原 著

Photodynamic Diagnosis of Squamous Cell Carcinoma
in Mouse Tongue Using 5-aminolevulinic AcidNarihiro KAMASAKI¹⁾, Hisazumi IKEDA¹⁾, Kazuo SANO²⁾,
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Abstract

Photodynamic diagnosis (PDD) is a form of cancer detection based on the administration of an exogenous photoactivated compound that accumulates in malignant tissues, followed by adequate photo irradiation. 5-aminolevulinic acid (ALA) is a prodrug of photosensitizer protoporphyrin IX (PpIX). The efficacy and optimal timing of ALA-based PDD was evaluated in mouse NR-S1 squamous cell carcinoma of the tongue. Fluorescence was induced with a pulsed Nd : YAG laser after administration of ALA (200 $\mu\text{g kg}^{-1}$ intraperitoneally, or 400 mg kg^{-1} orally). ALA-induced PpIX fluorescence intensities of about 635 nm in the tumor and adjacent normal tissues were measured. Maximum fluorescence intensities in the tumors were recorded 1 hour after intraperitoneal administration and 2 hours after oral administration of ALA. The PpIX fluorescence (i. p. and p. o. routes of ALA administration) had returned to background levels within 24 hours. PDD following administration of ALA may provide an effective approach for detection of oral cancer.

Keywords : 5-Aminolevulinic acid (ALA), Protoporphyrin IX (PpIX), Photodynamic diagnosis (PDD), Neoplastic lesion, Oral cavity

1. Introduction

Photodynamic diagnosis (PDD) is a promising method for detection of oral cancer.¹⁾ It is based on the administration of a photosensitizer to cells and tissues, the subsequent

exposure of the tissue to light of specific wavelength and the detection of a characteristic fluorescence emission.²⁾ In general, the typical strong porphyrin fluorescence is seen in the malignant cells and tissues, on the

other hand, little fluorescence was visualized in the normal cells and tissues. This difference is known to be caused by lower ferrochelatase activity in the tumor cells compared with normal cells^{(3), (4)} in combination with higher levels of porphobilinogen deaminase activity in tumor.⁽⁵⁾

Since the oral cavity is easily accessible with laser light, it is a suitable site for PDD. Laser-induced fluorescence of tissue after administration of photosensitizer has been used to distinguish between malignant/premalignant lesions and normal tissues in various tumors.⁽⁶⁻¹⁵⁾ The photosensitizers most commonly used to date consist of hematoporphyrin and its derivatives, in particular, porfimer sodium (Photofrin), a mixture of various porphyrins.^(11, 12) However, low selectivity and prolonged cutaneous sensitivity of at least 7-10 weeks are severe disadvantages of these agents.⁽¹³⁾ Recently, 5-aminolevulinic acid (ALA) has been advocated as a photosensitizing agent for photodynamic diagnosis (PDD) and therapy (PDT).⁽¹⁰⁾ The main advantages of using ALA as a photosensitizing agent lie in its rapid elimination from the body.

ALA is a natural precursor to protoporphyrin IX (PpIX) in the haem biosynthesis pathway of biological systems.⁽¹⁶⁾ After administration of ALA, the induced PpIX is characterized by preferential accumulation in malignant tissue as compared with normal tissue, and the short-term photosensitization usually lasts less than 24 hours. This tissue-specific photosensitization provides a basis for using ALA-induced PpIX not only for PDT but also for diagnostic purposes.^(6, 7, 17, 18)

ALA can be administered by injection, orally, or topically.⁽¹⁹⁾ Oral administration

of ALA would be simple, safe, economical and more acceptable to patients.⁽¹⁸⁾

The aim of this study was to investigate the feasibility of ALA-based PDD and determine its optimal route of administration.

2. Materials and methods

2.1. Animals and tumor models

Sixty male C3H/HeNCrj mice (Charles River, Osaka, Japan), aged 4-6 weeks and weighing of 20-25 g, were used in this study. They were housed in a room with subdued lighting and fed a standard pellet diet (Oriental Yeast, Osaka, Japan) and water.

Transplantable mouse squamous cell carcinoma, NR-S1,⁽²⁰⁾ was originally obtained from the National Institute of Radiological Sciences (Chiba, Japan) and was maintained by serial passage on the hind flanks of mice. Each mouse in this study was slightly anesthetized by inhalation of diethyl ether, and tumor cells were transplanted to the tongue. The tumors had reached the size of 5 × 5 mm in about 6 days after transplantation. At the time of the fluorescence measurements, the tumors were 5-6 mm in diameter.

2.2. Photosensitizing agent

5-aminolevulinic acid (ALA) hydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan).

