ORIGINAL ARTICLE

# Thioredoxin upregulation by 5-aminolaevulinic acid-based photodynamic therapy in human skin squamous cell carcinoma cell line

Tomoe Kuhara, Daisuke Watanabe, Yoichi Akita, Tomohiro Takeo, Natsuko Ishida, Aki Nakano, Noriko Yamashita, Yuichiro Ohshima, Morihiro Kawada, Takeshi Yanagishita, Yasuhiko Tamada & Yoshinari Matsumoto

Department of Dermatology, Aichi Medical University School of Medicine, Aichi, Japan

**Key words:**
5-aminolaevulinic acid; cancer; photodynamic therapy; reactive oxygen species (ROS); thioredoxin

**Correspondence:**
*Tomoe Kuhara, Department of Dermatology, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan.*
*Tel: +81 561 623311*
*Fax: +81 561 639914*
*e-mail: tomoe@aichi-med-u.ac.jp*

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**Conflicts of interest:**
None declared.

## Summary

***Background/purpose:*** 5-aminolaevulinic acid-based photodynamic therapy (ALA-PDT) is widely performed in the clinical setting for superficial skin cancers, giving favorable results, but residual tumor and recurrence occur occasionally. Thioredoxin is a common antioxidant that suppresses apoptosis and facilitates cell growth. We investigated the expression of thioredoxin following ALA-PDT in human skin squamous cell carcinoma cell line, HSC-5.

***Methods:*** ALA-PDT was performed in HSC-5 cells using low-dose ($5\,J/cm^2$, $100\,mW/cm^2$) or high-dose ($30\,J/cm^2$, $100\,mW/cm^2$) irradiation, and the expression of thioredoxin was measured by Western blotting. An MTT assay was used to assess cell growth following a low dose of multiple irradiations. Cell death was examined by Western blotting for caspase-3 and PARP. Immunofluorescence double staining using annexin V and propidium iodine was also performed.

***Results:*** Expression of thioredoxin was only observed following low-dose exposure ALA-PDT. Multiple low-dose exposure ALA-PDT significantly proliferated cell growth. With high-dose exposure ALA-PDT, caspase-3 and PARP expression were seen, and cell death due to apoptosis and/or necrosis was observed, but thioredoxin was barely detected.

***Conclusion:*** Low-dose exposure ALA-PDT increased the expression of thioredoxin and facilitated the growth of HSC-5 cells.

Superficial skin cancers such as actinic keratosis, basal cell carcinoma and Bowen's disease (1, 2) have been treated by 5-aminolaevulinic acid-based photodynamic therapy (ALA-PDT). The antitumor mechanism of ALA-PDT has not been fully clarified, but it has been shown that the production of reactive oxygen species (ROS) generates oxidative stress to induce cytotoxic effects, and subsequent apoptosis and/or necrosis lead to cell death (3–5). However, there have been cases of residual tumor and recurrence following ALA-PDT (6), which may be caused by insufficient laser irradiation or oxidative stress resistance.

Thioredoxin (13 kDa) is a common antioxidant, which was discovered as a coenzyme that donates a hydrogen ion to ribonucleotide reductase, an enzyme essential for DNA synthesis in *Escherichia coli* (7). Human thioredoxin was cloned as an adult T-cell leukemia-derived factor by Tagaya *et al.* in 1989 (8). The active site of thioredoxin (–Cys–Gly–Pro–Cys–) is highly conserved from *E. coli* to mammals. In oxidized thioredoxin, the two cysteine groups of the active site form a disulfide bond (S–S). On the other hand, they form a dithiol (–SH–SH) as the reduced form. Thioredoxin itself eliminates singlet oxygen and hydroxy radicals; in the presence of peroxiredoxin, it eliminates intracellular ROS. It also functions as a cofactor for ribonucleotide reductase to facilitate DNA synthesis and cell growth (9), and indirectly controls the DNA activities of various transcription factors such as NF-kB and p53 through redox factor 1 (Ref-1/HAP1) and AP-1 (Fos/Jun heterodimer) (10–12). Furthermore, thioredoxin regulates the activity of enzymes including apoptosis signal-regulated kinase 1 (ASK-1) by binding in a redox-dependent manner (13). Thioredoxin is expressed in response to ultraviolet light and has been shown to aid cancerization by facilitating cell growth and suppressing apoptosis (14, 15).

