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ORIGINAL ARTICLE

Influence of administration methods on the accumulation of ALA-induced Pp-IX in mouse tongue tumors

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OBJECTIVE: 5-Aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) has been used as a photosensitizer in photodynamic therapy (PDT) for oral cancer. This study investigates the optimal method of administrating ALA by analyzing PpIX fluorescence in tongue tumor tissue.

METHODS: Protoporphyrin IX intensities in the mouse (C3H)-transplanted tongue cancer (NR-S1) were compared with those in normal tongue after intraperitoneal (i.p.), oral (p.o.) or topical administration of ALA. Tongues were sampled at various times after ALA administration. PpIX intensities were obtained from frozen sections of each sample by using a spectrophotometer.

RESULTS: Protoporphyrin IX intensity in the tumor group peaked at 3 h after the i.p. and 5 h after the p.o. administration of ALA, and these levels were about twice as high as those in the normal group. Maximum PpIX accumulation in the tongue tumor tissue was seen at 5 h after p.o. administration of ALA. In contrast, the topical administration of 20% ALA cream was associated with the lowest PpIX accumulation in the tumor throughout the experiments.

CONCLUSION: These results suggested that p.o. administration of ALA was the most effective method in ALA-PDT for oral cancer.

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Keywords: 5-aminolevulinic acid; protoporphyrin IX; photodynamic therapy; tongue cancer; spectroscopy; pharmacokinetic

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Introduction

Tongue cancer is one of the most common malignancies in the oral region. Surgery with radiotherapy and/or chemotherapy has been used as the conventional treatment for tongue cancer. However, this treatment causes cosmetic and functional disturbances, especially in the head and neck region.

Photodynamic therapy (PDT) is a promising cancer treatment in which a photosensitizing drug accumulates in tumors and is subsequently activated by visible light of an appropriate wavelength matched to the absorption (Date *et al*, 2003). The advantages of this method, as compared to other conventional cancer treatment modalities, are its low systemic toxicity and its ability to destroy tumors selectively (Gaullier *et al*, 1997). Photofrin is the most widely used photosensitizer in clinical PDT trials and is the only agent that has been approved for cancer treatment in many countries. However, photofrin remains in the skin and causes photosensitivity lasting several weeks, and the tumor selectivity of this agent is poor (Peng *et al*, 1997a). 5-Aminolevulinic acid (ALA) is a precursor of protoporphyrin IX (PpIX) in the biosynthetic pathway for heme, and PpIX is an efficient photosensitizer. Today ALA–PDT is successfully used for the treatment of a variety of neoplastic and non-neoplastic diseases (Peng *et al*, 1997b). ALA-derived PpIX can be cleared from the body within 24–48 h after systemic ALA administration (Grant *et al*, 1993), and because of this rapid clearance, ALA-based PDT would reduce the risk of prolonged skin phototoxicity (Webber *et al*, 1997).

The kinetics of ALA-induced PpIX production in different tissues has been studied, typically by means of fluorescence spectroscopic techniques (Stolic *et al*, 2002). However, the relationship between the PpIX fluorescent accumulation in oral tumor tissue and the ALA administration methods has not been elucidated. This study investigated the optimal method for administrating ALA in PDT by analyzing PpIX fluorescence in tongue tumor tissue.

Materials and methods

Animals and tumors

Male C3H/HeNCrj mice, 6–8 weeks old, 22–26 g (Charles River, Osaka, Japan) were used in all experiments.

The animals were housed with subdued lighting and had free access to food and water. An NR-S1 mouse squamous cell carcinoma (Usui *et al*, 1976) (National Institute of Radiological Sciences, Chiba, Japan) was transplanted to the mouse tongue, and when the tumor reached a size of at least 3 mm × 3 mm, the photosensitizer was administered. The photosensitizer was also administered to normal mice as a control.

Chemicals and ALA administration route

5-Aminolevulinic acid was obtained as a hydrochloride in 98.0% pure powder from Cosmo Oil (Tokyo, Japan).

In the intraperitoneal (i.p.) ALA administration group, ALA was freshly dissolved in 0.2 ml of saline and injected at a dose of 250 or 500 mg kg^{-1}.

In the oral (p.o.) ALA administration group, animals were given 250 or 500 mg kg^{-1} of ALA freshly dissolved in 0.5 ml of saline by means of a gastric tube.

