Photodynamic therapy suppresses the migration and invasion of head and neck cancer cells in vitro

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Summary  Head and neck cancer is highly invasive. It has a tendency to metastasise to regional or distant sites after incomplete treatment. Photodynamic therapy (PDT) is effective in the treatment of head and neck cancers. To investigate the effect of sublethal PDT on the invasiveness of head and neck cancer cells and to elucidate the possible mechanisms, we initiated this study. Two head and neck cancer cell lines, KJ-1 and Ca9-22, were used in this study. Wound healing assay, migration assay, and matrigel invasion assay were used to evaluate the cell migration and invasion. Immunoblotting was performed to investigate the possibly involved signaling pathways. Sublethal PDT significantly suppressed the migration and invasion of both KJ-1 and Ca9-22 cells. Phosphorylation of the focal adhesion kinase (FAK) and its down-stream Src kinase and extracellular signal-regulated kinase (ERK) were also inhibited after sublethal PDT. Sublethal PDT suppresses the migration and invasion of Ca9-22 and KJ-1 cells. Inhibited phosphorylation of the FAK-Src kinase-ERK signaling pathway may be involved in the PDT-induced migration/invasion suppression.

Introduction

Photodynamic therapy (PDT) is now an established treatment for neoplastic diseases. It involves a tumour localising photosensitiser that induces cytotoxicity when activated by light of an appropriate wavelength. Singlet oxygen and oxygen free radicals are responsible for the PDT-induced cytotoxicity.1 It has been demonstrated that tumour cells have higher accumulation of photosensitiser than the surrounding normal tissue.2 As a result, when the photoactivation occurs upon the sensitisier, tumour cells were selectively destroyed compared to normal cells. The benefit is more obvious in
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the head and neck region, where traditional treatment modalities such as surgery and irradiation inevitably cause destruction of surrounding tissue and thus to carry severe aesthetic and functional impairments. In contrast, PDT has the advantage of selective eradication of tumour cells and better preservation of local tissue without sacrificing the efficacy of tumour control.3–5 In addition, photodynamic therapy does not have the cumulative toxicity associated with ionising radiation and can be applied safely to previously irradiated tissues.6 Thus, it has considerable potential for patients with locally persistent or recurrent disease after surgery or radiotherapy.

5-Aminolevulinic acid (5-ALA) is an endogenous cellular component and is metabolised within the haem biosynthetic pathway to produce protoporphyrin IX (PpIX), a potent endogenous photosensitiser. This metabolic pathway is well regulated by feedback control arising from the presence of cellular concentrations of haem. As a consequence, haem biosynthesis is under such tight feedback control that only small amounts of PpIX are present in most cells at any given time. However, if excessive amounts of ALA are introduced midway into the cycle, presumably through drug delivery intervention, intermediates, such as PpIX, back-accumulate in cellular compartments in neoplastic cells. These cells are known to have a reduced capacity to metabolise PpIX to haem. The insertion of iron by ferrochelatase is a rate-limiting step and further adds to this accumulation of cellular PpIX. As a result, selective cells become photosensitised to the action of light.7

Head and neck cancer is locally aggressive and highly invasive. It has a tendency to invade surrounding tissues and/or metastasise to regional or distant sites after incomplete resection. However, in a recent paper, we reported our experience of treating 45 locally recurrent head and neck cancer patients with PDT.6 Among them, 15 patients received more than one PDT treatment and none had developed distant metastasis during the follow up period. So, anecdotally, PDT seems to have the potential of inhibiting the invasiveness and metastasis of cancer cells. In this paper, we studied the effect of 5-ALA PDT on the migration and invasion of head and neck cancer cell lines. Possible underlying mechanisms were also investigated. Our results showed that PDT suppressed the migration and invasion of head and neck cancer cells, probably due to inhibition of tyrosine phosphorylation of the focal adhesion kinase (FAK) and its down-stream Src kinase-extracellular signal-regulated kinase (ERK) signaling pathway.