Residual malignant tumor or recurrence following insufficient ALA-PDT might involve thioredoxin. Here, we have measured the expression of thioredoxin following low-dose ($5\,J/cm^2$, $100\,mW/cm^2$) or high-dose ($30\,J/cm^2$, $100\,mW/cm^2$) exposure ALA-PDT using the human squamous cell carcinoma cell line, HSC-5.

## Materials and methods

### Cell line

The human skin squamous cell carcinima cell line, HSC-5, was obtained from the Human Science Research Resources Bank

(HSRRB, Osaka, Japan) and maintained in RPMI 1640 supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), glutamine (2 mmol/l) and fetal bovine serum (10%). This cell line was used for detecting thioredoxin caspase-3 and PARP expression for *in vitro* ALA-PDT.

### ALA-PDT

HSC-5 cells were plated in 12-well plates at a density of $2.0 \times 10^4$ cells/cm$^2$. The medium was replaced with medium without serum but containing ALA (50 μmol/l) for 2 h in a humidified atmosphere of 5% $CO_2$ at 37 °C. Each experiment was conducted at least three times to confirm its reproducibility.

To determine the effective light dose for ALA-PDT, the cells were irradiated with 1, 5, 10, 20, 30 and 40 J/cm$^2$ (each 100 mW/cm$^2$) at 630 nm, using an eximer dye laser PDT EDL-1 (Hamamatsu Photonics K.K., Hamamatsu, Japan). This experiment was evaluated by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method using the Tetra Color ONE reagent (Seikagaku Corp., Tokyo, Japan).

The experimental conditions for detecting thioredoxin, caspase-3 and PARP were determined from the results of the previous test. The cells were incubated with 50 μmol/l of ALA for 2 h, and then irradiated with low-dose or high-dose exposure. After irradiation, the cells were incubated in culture medium for various lengths of time (0, 4, 8, 16 or 24 h for thioredoxin; 0, 2, 4, 8, 16 or 24 h for caspase-3 and PARP) and harvested with a cell scraper.

Multiple-exposure experiment was also performed. First ALA-PDT was performed with low-dose or high-dose exposure. Twenty-four hours after first PDT, a second ALA-PDT was performed with the same dose each (low dose:low dose or high dose:high dose). Before each irradiation, ALA (50 μmol/l) was added to the cells and incubated for 2 h. After second irradiation, the cells were incubated in culture medium for 24 h. Cells without ALA were not subjected to irradiation but were harvested with a cell scraper.

### Microscopic analysis

Immunofluorescence double staining evaluated by fluorescence microscopy demonstrated early apoptosis (annexin V positive) and confirmed necrosis [propidium iodide (PI) positive] in the cells at 3 h after ALA-PDT. Control cells were not subject to laser irradiation.

### Western blot analysis

The cell samples for Western blot analysis of thioredoxin, caspase-3 and PARP were prepared as described above. To prepare whole-cell samples, HSC-5 cells were harvested and lysed in sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris pH 6.8, 10% glycerol, 10 mM NsF and bromophenol blue). For thioredoxin, the concentration of dithiothreitol in the sample buffer was 1 mM. Aliquots were subject to Western blot analysis. Subcellular fractions were electrophoresed on SDS-polyacrylamide gel (4–20%) and transferred to PVDF membranes, which were probed with a primary antibody. The antibodies used were: anti-thioredoxin (human from Redox Bio Science Inc., Kyoto, Japan) anti-caspase-3 (human, mouse from R&D Systems Inc., Minneapolis, MN, USA) and anti-PARP (human, mouse and rabbit from Cell Signaling Technology Inc., Danvers, MA, USA). After probing, the membranes were washed three times and then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibodies (rabbit for caspase-3 and PARP, mouse for thioredoxin). Interactions were detected with Super Signal West Dura Extended Duration Substrate (PIERCE Biotechnology Inc, Rockford, IL, USA). Western blots were also performed using mouse anti-β-actin antibody (Sigma-Aldrich Japan Inc., Tokyo, Japan).

### Growth kinetics

The HSC-5 cells were seeded in 12-well plates and then incubated in medium lacking serum but containing ALA (50 μmol/l) for 2 h. The cells then received low-dose irradiation. After irradiation, the cells were incubated in culture medium for 24 h, and then incubated again with ALA (50 μmol/l) for 2 h before being subjected to low-dose irradiation. After irradiation, the cells were incubated in culture medium for various lengths of time (24, 48, 72 h). ALA-alone cells were not subject to irradiation; controls received no treatment. ALA-alone cells and controls were also incubated in culture medium for various lengths of time (24, 48, 72 h). Viable adherent cells were collected and counted in quadruplicate under a phase-contrast microscope by trypan blue exclusion at appropriate intervals. Day 1 was defined as 24 h after the second irradiation; cell proliferation was determined up to day 3. Results are the mean of three independent experiments.

