolevulinic acid; light-induced fluorescence; oral cancer; oral dysplasia

INTRODUCTION

Cancer cure and survival rate relate directly to the stage of the cancer at the time of diagnosis. Early detection permits minimally invasive treatment modalities and greatly improves the long-term prognosis for the patient. However, early recognition of oral malignancy is problematic because of the inability to perform screening in high-risk sectors of the population and the frequent lack of gross signs and/or obvious symptoms, exacerbated by poor visual access in many cases.

Oral cancer will claim approximately 10,000 lives in the U.S. this year [1,2]. Accounting for 96% of all oral cancers, squamous cell carcinoma is usually preceded by dysplasia presenting as white epithelial lesions on the oral mucosa (leukoplakia). Leukoplakias develop in 1–4% of the population [1]. Malignant transformation, which is quite unpredictable, occurs in 1–40% of patients over 5 years [1]. Tumor detection is complicated by a tendency towards field cancerization, leading to multicentric lesions [3]. Current techniques detect malignant change too late. Of all oral cancer cases documented by the National Cancer Institute Surveillance, Epidemiology and End Results Program, advanced lesions outnumbered localized lesions by more than 2:1. Five-year survival rate is 75% for those with localized disease at diagnosis as compared to only 16% for those with cancer metastasis [1,2].

Oral cancer can be detected by dentists and physicians, but physicians do not routinely inspect their patients for suspicious oral lesions, and dentists are also remiss in the early diagnosis and referral for oral cancer. Since 11% of dentists and 45% of physicians do not feel adequately trained to complete an effective oral cancer examination, this results in their failure to examine for oral cancer [2]. The current approach to detecting the transformation of leukoplakia to squamous cell carcinoma is regular surveillance combined with biopsy or surgical excision. However, these techniques are invasive and unsuitable for regular screening of high risk sectors of the population. Moreover, clinical experience is necessary to adequately perform

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these examinations. Therefore, new techniques for non-invasive early detection and diagnosis of oral dysplasia and squamous cell carcinoma are required.

Topical or systemic application of chemical agents called photosensitizers can render pathologic tissues fluorescent when exposed to specific wavelengths of light [4,5]. While several studies have demonstrated the use of various porphyrins as photosensitizers [5–7], their accumulation in skin after systemic administration can cause phototoxic reactions upon exposure to sunlight. An alternative approach is to stimulate synthesis of photosensitizing agents in situ with a photoinactive precursor. The photosensitizer protoporphyrin IX (PpIX) is an immediate precursor of heme in the biosynthetic pathway for heme. In certain types of cells and tissues, the rate of PpIX synthesis is determined by the rate of synthesis of 5-Aminolevulinic acid (ALA), which in turn is regulated via a feedback control mechanism governed by the concentration of free heme. The presence of exogenous ALA bypasses the feedback control of this process, inducing the intracellular accumulation of photosensitizing concentrations of PpIX [4,8]. A selective accumulation of PpIX occurs in areas of increased metabolism such as tumor cells [4,5,8]. The resulting tissue-specific photosensitization provides the basis for using ALA-induced PpIX for photodynamic diagnosis and therapy, whereby far lower light doses are used for photodynamic diagnosis.

Using ALA-induced PpIX fluorescence in the rat colon [10] and in the bladder [11,12], malignant tissues were found to fluoresce with significantly greater intensity in the red spectral region than in the surrounding healthy tissues. The ratio of fluorescence levels in normal mucosa versus that of the viable tumor was 1:6 [12]. Four hours post-ALA application was the optimal detection time point regardless of administration method in both the studies cited. Photosensitization using ALA-induced PpIX was shown to be effective for mapping tumour presence and for photodestruction of bladder carcinoma [11,12]. An investigation by Svanberg et al. on head and neck cancer determined that the dosage of ALA must be kept low for maximal tumor demarcation. A higher dosage can cause excess bioavailability in normal tissue. An ALA-dose of 5–7.5 mg/kg was determined to be optimal for tumor differentiation [13].

Abels et al. investigated time-based porphyrin accumulation in neoplastic and surrounding tissue by measuring the kinetics and spectra of ALA-induced fluorescence in vivo. Using amelanotic melanoma A-Mel-3 cells grown in the Golden Syrian hamster, the optimal time point for photodynamic diagnosis and therapy post-i.v. administration of 500 mg/kg of ALA was found to be 90 minutes and 150 minutes. Maximum fluorescence intensity in neoplastic tissue occurred at 150 minutes [14].