Materials and methods

Materials

5-ALA was obtained from Sigma (St. Louis, MO, USA). Antibodies used in all experiments included anti-FAK (BD, NJ, USA), anti-pFAK-Y397 (Upstate, NY, USA) anti-pFAK-Y925 (Cell signaling, MA, USA), anti-Src (Upstate, NY, USA), anti-phosphotyrosine (Sigma, St. Louis, MO, USA), anti-p130cas, anti-ERK, and anti-pERK (Santa Cruz, CA, USA). All the chemicals were obtained from Sigma (St. Louis, MO, USA) unless specifically mentioned.

Cell culture and 5-ALA incubation

Nasopharyngeal carcinoma (NPC) cell line KJ-1 and oral squamous cell carcinoma cell line Ca9-22 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) at 37°C in a humidified 5% CO2 incubator. The cells for PpIX measurement or PDT treatment were incubated with 1 mM 5-ALA, which was diluted in serum-free medium and neutralized to pH 7.2 with NaOH immediately before use.

Measurement of the PpIX fluorescence

The measurement of PpIX fluorescence was described before.8 In brief, the cells were plated at a density of 5 × 103 in 6 cm petri dish overnight, and then incubated with 5-ALA for 1, 2, 3 or 4 h, respectively. After incubation, the cells were washed with PBS, scraped off from the petri dish, and then centrifuged at 1800 g for 10 min after the addition of 0.1% Triton TX-100. The fluorescence of extracted PpIX was measured in the Amino-Bowman series 2 spectrophotometer (SLM Instruments, Urbana, IL) at an excitation wavelength of 405 nm and an emission wavelength of 610 nm. PpIX concentration was deduced from a standard curve of PpIX (6.25–100 ng/ml). The protein content of the cells was determined by the Pierce Micro BCA protein assay method, and the PpIX content was expressed as nmole/mg protein.

Photodynamic treatment

For photodynamic treatment, the cells were incubated with 5-ALA for 3 h, washed thoroughly in PBS, and then exposed to various doses of light. After light treatment, the cells were incubated with fresh complete medium for further experiments. The cell viability was determined by using the [3(4,5-dimethyl-thiazoyl-2-yl) 2,5 diphenyl-tetrazolium bromide] (MTT assay) 24 h after PDT. The light doses used in the wound healing, cell migration and matrigel invasion assays were that to kill 10–20% of the cells. The light source for 5-ALA activation was a diode laser with an emission of red light at a wavelength of 633-nm (CeramOptec GmbH, Germany). Control groups consisted of cells that were not incubated with 5-ALA and had no light treatment, the cells that were not incubated with 5-ALA but had light treatment, and the cells that were incubated with 5-ALA but without light treatment. The results were obtained from at least three individual experiments.

Wound healing assay

The cells were grown to confluence in 3.5 cm petri dishes and then subjected to PDT. After PDT, the cell monolayer was lesioned using a 1.2 mm cell scraper without damaging the dish surface. Lesion areas were imaged at 0 h and 16 h after PDT. The photos were analysed in imaging software. The distance of cell migration was calculated by subtracting the distance between the lesion edges at 16 h from the distance measured at 0 h. The values were expressed in millimeter.

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Cell migration assay

Migration of KJ-1 and Ca9-22 cells through 8-μm pores was assessed using the Transwell cell culture chamber (Corning Costar, Tokyo, Japan) as described previously. In brief, cells at a density of $1 \times 10^4$ were seeded in the upper surface of the transwell insert. The upper chambers were supplemented with serum-free media while the larger lower chambers were supplemented with complete media. The cells in the upper chambers were then subjected to PDT. After 24 h, the cells on the upper surface of the insert membrane were removed with a cotton swab and the migrated cells on the underside of the insert membrane were fixed using 100% ethanol and stained with haematoxylin. The number of cells that had migrated to the underside of the insert membrane was counted in five random high-power fields under a light microscope. For each experimental condition, the assay was performed at least in triplicates.