### Statistical analysis

The data were expressed as mean ± standard deviation (SD) or median (range). ANOVA (Stat View ver. 5; SAS Co Ltd., Cary, NC, USA) was used where appropriate for statistical analysis. *P*-values $< 0.05$ were considered to be statistically significant.

## Results

### Induction of cell death by ALA-PDT

HSC-5 cells were subjected to ALA-PDT at different levels of exposure, and an MTT assay was performed to count viable cells 24 h after irradiation. The suppression of cell growth with 1%, 5%, 10%, 20%, 30% and 40 J/cm$^2$ irradiation was 2%, 5%, 31%, 67%, 90% and 92%, respectively (Fig. 1). As a result, low- and high-dose exposures were defined as 5 and 30 J/cm$^2$, respectively.

### Expression of thioredoxin following ALA-PDT

After ALA-PDT, the expression of thioredoxin in subcellular fractionated samples was measured by Western blotting. With

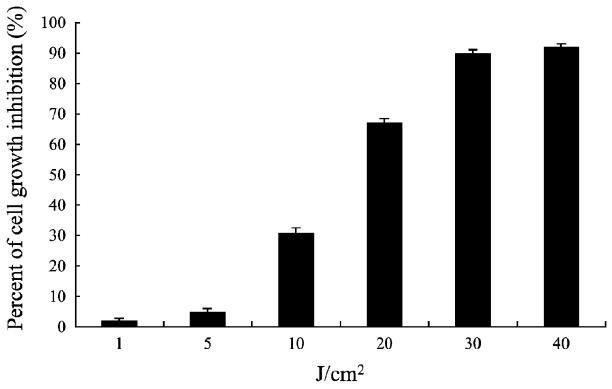


**Fig. 1.** HSC-5 cell death using eximer dye laser (630 nm), evaluated by MTT assay. Up to 5 J/cm$^2$ most cells did not die, but with 30 J/cm$^2$, cell growth was suppressed by 90%. Values are the mean of three independent experiments.

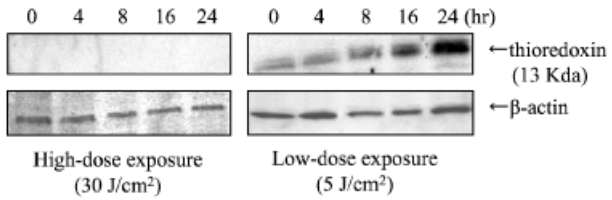


**Fig. 2.** HSC-5 cells were subjected to low-dose exposure (5 J/cm$^2$) or high-dose exposure (30 J/cm$^2$) ALA-PDT, and the expression of thioredoxin was measured at different times. The expression of thioredoxin increased with time following low-dose exposure ALA-PDT (right), but was absent following high-dose exposure ALA-PDT (left). ALA-PDT, 5-aminolaevulinic acid-based photodynamic therapy.

low-dose exposure ALA-PDT, thioredoxin expression increased with time (Fig. 2, right-hand panel). However, hardly any thioredoxin was detected following high-dose exposure ALA-PDT. Cells were subject to a second ALA-PDT 24 h later with the same dose each, and the samples obtained 24 h after this were analyzed by Western blotting. The expression of thioredoxin after the second low-dose exposure ALA-PDT was higher (Fig. 3, left-hand panel), while the results following the second high-dose exposure ALA-PDT were the same as those following a single irradiation, in which thioredoxin was barely detected (Figs 2 and 3).

### Chronological changes in cell growth

HSC-5 cells were divided into the following three groups to compare chronological changes in cell growth: a control group (no ALA or laser irradiation), an ALA group (ALA but not laser irradiation) and a PDT group (low-dose exposure ALA-PDT performed twice at a 24 h interval). The results showed a significant difference in cell growth between the PDT group and the other two groups 72 h after PDT ($P < 0.05$) (Fig. 4).

### Detection of cell death by apoptosis and/or necrosis

HSC-5 cells were subjected to low- or high-dose exposure ALA-PDT and then double immunofluorescence stained using annexin V and PI. Under a fluorescent microscope, apoptotic cells (annexin V) and necrotic cells (PI) were detected (Fig. 5). The number of apoptotic and/or necrotic cells increased in a dose-dependent manner.