For the intraperitoneally ALA-based PDD group (i. p. group), ALA was freshly dissolved in saline to a concentration of 10 mg ml⁻¹ and injected at a dose of 200 mg kg⁻¹ body weight (n = 25).

For the orally ALA-based PDD group (p. o. group), each mouse (n = 30) was given 400 mg kg⁻¹ of ALA freshly dissolved in 0.5 ml of saline by means of gastric tube.

2.3. ALA-induced PpIX fluorescence spectroscopy

At 0.5, 1, 2, 4 and 24 hours after the i. p. administration, and 0.5, 1, 2, 4, 8 and 24 hours (5 mice for each time point) after the oral administration of ALA, the mice were sacrificed, and the tongues were excised. The tongue tumors were then cut into two halves, and the fluorescence spectra were measured. For the control group, the tumor and the adjacent normal tissue of the mice ($n = 5$) without ALA administration were also measured.

The excitation source was a laser system consisting of a pulsed Nd : YAG laser (Quanta-Ray® DCR-3 and PDL-2, Spectra Physics, Mountain View, California, USA) tuned to 532 nm. The PpIX fluorescence emission spectra of the mouse tongues were recorded with a fiber-guided spectrometric multichannel analyzer (SMA System, Tokyo Instruments, Tokyo, Japan). The fiber tip was held about 2 cm away from the tissue, and excitation light was focused onto the tissue in a diameter of 1 mm. The fluorescence was measured at 4 points in the tumor

Fig. 1 Tongue tumors were cut into two halves ; the figure represents the interior of the tongues. The fluorescence spectra were measured at the points of the tumor center, the tumor-muscle border, and 3 and 5 mm away from the border.

and in the healthy region. These points included the tumor center, the tumor-muscle border, and points at 3 and 5 mm away from the border (Fig. 1). The excised tongue tumor represents white, while the surrounding healthy muscle represents dark red color under white light. The induced fluorescence was guided back through the same fiber and the fluorescence spectrum was recorded in the range of 400-800 nm. The peak fluorescence value at 635 nm was used for analysis. The fluorescence intensities in all spectra are represented in arbitrary units.

2.4. Fluorescence imaging

After the elapse of 2 hours from oral ALA (400mg kg^{-1}), a 100 W mercury light source was used in the dark room to excite PpIX at a wavelength of 400-440 nm. AY-48 yellow filter (Toshiba, Tokyo, Japan) was set in front of the lens in order to absorb fluorescence below 480 nm.

2.5. Statistical analysis

Differences in fluorescence intensities for each point at different times were analyzed using analysis of variance (ANOVA). Statistical significance was taken as $P < 0.05$. If a significant overall difference was present, multiple comparisons were performed using Fisher's protected least significant difference (PLSD) multiple comparison procedure. Data are presented as means $\pm$ standard error of the mean (SEM).

3. Results

3.1. PpIX fluorescence spectrum from tumor and normal region

No PpIX fluorescence was observed in the tumor and the normal region without ALA administration. There was no significant difference in the autofluorescence between

from tumor and normal tissue.

The PpIX fluorescence feature peaking at 635 nm was detected in the tumor and the normal region at 1 hour after i. p. administration of ALA (200mg kg⁻¹). The PpIX fluorescence was much more pronounced in the tumor as compared with the adjacent normal

Fig. 2 The fluorescence spectrum in the tumor of a mouse tongue and in adjacent normal tissue at 1 hour after i. p. (200mg kg⁻¹) administration of ALA. Peak PpIX fluorescence at about 635 nm was recorded from the tumor.

region (Fig. 2).

3.2. Time courses of PpIX fluorescence after ALA administration

For the i. p. group, the PpIX fluorescence intensity reached a maximum at 1 hour after ALA injection, then decreased rapidly and returned to baseline levels within 24 hours (Fig 3-a). Significant differences ($P < 0.01$) between the point of tumor and the other regions were demonstrated only at 1 hour after i. p. injection.

For the p. o. group, the PpIX fluorescence intensity in the tumor increased rapidly and plateaued at 1-4 hours (maximum at 2 hours), then gradually decreased and returned to baseline levels within 24 hours. There were significant differences between the tumor and the points located 3 mm and 5 mm away from the border at 0.5, 1, 2, and 4 hours ($P < 0.01$ at 0.5, 4 hours, $P < 0.05$ at 1, 2 hours) after p. o. administration (Fig. 3-b).

PpIX fluorescence ratios (tumor/normal

Fig. 3-a PpIX fluorescence at each point after ALA i. p. injection (200 mg kg⁻¹). Maximum PpIX fluorescence intensity in the tumor was at 1 hour after injection, and the fluorescence declined rapidly after reaching the maximum level. There were significant differences between the tumors and the other regions only at 1 hour after administration. The asterisk indicates $P < 0.01$. The PpIX fluorescence returned to background levels within 24 hours. Data are represented as averages $\pm$ SEM from 5 animals.