In the case of topical ALA administration, an oil-in-water emulsion containing 20% ALA was freshly prepared prior to use. After topical administration of ALA cream to the tongue, animals were maintained under deep anesthesia by pentobarbital sodium to prevent the ALA cream from being washed out or swallowed. Furthermore, two kinds of ALA ester derivative (ALA methyl ester and ALA pentyl ester; Cosmo Oil) were also administrated and compared with topical application. These ALA ester derivatives are more lipophilic than ALA and thus may penetrate more easily through the keratinized layer and deeper into tumors than ALA itself (Juzenas *et al*, 2002).

Mice were killed at 1, 3, 4, 5, 6 and 8 h after ALA administration ($n = 4$ animals per time point) and mouse tongue samples were excised. Serial frozen sections (10-μm thick) of each sample were prepared for exact histologic localization and quantitative measurement of concentration of PpIX. PpIX localization was confirmed by fluorescence microscopy (PROVIS-AV80type; Olympus, Tokyo, Japan) by comparing with a hematoxylin–eosin (H–E)-stained section. The wavelength width of the excitation filter was in the blue violet region (400–440 nm) and the observation wavelength was more than 475 nm.

Quantitative measurement of PpIX fluorescence

Protoporphyrin IX intensities in the mouse-transplanted tongue cancer were compared with those in the normal tongue after i.p., p.o. or topical administration of ALA. Levels of PpIX fluorescence were measured with a spectrophotometer. The details of this measurement method have been described by Miyoshi *et al* (2004).

Fluorescence emission spectra excited by 410-nm light were obtained from a total of five serial frozen sections (10-μm thick) from each sample by using a spectrophotometer (850 type; Hitachi, Tokyo, Japan) equipped with a holder for the particle sample.

The subtracted spectrum was obtained by subtracting the background spectrum from the sample raw spectrum.

A typical fluorescence spectrum from a tumor showed prominent emission bands at $\lambda = 635$ and 705 nm, which corresponded to the standard PpIX spectrum (Figure 1).

Protoporphyrin concentration (μM) was calculated from the fluorescence intensity at the 635-nm peak of the sample emission spectrum and a calibration curve of the known concentrations of standard PpIX solution (Figure 2). Standard PpIX aqueous solution was

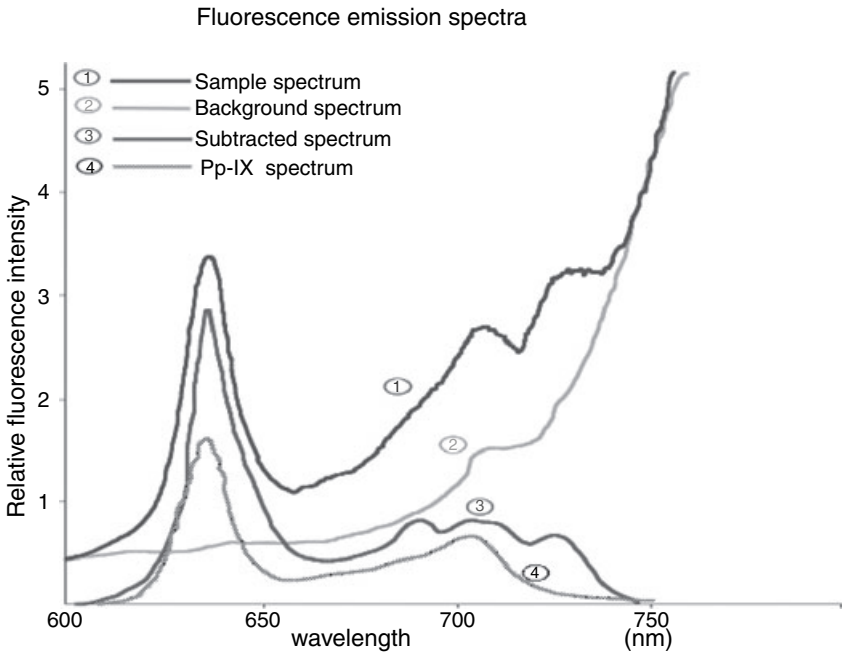

Figure 1 Fluorescence emission spectra excited by 410-nm light are obtained from a total of five serial frozen sections (10-μm thick) from each sample by using a spectrophotometer. The no. 3 subtracted spectrum was obtained by subtracting the no. 2 background spectrum from the no. 1 sample raw spectrum. In addition, we confirmed that the no. 3 subtracted spectrum pattern corresponded to the no. 4 standard protoporphyrin IX (PpIX) spectrum