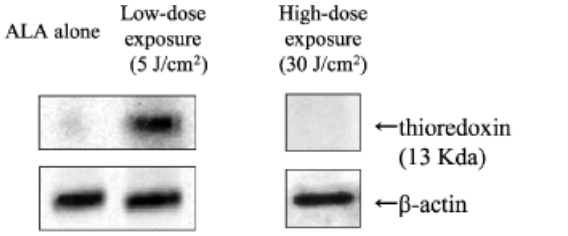


**Fig. 3.** ALA-PDT was performed twice, 24 h apart, and the expression of thioredoxin was investigated 24 h after the end of the second irradiation. With low-dose exposure ALA-PDT, the expression of thioredoxin was marked (left). However, with high-dose exposure ALA-PDT, the expression of thioredoxin was absent (right). ALA-PDT, 5-aminolaevulinic acid-based photodynamic therapy.

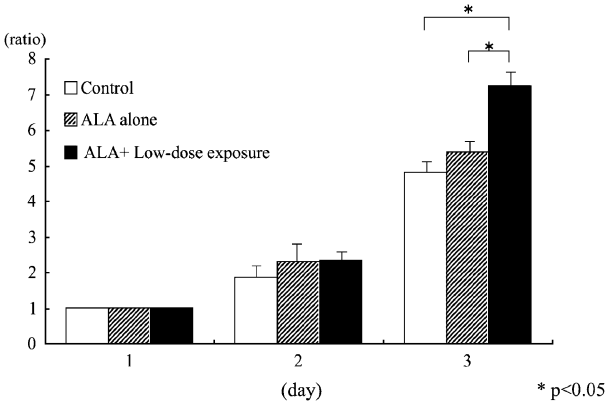


**Fig. 4.** HSC-5 cells were divided into the following three groups to compare the chronological changes in cell growth: control group (no ALA or laser irradiation), ALA group (ALA but not laser irradiation) and PDT group (low-dose exposure ALA-PDT was performed twice with a 24-h interval). At 72 h after PDT, there was a significant difference in cell growth between the PDT group and the other two groups ($P < 0.05$). ALA-PDT, 5-aminolaevulinic acid-based photodynamic therapy.

### Detection of caspase-3 and PARP

HSC-5 cells were subjected to ALA-PDT, and Western blotting was performed to detect active forms of caspase-3 and PARP. With high-dose exposure ALA-PDT, cleaved forms of caspase-3 and PARP were detected starting 4 h after irradiation (Fig. 6a and b, right-hand panel). However, with low-dose exposure ALA-PDT, activated caspase-3 and PARP were barely seen (Fig. 6a and b, left-hand panel).

## Discussion

Topical ALA-PDT is a promising and useful method for the treatment of superficial skin cancers because the affected sites are accessible to light exposure and the method has few side

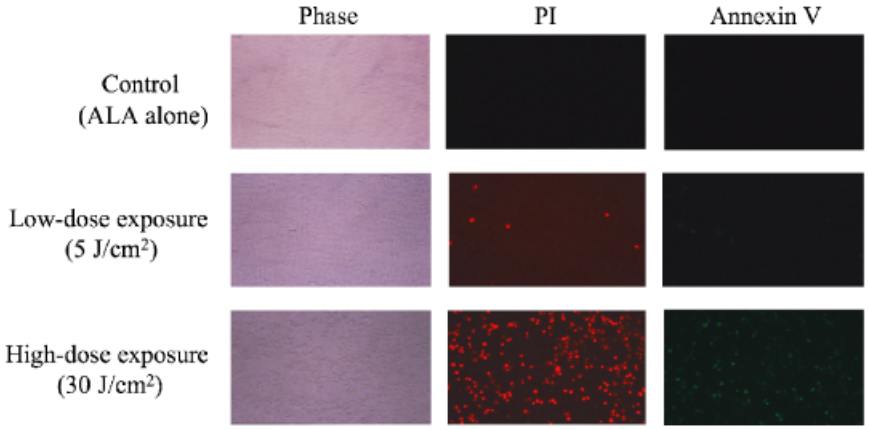


**Fig. 5.** HSC-5 cells were subjected to low-dose exposure or high-dose exposure ALA-PDT and then immunofluorescense double stained using annexin V and PI. Under a fluorescent microscope, apoptotic cells (annexin V) and necrotic cells (PI) were detected. The number of apoptotic and/or necrotic cells increased in a dose-dependent manner. ALA-PDT, 5-aminolaevulinic acid-based photodynamic therapy; PI, propidium iodide.