Matrigel invasion assay

The invasiveness of cells before and after PDT was measured by using the matrigel invasion assay as described previously. Briefly, transwell inserts with 8-μm pores (Becton Dickinson, Franklin Lakes, NJ) were coated with Matrigel (0.77 g/μl; Becton Dickinson, Bedford, MA). Cells at a density of $1 \times 10^4$ were seeded in the upper chambers with 150 μl of serum-free medium. Medium supplemented with 10% FCS was placed in the lower wells. The cells in the upper chambers were subjected to PDT and then cultured for 24 h. Cells that had invaded to lower surface of the matrigel-coated membrane were fixed with 70% ethanol, stained with haematoxylin, and counted in five random high-power fields under a light microscope. For each experimental condition, the assay was performed at least in triplicates.

Immunoblotting

Proteins from cell extracts or immunoprecipitates were separated on SDS-PAGE and transferred electrophoretically to PROTRANBA85 nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH). The membranes were blocked with 5% skimmed milk and the proteins of interest detected using specific monoclonal or polyclonal antibodies as indicated, followed by appropriate secondary antibodies. Immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham Bioscience, England, UK).

Results

Photodynamic treatments

To determine the best time to perform PDT, we measured the cellular PpIX levels in Ca9-22 and KJ-1 cells after 5-ALA incubation. As shown in Figure 1, the pattern of cellular PpIX levels in both cell lines was similar. It gradually increased after incubation with 5-ALA, peaked at 3 h, and then decreased afterwards. Thus we used 3 h as the incubation time for 5-ALA in subsequent PDT experiments.

Figure 1 The PpIX fluorescence in KJ-1 and Ca9-22 cells. The cells were incubated with 1 mM 5-ALA and the PpIX fluorescence was measured as described in the "Materials and Methods". Each point represented the mean value obtained from three independent experiments.

Figure 2 Cytotoxicity of 5-ALA PDT on Ca9-22 and KJ-1 cells. Ca9-22 (A) and KJ-1 (B) cells were incubated with 1 mM 5-ALA for 3 h and then exposed to light irradiation at different light doses. Cell viability was assessed by MTT assay 24 h after light irradiation. Data were obtained from three independent experiments. Bars, SE.
The cytotoxic effects of 5-ALA PDT on the cells were shown in Figure 2. It seemed that the Ca9-22 cells were more sensitive to 5-ALA PDT than the KJ-1 cells. The light dose that caused 50% of cell death (LD50) was around 0.5 J/cm² for Ca9-22 cells, while the LD50 for KJ-1 was around 3 J/cm².

PDT suppressed the migration and invasion of Ca9-22 and KJ-1 cells

To evaluate the effects of PDT on the migration and invasion of cancer cells, a sublethal dose that killed only 10–20% of cancer cells, a sublethal dose that killed only 10–20% of

**Figure 3** 5-ALA-PDT suppressed cell migration in wound healing assay. (A) monolayer of Ca9-22 and KJ-1 cells were lesioned by a scraper in petri dish. Repair of lesion by cell migration with or without PDT was photographed 16 h later. The total migrating distance of Ca9-22 (B) and KJ-1 (C) cells from the edges of the lesion after 16 h was measured. Data represented mean ± SE and were obtained from three independent experiments. *p < 0.05, **p < 0.001.
the cells was used. Accordingly, the light dose we used in this study was 0.25 J/cm² and 1.0 J/cm² for Ca9-22 and KJ-1 cells, respectively. In the wound-healing assay, PDT significantly suppressed the migration of both Ca9-22 and KJ-1 cells (Fig. 3A). The migrating distance of Ca9-22 cells decreased from 0.83 ± 0.1 mm in the control cells to 0.43 ± 0.01 mm in PDT-treated cells (p < 0.05) (Fig. 3B). In the KJ-1 cells, the migrating distance was 0.442 ± 0.134 mm in the control group and 0.037 ± 0.035 mm in the PDT group (p < 0.001) (Fig. 3C). In the cell migration assay, the number of either Ca9-22 or KJ-1 cells migrating through the pores of transwell cell culture chamber was also reduced after sublethal dose PDT (data not shown). Matrigel invasion assay showed that PDT significantly suppressed the invasiveness of both cancer cells (Fig. 4). The average cell counts crossing matrigel-coated membrane in one high power field was 69.8 ± 4.9 for the control group and 1.3 ± 1.0 for the PDT group of KJ-1 cells (p < 0.001); 75.7 ± 8.1 for the control group and 40 ± 5.3 for the PDT group of Ca9-22 cells (p < 0.05).