The use of ALA-induced PpIX fluorescence for the early detection of laryngeal and lung cancer is also promising [15,16]. For this application, the preferred method of administration is via the medical nebulizer. Investigators found a strong ALA-induced fluorescence in carcinoma, carcinoma in situ, and dysplasia [15,16]. Both of these

studies determined a correlation between red fluorescence intensity and malignancy.

In a clinical study, oral use of a 15-minute ALA mouth rinse resulted in varying levels of PpIX fluorescence. Differing levels of intensity were attributed to variations in rinsing procedure in each patient and to varying localization and histopathology of the tumors under investigation. Optimal conditions for fluorescence-based tumor localization occurred within 2 hours of ALA-rinsing. PpIX fluorescence was limited to the surface layers of the epithelium (penetration depth less than 1mm) [17,18]. This leaves unresolved issues pertaining to tumor depth and the relationship between histopathological staging and fluorescence.

Protoporphyrin IX fluorescence was significantly higher in dysplastic than in healthy oral tissue in a study comparing tumor discrimination and delineation ability of ordinary white light inspection, versus autofluorescence and versus ALA-induced PpIX fluorescence [19]. Investigators found that a combined fluorescence diagnosis (CFD) comprising a combination of autofluorescence and ALA-induced PpIX fluorescence, showed the most favorable contrast in the red-green spectra between malignant and healthy tissues [19]. Although the CFD was hindered by tumor keratinization, it was found to be independent of tumor grading, staging, or localization.

The safety of ALA when used as a topical or systemic photosensitizer has been established in multiple clinical trials [8–27]. ALA and PpIX are normally present in certain tissues of the body. ALA-induced PpIX is cleared from the body within 24 hours of its induction, whether the route of administration is systemic or topical [8]. Thus protection from exposure to sunlight is only necessary for 24 hours after ALA application.

The purpose of this study was to investigate in the standard hamster cheek pouch model (1) the ability of light-induced fluorescence (LIF) following application of ALA to differentiate between healthy tissue and the different stages of oral premalignancy and malignancy and (2) the effectiveness of ALA application at 90 minutes versus 180 minutes for diagnostic purposes.

MATERIALS AND METHODS

Animal Model

Using the Golden Syrian Hamster (*Mesocricetus auratus*, Harlan Sprague Dawley, San Diego, CA) cheek pouch model, thrice weekly application of 0.5% DMBA (9,10 dimethyl-1,2-benzanthracene) (Sigma-Aldrich, St. Louis, MO) in mineral oil in 38 animals produced dysplastic leukoplakia after 4–6 weeks, progressing to squamous cell carcinoma after 8–20 weeks. Histological features in the hamster cheek pouch model correspond closely with premalignancy and malignancy in human oral mucosa. The animals were treated in accordance with ARC guidelines at UCI (IACUC 97-1972).

Standard DMBA carcinogenesis was applied to the right cheek pouch in 18 hamsters for 0–20 weeks. In the control left cheek pouch of these hamsters, only mineral oil

(E.R. Squibb & Sons, Inc., Princeton, NJ) was applied. The left cheek of hamsters receiving DMBA carcinogenesis was used as control as previous studies have shown that the process of carcinogenesis in one hamster cheek pouch does not affect histological or fluorescence characteristics of the untreated cheek pouch in the same animal [18,19]. Prior to sacrifice, 1 g of 20% ALA (5-Aminolevulinic Acid HCl, Sigma-Aldrich) adjusted to a pH of 5.5 using 1 N sodium hydroxide in Eucerin (Beiersdorf Inc., Norwalk, CT) was applied to both cheek pouches for 90 or 180 minutes.

After hamsters were euthanized, both cheek pouches were excised, mucosal specimens of 3–4 mm each were retrieved and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A. Inc., Torrance, CA). The blocks were rapidly frozen in liquid nitrogen and stored at -70°C in the dark.

Standard 6 μm cryosections (Cryostat microtome, AO Reichert, Buffalo, NY) were prepared in low, diffuse light. After acquisition of fluorescence images, sections were stained with H&E, and the same microscopic areas were then imaged using bright-field techniques to evaluate histology (see below).