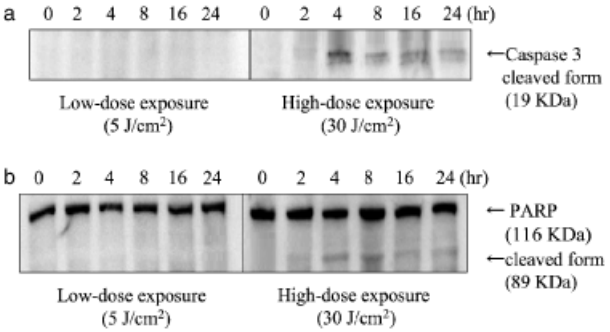


**Fig. 6.** HSC-5 cells were subjected to ALA-PDT, and Western blotting was performed to measure caspase-3 and PARP activities. With high-dose exposure ALA-PDT, caspase-3 and PARP activities were detected starting 4 h after irradiation (a and b, right). However, with low-dose exposure ALA-PDT, caspase-3 and PARP activities were not detected (a and b, left). ALA-PDT, 5-aminolaevulinic acid-based photodynamic therapy.

effects (16–19). However, in some cases, disease recurrence after treatment is a major problem (20, 21). The distribution of ALA in skin is dependent on many parameters such as drug penetration through the stratum corneum, diffusion through the epidermis and dermis, drug clearance rate and conversion of ALA to protoporphyrin IX (22). Furthermore, it is thought that the laser light does not reach lesions encasing adnexal structures or proliferative superficial skin tumors with atypical cell infiltration (23) and the resulting insufficient exposure might lead to residual tumor and recurrence. Here, in order to analyze the mechanism of prolonged therapy due to insufficient exposure, HSC-5 cells were subjected to ALA-PDT with low- (5 J/cm$^2$) or high-dose (30 J/cm$^2$) exposure, and then cell death and the expression of antioxidants hindering therapy were assessed.

Thioredoxin is a low-molecular-weight (13 kDa) redox protein that protects cells against oxidant damage. Ultraviolet A (UVA) and B (UVB) radiation increases thioredoxin expression in human keratinocytes and skin fibroblasts increased expression of thioredoxin-1 protects human skin fibroblasts from UVA-radiation-induced DNA damage (24–27). Thioredoxin provides reducing equivalents to cytoplasmic thioredoxin peroxidases that protect cells against oxidant-induced apoptosis by scavenging hydrogen peroxide and organic hydroxides (28).

There are several reports arguing apoptotic cell death induced by different doses of irradiations, but the results are controversial. Takasawa and Tanuma observed that in human keratinocyte HaCaT cells, apoptosis was induced by UVB irradiation with a high dose (500 J/cm$^2$), but not with a low dose (150 J/cm$^2$), because low-dose UVB irradiation resulted in blocking the apoptosis signal cascade at the caspase activation step and so the cells survived (29). The clinical effect of different fluence rates of ALA-PDT was compared using hamster subcutaneous amelanotic melanoma model (30). In the study, only high-dose PDT induced complete remission of all tumors with a significant decrease of partial oxygen pressure ($pO_2$). In contrast, Bisland *et al.* (31) reported that low-dose ALA-PDT induced apoptosis more effectively than high-dose PDT by using a rat malignant blain tumor model. Taken together, the optimal irradiation dose or fluence rates might be dependent on the kind, location and environment of the target tumor.

In our study, high-dose exposure ALA-PDT induced extensive number of cell deaths with the expression of caspase-3, PARP and annexin V, but thioredoxin expression was hardly detected. On the other hand, in low-dose exposure ALA-PDT, the number of dead cells was limited and the expression of caspase-3 and PARP was hardly detected, but the induction of thioredoxin was observed. In some clinical cases, ALA-PDT is repeated several times against target tumors. To mimic the clinical ALA-PDT condition, we also examined multiple ALA-PDT with different doses of irradiation. The expression of thioredoxin was observed only in low-dose irradiation cells. It was also interesting that marked HSC-5 cell growth was observed by low-dose exposure ALA-PDT at 48 and 72 h after irradiation. When laser irradiation was performed without ALA, cell growth was not detected (data not shown). These results are consistent with the previous observations that an increase of thioredoxin is associated with cell proliferation and apoptosis inhibition (9–14).

In conclusion, this is the first study to show that the expression of thioredoxin increases and cell growth is facilitated with low-dose exposure ALA-PDT. However, high-dose exposure ALA-PDT induced marked cell death. Therefore, as one of the factors for residual tumor and recurrence, insufficient exposure might increase the expression of thioredoxin and might actually facilitate tumor cell growth due to the proliferation effects of thioredoxin.

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