Phosphorylation of the FAK, Src kinase, and ERK was inhibited after PDT

The FAK signaling pathway plays a central role in the migration and invasion of cancer cells.11 FAK signaling involves its phosphorylation on different tyrosine residues and its binding to Src kinase to form Src-FAK signaling complex.12,13 Our Western blot analyses showed PDT significantly inhibited the phosphorylation of FAK (at both Y397 and Y925), Src kinase and ERK at 30 min after treatment (Fig. 5). The phosphorylation of Src kinase returned to baseline level at 3 h after PDT while the phosphorylation of FAK (Y925) and ERK was still inhibited at this time point. At 24 h after PDT, when the phosphorylation of FAK has resumed the baseline level, prolonged inhibition of the ERK phosphorylation was still noted (Fig. 5).

![Figure 4](image-url)  
*Figure 4 Matrigel invasion assay. (A) Image of cells invading through matrigel-coated pored membrane in the control and PDT-treated group. (B) Average cell count of invaded cells per 5 HPFs. The data were obtained from at least three independent experiments. *p < 0.05, **p < 0.001. Bars, SE.*
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Since the migration/invasion of NPC cells was significantly suppressed, PDT might be a useful addition to the armamentarium of the combined treatment modalities for NPC.

The therapeutic benefit of 5-ALA-mediated PDT in some cancers was well-documented. Its mechanism was based on the selectively higher accumulation of photosensitiser PpIX in cancer cells. The hypothesised explanations include reduced ferrochelatase content in tumour cells to metabolise PpIX into haem and poorly developed vascular drainage around tumour impeding efflux of PpIX. In order to acquire the maximal photosensitising effect, the timing of peak PpIX accumulation should be determined before light irradiation. We measured the fluorescence of PpIX and found the concentration of PpIX peaked at 3 h after incubation with 1 mM 5-ALA in both KJ-1 and Ca9-22 cells (Fig. 1). Other studies investigating the intracellular PpIX levels in different cell lines after exogenous 5-ALA administration have also revealed the time for peak intracellular PpIX accumulation was around 3–8 h after incubation. The efficacy of 5-ALA-mediated PDT may depend on both the amount of PpIX accumulated and the light dose applied. Since the intracellular PpIX level was higher in the KJ-1 cells than in the Ca9-22 cells (Fig. 1), it is reasonable to expect the light dose needed to achieve a certain level of cell killing should be lower in the KJ-1 cells. However, results from the cytotoxicity assay showed that KJ-1 cells were more resistant to 5-ALA-PDT than the Ca9-22 cells (Fig. 2), suggesting that factors other than PpIX concentration contributed to the 5-ALA-PDT-mediated cytotoxicity. One reasonable explanation is the involvement of different death pathways in the 5-ALA-PDT-mediated cytotoxicity in different cell lines.

These results underscored the complex interplays between 5-ALA metabolism, PpIX accumulation, and cytotoxicity in 5-ALA-PDT.