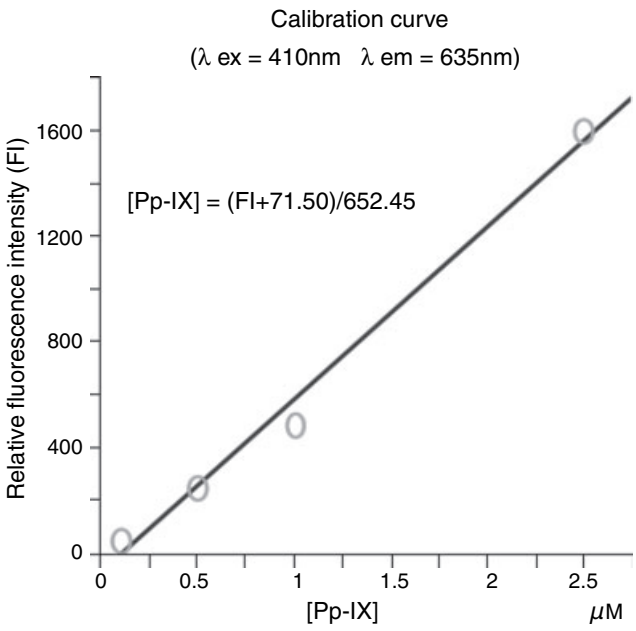

Figure 2 Protoporphyrin IX (PpIX) concentration (μM) was calculated from the fluorescence intensity at the 635-nm peak of the subtracted spectrum and a calibration curve of known concentrations of standard PpIX

prepared with phosphate-buffered saline solution, cationic surfactant, acetyl-trimethyl-ammonium-bromide and PpIX.

Statistics

Groups of normally distributed data were compared using Student's *t*-test, while the non-parametric Mann–Whitney test was otherwise employed. Values of <0.05 were considered to indicate statistical significance.

Results

The fluorescence microscopic image showed that the red fluorescence emission of PpIX was distributed strongly and homogeneously in the tongue tumor tissue at 5 h after p.o. administration of ALA (Figure 3). However, PpIX accumulation was not seen in the necrotic area of the tumor tissue. In addition, there was very weak PpIX accumulation in the normal lingual muscle after administration of ALA.

The tumor group showed constantly higher PpIX intensities than the normal group throughout the

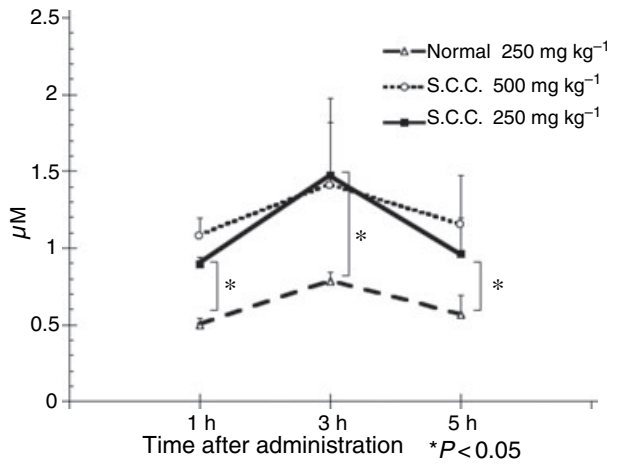

Figure 4 Protoporphyrin IX intensity in tongue tissue after intraperitoneal administration of 5-aminolevulinic acid

experiments following the i.p. and p.o. administration of ALA. PpIX intensity in the tumor group peaked at 3 h after i.p. and 5 h after p.o. administration of ALA, and these peak values were about twice as high as those in the normal group. However, the PpIX intensity in the tumor group was not enhanced by an increase in the administrated dose of ALA from 250 to 500 mg kg^{-1}(Figures 4 and 5). Maximum PpIX accumulation in the tongue tumor tissue was seen at 5 h after the p.o. administration of ALA (Figure 6). In contrast, the topical administration of 20% ALA cream was associated with the lowest PpIX accumulation in the tumor throughout the experiments (Figure 7). Furthermore, the topical administration of 20% ALA ester derivatives cream (ALA methyl ester and ALA pentyl ester) also resulted in low PpIX accumulation in the tumor, which was not different from the case of topical administration of 20% ALA cream.