Low-Light Level Fluorescence Microscopy

Excitation/detection technique. A low-light level fluorescence microscopy (LLFM) system comprised of a slow-scan cooled CCD camera (576×384 pixels, model 57–180, Princeton Instruments, Trenton, NJ) with 16-bit per-pixel dynamic range of data acquisition interfaced with a personal computer (Power Macintosh 8600/300, Apple Computer, Inc., Cupertino, CA) attached to a Zeiss Axiovert 10 inverted microscope (Zeiss, Oberkochen, Germany) with a $10\times$ Zeiss Plan-NEOFLUAR objective (N.A. = 0.3) (Zeiss, Oberkochen Germany). Excitation was provided by a 100 W Hg-lamp. For ALA-induced PpIX fluorescence, the excitation light at 405 nm was provided through a 405 nm bandpass filter, and the emission light was isolated with a 635 nm broad bandpass filter.

Bright field, phase contrast, and fluorescence images could be acquired with this system and the software (IPLab, Ver.3.2, Signal Analytics Corp., Vienna, VA), permitting a direct quantification of tissue fluorescence characteristics and comparison with histological status.

Fluorescence measurement. After fluorescence acquisition, slides underwent routine H&E staining and re-acquisition of H&E stained images. The pre-standardized first investigator assessed histological status from the H&E stained images. In five slides from each animal, up to five areas of each histological diagnosis were selected. Fluorescence was measured by a second investigator in the areas designated by the first investigator. The second investigator was blinded to the histopathological diagnosis of all areas being measured. Fluorescence was quantified digitally by superimposing a square (12×12 pixels, $29.6 \mu\text{m}^2$) onto the fluorescence image from the bright-field image and measuring intensity digitally within the bound areas of the square. In each area five fluorescence measurements were performed, providing a total of up to

125 fluorescence measurements per histological diagnosis per animal.

Histological Evaluation

Histological evaluation of each section was quantified by the same blinded, pre-standardized investigator (first investigator) according to standard criteria [28], whereby each characteristic listed below was assessed.

The following numerical grading system was used for each area on a slide: 0-healthy, 1-hyperkeratosis, 2-mild dysplasia, 3-moderate dysplasia, 4-severe dysplasia, 5-carcinoma in situ, 6-squamous cell carcinoma. Criteria for hyperkeratosis included thickening of the keratin layer or prickle cell layer (hyperplasia) without morphologic changes in the cells (no dysplasia). Criteria for oral epithelial dysplasia included drop-shaped rete ridges, irregular epithelial stratification, keratinization of cells below the keratinized layer, basal cell hyperplasia, loss of intercellular adherence, loss of polarity, hyperchromatic nuclei, increased nucleocytoplasmic ratio, anisocytosis and anisonucleosis, pleomorphic cells and nuclei, increased level of mitotic activity, presence of bizarre mitoses. If only basal layer hyperplasia was present, mild dysplasia was diagnosed. Presence of abnormal mitosis and/or pleomorphism in cells was considered indicative of severe dysplasia. Carcinoma in situ was diagnosed if dysplasia extended the entire thickness of the epithelium. It is histologically similar to squamous cell carcinoma except that there is no invasion of the connective tissue. Squamous cell carcinoma was diagnosed when epithelial cells disrupted the basement membrane layer and invaded the underlying connective tissues.

Statistical Analysis

Data were analyzed using analysis of variance methods. Fluorescence data were transformed using a log transformation prior to analysis to correct for non-normality. Differences in fluorescence between groups defined by histopathology were assessed using pairwise *t*-tests with Bonferroni correction for multiple comparisons (16 precise comparisons). To ensure clinical relevance, differences in fluorescence between histopathological grades were evaluated within each animal, as well as for all animals.

RESULTS

Application of ALA cream for 90, 180 minutes was unproblematic, although it was not possible to ascertain how much of the cream was licked off or swallowed by the animals. No signs of oral irritation by the cream were visible.

Histopathology

After approximately 4 weeks of DMBA carcinogenesis, hyperkeratosis was observed in the epithelium of the oral mucosa. At approximately 6 weeks, mild dysplasia occurred. At approximately 8–12 weeks, moderate and severe dysplasia were observed. At approximately 14–16 weeks carcinoma-in-situ was seen. At approximately

a **b**

Fig. 1. **a:** H&E stained, enhanced fluorescence and normalized fluorescence images of healthy tissue, hyperkeratosis and mild dysplasia after 90 minutes ALA application. Arrows identify the mucosal surface. **b:** H&E stained, enhanced fluorescence and normalized fluorescence images of moderate, severe dysplasia and carcinoma after 90 minutes ALA application. Arrows identify the mucosal surface.

18–20 weeks, squamous cell carcinoma was observed (Figs. 1 and 2).