Cell migration is essential for the invasion and metastasis of cancer cells. It involves the assembly and disassembly of the focal adhesion complex. These integrin-linked complexes are the primary sites of adhesion between cells and the surrounding extracellular matrix (ECM). FAK plays a central role that serves to regulate the turnover of these adhesion sites. FAK controls the dynamic regulation of integrin-linked adhesions and is an important regulator of cell migration. In normal cells, FAK mRNA level is usually very low. Increased FAK expression and activity are frequently correlated with malignant or metastatic disease and poor patient prognosis. In response to external signals, such as integrin clustering or growth receptor stimulation, autophosphorylation of FAK on tyrosine (Y) residue 397 is activated and creates a high affinity binding site for the Src homology 2 (SH2) domain of several proteins including the upstream Src kinase itself. The association of Src with FAK leads to a conformational change and formation of Src-FAK signaling complex, resulting in the activation of the kinase activity of Src. The ensuing phosphorylation of FAK by Src is required for the full enzymatic activity of FAK. In this study, our results showed that the phosphorylation of FAK on tyrosine residue 397 (pFAK-Y397) was significantly suppressed at 30 min after PDT, and then gradually recovered afterwards (Fig. 5). Decreased phosphorylation of FAK-Y397 should have a negative effect on the formation of Src-FAK signaling complex resulting in the reduced cell migration.

Discussion

PDT is a superficial treatment because the light fluence rate in tissues decreases with the logarithm of the distance from the site of illumination. Thus, a major problem of PDT in clinical cancer treatment is that the periphery of tumours might receive insufficient light dose to completely eradicate the cancer cells. This may lead to recurrence and even metastasis of the tumour. However, our previous experience anecdotally suggested that PDT might have the potential of inhibiting the invasiveness and metastasis of incompletely treated cancer cells. A recent study by Linsjak et al. also showed that 5-ALA PDT decreased the angiogenesis and metastasis of cancer cells in a mice Lewis lung carcinoma model. To look for a mechanistic support to our clinical observation, we studied the effects of sublethal PDT on the migration and invasion of head and neck cancer cells. Our results showed that sublethal PDT significantly suppressed the migration and invasion of cancer cells (Figs. 3 and 4). Although Ca9-22 cells were more sensitive to 5-ALA PDT than the NPC KJ-1 cells (LD50 0.5 J/cm² for Ca9-22 cells, 3 J/cm² for KJ-1 cells), the KJ-1 cells were more susceptible to PDT-induced migration/invasion-suppression. The invasion rate after PDT (cells invaded through matrigel in the PDT group/cells invaded through matrigel in the non-PDT control group) was 53% and 2% for the Ca9-22 and KJ-1 cells, respectively (Fig. 4). It is known that NPC has a high propensity for local invasion and regional and distant metastasis, which is responsible for most treatment failures.

Figure 5  PDT inhibited the phosphorylation of FAK, Src kinase, and ERK. KJ-1 cells were treated with sublethal dose of PDT, collected at 30 min, 3 h and 24 h after PDT, and then subjected to Western blot analyses.

By 3 h, the phosphorylation of FAK on tyrosine residue 397 (pFAK-Y397) was significantly lower in the PDT group than in the control group (Fig. 5). The downstream targets of FAK include Src and ERK. Src is activated and creates a high affinity binding site for the SH2 domain of several proteins, including the upstream Src kinase itself. The association of Src with FAK leads to a conformational change and formation of Src-FAK signaling complex, resulting in the activation of the kinase activity of Src. The ensuing phosphorylation of FAK by Src is required for the full enzymatic activity of FAK. In this study, our results showed that the phosphorylation of FAK on tyrosine residue 397 (pFAK-Y397) was significantly suppressed at 30 min after PDT, and then gradually recovered afterwards (Fig. 5). Decreased phosphorylation of FAK-Y397 should have a negative effect on the formation of Src-FAK signaling complex resulting in the reduced cell migration.