Discussion

If the photosensitizer that is administered before light illumination accumulates more highly in tumor tissue, the efficacy of PDT for cancer might be improved. Although photofrin should be used only via intravenous administration, ALA can be used via various administration routes. There have been numerous studies on ALA-induced PpIX fluorescence after a variety of administration routes in various organs. Systemic

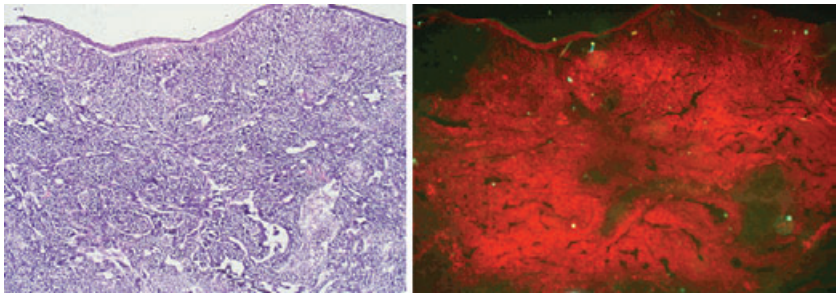

Figure 3 Fluorescence image and corresponding hematoxylin–eosin-stained image of tongue tumor tissue at 5 h after oral administration of 5-aminolevulinic acid

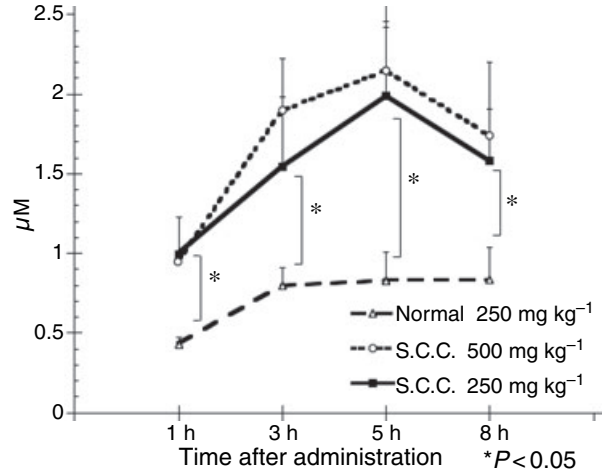

Figure 5 Protoporphyrin IX intensity in tongue tissue after oral administration of 5-aminolevulinic acid

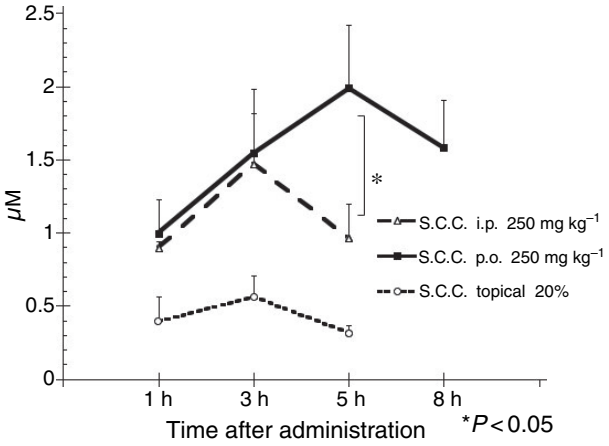

Figure 6 Protoporphyrin IX intensity in tongue tumor tissue after various types of 5-aminolevulinic acid administration

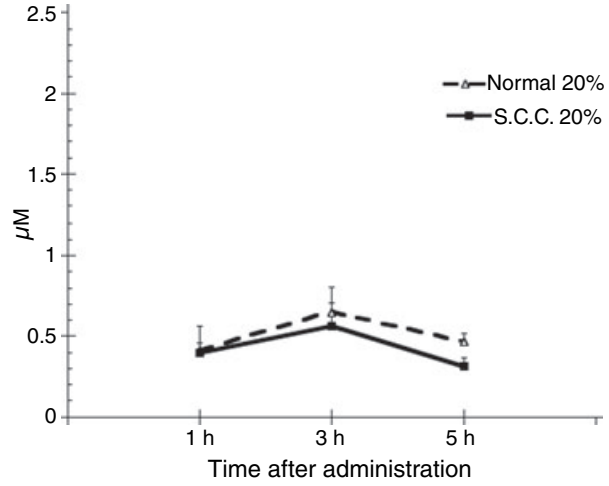

Figure 7 Protoporphyrin IX intensity in tongue tissue after topical administration of 5-aminolevulinic acid

(intravenous or p.o.) ALA-based PDT has also been reported for treatment of oral neoplastic lesions (Grant *et al*, 1993; Fan *et al*, 1996). However, the optimal administration method of ALA in PDT for oral cancer is still not established. The present study was carried out to determine the most effective route and optimal timing of ALA administration with respect to subsequent therapeutic illumination.