Fluorescence

Enhanced and normalized fluorescence images of each type of histopathology after 90 or 180 minutes ALA application are shown in Figures 1 and 2. In the enhanced images, fluorescence was amplified to optimize image visualization. Thus apparent fluorescence intensities shown are not to scale and there is no apparent difference in fluorescence intensities in different tissues. In the normalized images, the same intensity scale and range are used in all images to permit numerical and visual comparison of fluorescence intensities between locations and slides. The normalized image of healthy tissue appears black as the scale selected for this series of images had to accommodate the very bright fluorescence generated in pathological tissues. Therefore, this scale does not show the very low level of fluorescence generated by healthy tissue.

In general, PpIX fluorescence was greater at 90 minutes than at 180 minutes in comparable tissues (Table 1). The normalized fluorescence images of healthy tissue after 90 or 180 minutes ALA application showed weak fluorescence

intensity. With increasing severity of pathology, fluorescence intensities became progressively stronger. Moreover, the presence and intensity of fluorescence “hotspots,” small localizations of very strong fluorescence within the tissues became increasingly common in severe dysplasia and carcinoma (Figs. 1 and 2).

Average fluorescence intensities of healthy tissue, hyperkeratosis, mild dysplasia, moderate dysplasia, severe dysplasia, and carcinoma after 90 and 180 minutes ALA application in all animals are shown in Table 1. Healthy tissue demonstrated the lowest fluorescence intensity (Tables 2 and 3, $P < 0.01$), and severe dysplasia and carcinoma evidenced significantly higher intensity than other tissue conditions (Tables 2 and 3, $P < 0.01$) after 90 and 180 minutes ALA application. PpIX fluorescence intensities at 90 and 180 minutes in hyperkeratotic tissue were higher than in healthy tissue, mild or moderate dysplasia (Tables 2 and 3), sometimes even equaling fluorescence intensities in severe dysplasia or carcinoma.

After 90 and 180 minutes ALA application, fluorescence intensity discriminated clearly between healthy tissue and (a) hyperkeratosis, (b) mild dysplasia, (c) moderate dysplasia, (d) severe dysplasia, and (e) carcinoma (Tables 2 and 3).

Fig. 2. **a:** H&E stained, enhanced fluorescence and normalized fluorescence images of healthy tissue, hyperkeratosis, and mild dysplasia after 180 minutes ALA application. Arrows identify the mucosal surface. **b:** H&E stained, enhanced fluorescence and normalized fluorescence images of moderate, severe dysplasia and carcinoma after 180 minutes ALA application. Arrows identify the mucosal surface.

The fluorescence intensity of mild and moderate dysplasia at both time points differed significantly from that of severe dysplasia and carcinoma (Tables 2 and 3). In severe dysplasia and carcinoma, “hotspots” of high fluorescence intensity (up to 10,000 f.u.) were seen at irregular intervals in the basal lamina area of the epithelium.

Average fluorescence intensities of healthy tissue, hyperkeratosis, mild dysplasia, moderate dysplasia, severe

dysplasia, and carcinoma after 90 minutes ALA application in individual animals are shown in Table 4. The same trends are shown in individual animals as those summarized for all animals in Tables 1–3.

DISCUSSION

In this study, a 405 nm excitation wavelength and a 635 nm detection wavelength were adopted. Optimal excitation of PpIX fluorescence occurs at approx. 405 nm [16]. ALA-induced PpIX fluorescence emits strongly in the red spectral region with maxima at approximately 635 and 710 nm. Animal studies have demonstrated earlier and stronger PpIX fluorescence after systemic application of ALA in colon [10], bladder [12], and skin tumors [21] than in adjacent healthy tissue. In cancer patients, application of ALA can induce strong PpIX fluorescence in tumors of the bronchi, skin, and mammary tissue [8,9,13,17,22]. High oral doses of ALA (30–60 mg/kg b.w.) have been successfully used for demarcation of colorectal and oral tumors [11,15]. However, in most of these studies systemic ALA was used, and no correlation was attempted between stage and type of malignancy and fluorescence signature.

Previous preliminary time-based pharmacokinetic studies in the hamster cheek pouch model have demonstrated

TABLE 1. Fluorescence Intensities in All Animals

	Mean fluorescence intensity (f.u.) $\pm$ SD ^a	
	After 90 min ALA application	After 180 min ALA application
Healthy tissue	110.09 $\pm$ 5.44	49.62 $\pm$ 4.98
Hyperkeratosis	372.74 $\pm$ 134.35	285.29 $\pm$ 103.31
Mild dysplasia	353.06 $\pm$ 162.29	240.03 $\pm$ 172.41
Moderate dysplasia	301.56 $\pm$ 103.88	222.92 $\pm$ 163.34
Severe dysplasia	452.72 $\pm$ 319.44	308.98 $\pm$ 211.71
Carcinoma	454.18 $\pm$ 292.91	295.40 $\pm$ 84.68

^an = 25 measurements/histopath grade/animal; 18 animals.