Fig. 3-b PpIX fluorescence at each point following ALA oral (400 mg kg^{-1}) administration. Maximum fluorescence of PpIX occurred at 2 hours after administration. There were significant differences between the tumor and the points located 3 mm and 5 mm away from the border at 0.5, 1, 2, and 4 hours after administration. The asterisk indicates $P < 0.01$ at 0.5, 4 hours, $P < 0.05$ at 1, 2 hours. However the PpIX fluorescence returned to background levels within 24 hours. Data are represented as averages $\pm$ SEM from 5 animals.

Fig. 4 PpIX fluorescence ratios (tumor/normal) after ALA i. p. injection (200 mg kg^{-1}) and oral (400 mg kg^{-1}) administration. The p. o. group maintained a higher ratio level than the i. p. group from 0.5-4h. Data are represented as averages $\pm$ SEM from 5 animals.

tissue) after both i. p. and p. o. administration are presented in Figure 4. The ratios were given for tumor center to the point at 5 mm away from the border (tumor center/the point at 5 mm away from the border). The fluorescence intensity in the tumor was

about 3 times higher than that in the normal tissue 1 h after both routes of ALA administration. The ratio of the p. o. group maintained a higher level (approximately 3-4) in the 0.5-4 hour period than that of the i. p. group. For the i. p. group, the maximum

ratio was recorded at 1 hour post-injection with a ratio of about 3 times, and then decreased immediately. The PpIX fluorescence ratio in both groups (i. p. and p. o. routes of ALA administration) returned to the background level within 24 hours (Fig. 4). Data are represented as averages $\pm$ SEM from 5 animals.

3.3. Localization of ALA-induced PpIX fluorescence

After oral ALA, the typical red fluorescence of PpIX was observed only in the tumor of the mouse tongue, and demarcation of the tumor was demonstrated (Fig. 5). The red PpIX fluorescence in the tumor could be seen with naked eye through the yellow filter.

4. Discussion

Many experimental or clinical trials for PDD using ALA have been performed in various types of tumor, including colonic,^{6, 15)} mammary,⁷⁾ dermatic,⁹⁾ liver,¹⁴⁾ and gastric¹⁷⁾ cancer. ALA can be administered intravenously, but has recently been used orally or topically.^{18, 19)} The characteristic ALA-induced PpIX fluorescence peaking at 635 nm was detected in the tumor, distinguishing it from the adjacent healthy tissue (Fig. 2). The excitation wavelength (532 nm), employed from previous article¹⁹⁾ was available to detect the PpIX fluorescence spectra.

This result suggests applicability of ALA for PDD in oral malignant lesions. Attenuation of fluorescence was shown to be slower for the p. o. group than for the i. p. group.

Some authors have reported the importance of the tumor-normal PpIX fluorescence ratio for fluorescence diagnosis.^{11, 15)} High intensity of PpIX fluorescence may increase the

Fig. 5 Fluorescence excitation (400-440 nm) 2 hours after oral administration of ALA (400mg kg^{-1}). Typical red fluorescence illumination of protoporphyrin IX was detected in the tumor.

sensitivity of PDD, but may also cause false positives resulting in decreased specificity. A high ratio would therefore be useful in enhancing the specificity of PDD. As shown in Figure 4, the p. o. group maintained a higher fluorescence ratio level than the i. p. group, indicating that PDD following oral administration of ALA might have higher specificity than that following i. p. administration. This might be caused by the difference of mechanism of ALA accumulation and metabolism in the tissues. ALA administered orally needs to pass through intestinal enterocytes before entering the portal and final systemic circulation. First-pass metabolism can occur in the intestinal lumen by the resident flora, within the wall of the gastrointestinal tract and in the liver. Due to first-pass metabolism, a higher oral dose of ALA is required to achieve the same level of photosensitization as intravenous administration.¹⁸⁾ However the exact reason of the difference in specificity is unknown. Furthermore, oral administration is safer, simpler, more convenient than administration by injection.

Fluorescence imaging of the tongue consisted with the results of spectroscopic observation. This suggests the capability of distinguishing a tumor from normal tissue in real time.

Point monitoring of ALA - induced PpIX fluorescence enables tumor detection, while fluorescence imaging might serve as an intraoperative guide for the surgical margin to allow a more radical tumor removal.

In conclusion, the present study suggests that ALA-based PDD is a useful, safe and promising technique for detecting oral cancer. Oral administration of ALA had higher specificity than i. p. injection in the present study.

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