FAK
pFAK-Y397
pFAK-Y925
Src
pSrc
ERK
pERK

Table 1  ALA-PDT-mediated cytotoxicity in different cell lines. The cytotoxicity assay showed that KJ-1 cells were more resistant to 5-ALA-PDT than the Ca9-22 cells. Other studies investigating the intracellular PpIX levels in different cell lines after exogenous 5-ALA administration have also revealed the time for peak intracellular PpIX accumulation was around 3–8 h after incubation. The efficacy of 5-ALA-mediated PDT may depend on both the amount of PpIX accumulated and the light dose applied. The intracellular PpIX level was higher in the KJ-1 cells than in the Ca9-22 cells, it is reasonable to expect the light dose needed to achieve a certain level of cell killing should be lower in the KJ-1 cells. However, results from the cytotoxicity assay showed that KJ-1 cells were more resistant to 5-ALA-PDT than the Ca9-22 cells (Fig. 2), suggesting that factors other than PpIX concentration contributed to the 5-ALA-PDT-mediated cytotoxicity. One reasonable explanation is the involvement of different death pathways in the 5-ALA-PDT-mediated cytotoxicity in different cell lines. These results underscored the complex interplays between 5-ALA metabolism, PpIX accumulation, and cytotoxicity in 5-ALA-PDT.

Cell migration is essential for the invasion and metastasis of cancer cells. It involves the assembly and disassembly of the focal adhesion complex. These integrin-linked complexes are the primary sites of adhesion between cells and the surrounding extracellular matrix (ECM). FAK plays a central role that serves to regulate the turnover of these adhesion sites. FAK controls the dynamic regulation of integrin-linked adhesions and is an important regulator of cell migration. In normal cells, FAK mRNA level is usually very low. Increased FAK expression and activity are frequently correlated with malignant or metastatic disease and poor patient prognosis. In response to external signals, such as integrin clustering or growth receptor stimulation, autophosphorylation of FAK on tyrosine (Y) residue 397 is activated and creates a high affinity binding site for the Src homology 2 (SH2) domain of several proteins including the upstream Src kinase itself. The association of Src with FAK leads to a conformational change and formation of Src-FAK signaling complex, resulting in the activation of the kinase activity of Src. The ensuing phosphorylation of FAK by Src is required for the full enzymatic activity of FAK. In this study, our results showed that the phosphorylation of FAK on tyrosine residue 397 (pFAK-Y397) was significantly suppressed at 30 min after PDT, and then gradually recovered afterwards (Fig. 5). Decreased phosphorylation of FAK-Y397 should have a negative effect on the formation of Src-FAK signaling complex resulting in the reduced cell migration.

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The cell migration and invasion is also controlled by the FAK-Src-ERK signaling pathway. Activated Src kinase phosphorylates FAK on Tyr925 (pFAK-Y925), which creates an SH2-binding site for the GRB2 adapter protein. GRB2 binding to FAK is one of several connections that lead to the activation of Ras and the ERK-2/mitogen-activated protein kinase (MAPK) cascade. In this study, the phosphorylation of Src kinase was transiently suppressed by PDT. However, prolonged suppression of its downstream pFAK-Y925 was observed at 3 h after PDT (Fig. 5). At 24 h after PDT, when the phosphorylation of FAK-Y925 has returned to the baseline level, its downstream ERK was still hypophosphorylated (MAPK) cascade. In this study, the phosphorylation of Src kinase, one of the major regulatory pathways to FAK is one of several connections that lead to the activation of Ras and the ERK-2/mitogen-activated protein kinase (MAPK) cascade. In this study, the phosphorylation of Src kinase was transiently suppressed by PDT. However, prolonged suppression of its downstream pFAK-Y925 was observed at 3 h after PDT (Fig. 5). At 24 h after PDT, when the phosphorylation of FAK-Y925 has returned to the baseline level, its downstream ERK was still hypophosphorylated.

In conclusion, this study demonstrates that sublethal 5-ALA-PDT inhibits the migration and invasion of oral cancer Ca9-22 and NPC KJ-1 cells. The mechanism by which PDT suppresses migration/invasion of cancer cells may be complex because cellular proteins, lipids, nucleic acids and intracellular organelles are all impacted by PDT. Nevertheless, our results demonstrated that the FAK-Src kinase-ERK phosphorylation may also be responsible for the PDT-induced migration/invasion suppression.

Conflict of interest statement
We declare no conflicts of interest.

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References
kinase in acute myeloid leukemia is associated with enhanced blast migration, increased cellularity, and poor prognosis. 

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