TABLE 2. Statistical Analysis: All Animals: Fluorescence Intensity After 90 Minutes ALA Application

	Healthy tissue	Hyperkeratosis	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Carcinoma
Healthy tissue		**	**	**	**	**
Hyperkeratosis	**		NS		NS	NS
Mild dysplasia	**	NS		NS	*	**
Moderate dysplasia	**	**	NS		**	**
Severe dysplasia	**	NS	*	**		NS
Carcinoma	**	NS	**	**	NS	

* $P < 0.05$.** $P < 0.01$.**TABLE 3. Statistical Analysis: All Animals: Fluorescence Intensity After 180 Minutes ALA Application**

	Healthy tissue	Hyperkeratosis	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Carcinoma
Healthy tissue		**	**	**	**	**
Hyperkeratosis	**		**	**	NS	NS
Mild dysplasia	**	**		NS	**	**
Moderate dysplasia	**	**	NS		**	**
Severe dysplasia	**	NS	**	**		NS
Carcinoma	**	NS	**	**	NS	

* $P < 0.05$.** $P < 0.01$.**TABLE 4. Fluorescence Intensities in Individual Animals**

Animal number	Histopathology	Mean f.u. $\pm$ SD after 90 min ALA application ^a
1	Healthy tissue	108.32 $\pm$ 4.04
1	Hyperkeratosis	372.74 $\pm$ 73.96
1	Mild dysplasia	380.42 $\pm$ 52.02
2	Healthy tissue	114.19 $\pm$ 6.13
2	Hyperkeratosis	242.37 $\pm$ 63.42
2	Mild dysplasia	262.19 $\pm$ 45.20
2	Moderate dysplasia	357.89 $\pm$ 69.21
3	Healthy tissue	105.93 $\pm$ 3.02
3	Hyperkeratosis	281.51 $\pm$ 74.32
3	Moderate dysplasia	262.04 $\pm$ 57.98
3	Severe dysplasia	372.74 $\pm$ 134.35
4	Healthy tissue	106.51 $\pm$ 5.27
4	Moderate dysplasia	353.06 $\pm$ 162.29
4	Severe dysplasia	563.58 $\pm$ 172.21
5	Healthy tissue	110.09 $\pm$ 5.44
5	Hyperkeratosis	372.74 $\pm$ 134.35
5	Moderate dysplasia	353.06 $\pm$ 162.29
5	Severe dysplasia	484.13 $\pm$ 209.86
5	Carcinoma	550.75 $\pm$ 214.65
6	Healthy tissue	110.09 $\pm$ 5.44
6	Mild dysplasia	372.74 $\pm$ 134.35
6	Moderate dysplasia	353.06 $\pm$ 162.29
6	Severe dysplasia	527.91 $\pm$ 339.76
6	Carcinoma	649.48 $\pm$ 261.30

Representative results are shown from six of the 18 animals, due to space constraints.

^an = 25 measurements/histopath grade/animal.

optimal fluorescence intensity ratios between healthy and pathological tissues 180–240 minutes after topical ALA application [18,19]. In this study, 90 minutes ALA application actually provided stronger fluorescence intensities than 180 minutes, thus even earlier time points will be considered in future studies. The weaker fluorescence levels at 180 minutes are probably related to the practical difficulties associated with maintaining ALA cream in contact with the hamster cheek mucosa over a prolonged period of time. It is likely that a considerable portion of the photosensitizer cream may have been washed off the mucosa, or diluted extensively by the saliva in the animal's mouth.

In this study, ALA was applied topically in a Eucerin vehicle. Other investigators have reported on systemic use of ALA in orange juice ingested in three separate doses at 0, 1, and 2 hours [19]. However, it is desirable to avoid systemic ALA application because of the possibility of dermal photosensitization in patients [29–31]. 5-ALA hydrochloride has also been used as a solution applied locally [17,18,32]. In that study, differences in fluorescence characteristics were observed between healthy and carcinomatous tissues. Any attempt at a more specific fluorescence-based diagnosis was not reported by the investigators involved in that study.

CONCLUSION

This investigation established (1) the ability of LIF following application of ALA to differentiate between healthy tissue and the different stages of oral premalignancy and malignancy and (2) the effectiveness for diagnostic purposes of ALA application at 90 minutes.

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