In most studies, the techniques used were based on a non-invasive method using a spectrofluorometer or the chemical technique of high-pressure liquid chromatography (HPLC) to measure *in vivo* PpIX fluorescence after administration of ALA. Spectrofluorometry is simple and non-invasive, but can detect only surface emission of the tissue. HPLC can measure the whole tissue, but the resulting values are an average for the whole tissue (more than 1 g) and have no relation to the histopathologic findings. Furthermore, the techniques required for this method are complicated (Miyoshi *et al*, 2004). We confirmed the direct detection of PpIX concentrations from the frozen section always in combination with histologic staining using cryosamples.

Previous studies have shown that the peak of PpIX fluorescence intensity varied between 1 and 6 h after ALA administration in different tissues (Grant *et al*, 1993; Henderson *et al*, 1995; Ma *et al*, 1999). Our present results showed that PpIX intensity in the tongue tumor tissue peaked at 3 h after i.p. administration of ALA. Another study has also shown that PpIX fluorescence in rat tongue cancer reached a maximum intensity at 3 h after ALA i.p. administration (Ma *et al*, 1999). However, in this study, the maximum PpIX accumulation in the tongue tumor tissue was confirmed at 5 h after p.o. administration of ALA. Although the reason for this finding is unclear, Mustajoki *et al* (1992) have shown that a high serum ALA level can be achieved in a human volunteer by continuous enteral infusion of ALA solution. Loh *et al* (1993) reported that the temporal fluorescence kinetics after p.o. administration were comparable with that after intravenous injection in the stomach, colon and bladder mucosa of normal rats. Oral administration is considered to be simpler and it does not require full buffering. ALA can be undertaken by patients themselves, prior to therapy and without supervision (Loh *et al*, 1993). The results of the present study suggested that p.o. administration was the most effective administration method in ALA-PDT for oral cancer.

Furthermore, there was no obvious difference in PpIX intensity between 250 and 500 mg kg^{-1} after both i.p. or p.o. administration of ALA. Accumulation of PpIX in tongue tumor tissues reaches a plateau after administering at least 250 mg kg^{-1} doses of ALA. Ma *et al* (1999) reported that early malignant lesions in rat tongue showed complete response to the i.p. administration of ALA-based PDT at both 250 and 1000 mg kg^{-1}. These results suggested that it is not necessary to administer a greater amount of ALA to achieve sufficiently high PpIX levels suitable for PDT in oral cancer.

Topical ALA-based PDT has been widely used in treating neoplastic lesions of the skin and bladder (Peng *et al*, 1997b), because local administration of ALA might increase the PpIX concentration in the tumor without unwanted general side effects. It is known that topical application of an oil-in-water emulsion of ALA on the skin lesion can permit penetration of ALA into the lesion and allow synthesis of PpIX (Kennedy and Pottier, 1992: Szeimies *et al*, 1994). Recently, in cases of oral cancer, topical administration of ALA as a rinsing solution has also been tried for ALA photodynamic diagnosis. However, because there has been no report on the use of topical administration ALA-PDT for oral cancer, we here tried topical administration of 20% ALA cream for tongue tumor. Our results showed that PpIX intensity in the tongue tumor after topical administration of ALA cream was not enhanced compared with that in the normal tongue. Several ALA esters have been synthesized and are more lipophilic than ALA. This higher lipophilicity might result in better penetration into the skin, higher PpIX levels and a more uniform and deeper PpIX distribution (van den Akker *et al*, 2000). Therefore, we attempted to apply the two kinds of ALA esters for topical administration. However, ALA esters also did not enhance the PpIX intensity in the tongue tumor tissue. Although the reason for this result is unclear, the neutral pH of saliva might cause the immediate degeneration of ALA in the oral mucosa (Loh *et al*, 1993; van den Akker *et al*, 2000). Based on these results, 5 h after p.o. administration of ALA was regarded to be the optimal time for light irradiation in ALA-PDT.

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