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Mechanisms involved in δ-aminolevulinic acid (ALA)-induced photosensitivity of tumor cells: Relation of ferrochelatase and uptake of ALA to the accumulation of protoporphyrin

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ARTICLE INFO

Article history:
Received 31 August 2005
Accepted 7 October 2005

Keywords:
Ferrochelatase
δ-Aminolevlinic acid
Protoporphyrin
Photo-damage
PDT

Abbreviations:
ALA, δ-aminolevlinic acid
DFO, desferrioxamine
DMEM, Dulbecco's modified Eagle's medium
FCS, fetal calf serum
HO-1, heme oxygenase-1
PBG, phorphobilinogen
PDT, Photodynamic therapy
PVDF, poly(vinylidene difluoride)
SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

ABSTRACT

Photodynamic therapy (PDT) using δ-aminolevulinic acid (ALA)-induced accumulation of protoporphyrin IX is a useful approach to the early detection and treatment of cancers. To investigate the role of ferrochelatase in the accumulation of protoporphyrin, we first made mouse fibroblast Balb/3T3 cells highly expressing ferrochelatase and examined the ALA-induced photo-damage as well as the accumulation of porphyrin in the cells. When the ferrochelatase-transfected cells were treated with ALA and then exposed to visible light, they became resistant to the light without accumulating porphyrins, with a concomitant increase in the formation of heme. The accumulation of protoporphyrin was also abolished in human erythroleukemia K562 cells stably expressing mouse ferrochelatase. When mouse fibrosarcoma MethA cells, mouse fibroblast L929 cells and Balb/3T3 cells were treated with ALA, the greatest accumulation of protoporphyrin and the greatest level of cell death in response to the light were observed in MethA cells. The expression level of ferrochelatase was the lowest in MethA cells, while that of porphobilinogen deaminase was similar among all three cell lines. Moreover, an iron-chelator, desferrioxamine, which sequesters iron preventing the ferrochelatase reaction, enhanced the photo-damage as well as the accumulation of protoporphyrin in ALA-treated L929 cells. Thus, the light-induced cell death was tightly coupled with the accumulation of protoporphyrin caused by a decrease in ferrochelatase. Finally, we examined the uptake of ALA by MethA, L929 and Balb/3T3 cells. The extent of the uptake by MethA and L929 cells was greater, indicating a greater accumulation of protoporphyrin than in the Balb/3T3 cells. Taken together, not only the low level of ferrochelatase but also the augmented uptake of ALA contributes to the ALA-induced accumulation of protoporphyrin IX and subsequent photo-damage in cancer cells.

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doi:10.1016/j.bcp.2005.10.019

1. Introduction

Photodynamic therapy (PDT) is a relatively new modality in the treatment of neoplasis. It involves pretreatment of a tissue with a photosensitizer which causes the release of singlet oxygen upon exposure to light, resulting in photo-damage and subsequent tissue destruction [1–3]. The photosensitizers most commonly used are hematoporphyrins and their derivatives [4]. One disadvantage of these photosensitizers is a general photosensitization with skin. ALA has received considerable attention as a precursor of the photosensitizer protoporphyrin in the heme biosynthetic pathway [5–7]. Upon its administration, ALA is converted enzymatically into protoporphyrin, which is effective as an endogenous photosensitizer produced by the cells, and which can be activated by visible light. The application of ALA following PDT treatment has been used in the treatment of skin diseases and has advantages over systemic administration in that the entire body does not face sensitization. ALA-induced PDT has been successfully applied in various medical fields including urology, gastroenterology and dermatology [8,9]. Although there are reports that ALA-induced PDT can also be used as a fluorescence detection marker for the photodiagnosis of tumors [8–10], the mechanisms involved in the specific accumulation of protoporphyrin in cancerous tissues have not been clearly demonstrated. The accumulation of protoporphyrin in tumor cells may be attributable to a difference in metabolizing ability of the porphyrin-heme biosynthetic pathway between cancerous and normal cells.

At the last step in the pathway to synthesize heme, ferrochelatase catalyzes the insertion of ferrous ions into protoporphyrin IX to form protoheme and the eukaryotic enzyme is located in the inner membrane facing the matrix of the mitochondrion [11]. It is known that ferrochelatase in erythroid cells is positively regulated at transcriptional and translational levels [12,13]. Moreover, how the expression of the enzyme is regulated in non-erythroid cells and cancer cells has not been elucidated. In the case of the addition of exogenous ALA, protoporphyrin may accumulate due to the limited capacity for a ferrochelatase reaction [9]. Although the enzyme activity was thought to decrease in cancer cells [8], no direct evidence of the involvement of the expression of the mammalian ferrochelatase in the accumulation of porphyrins in tumor tissues has been obtained. Furthermore, a systematic analysis of the ALA-induced accumulation of protoporphyrin and photosensitivity has not been made. To examine whether ferrochelatase plays a role in the accumulation of porphyrin, we tried to isolate cells highly expressing the enzyme through the transfection of ferrochlatase cDNA. Here, we obtained direct evidence of an inverse correlation of the expression of ferrochelatase to photosensitivity of cells via the accumulation of protoporphyrin.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA modifying enzymes were obtained from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Mesoporphyrin IX was purchased from Porphyrin Products (Logan, UT). Antibodies for bovine ferrochelatase and rat heme oxygenase-1 (HO-1) were, as previously described [13,14]. Anti-PBG deaminase was kindly provided by Dr. Shigeru Sassa. Anti-actin was a product of Santa Crutz Co. (Santa Crutz, CA). All other chemicals were of analytical grade.

2.2. Plasmids

The full-length cDNA of mouse ferrochelatase [15] was digested with *Sma*I and ligated into a *Eco*RV-digested pEF-neo vector [16]. The resulting plasmid pEF-mouse FECH was introduced into *Escherichia coli* XL1-Blue. The plasmid pEF-human FECH was also obtained by ligation of the *Xba*I-digested pEF with the entire human ferrochelatase cDNA [17] as described.

2.3. Cell cultures

Mouse fibroblast Balb/3T3 and fibroblast-like L929 cells, and mouse fibrosarcoma MethA cells were grown in DMEM supplemented with 10% FCS and antibiotics. Human erythroleukemia K562 cells were also grown in RPMI 1640 medium supplemented with 7% FCS and antibiotics. The cells (5×10^5) in a 3.5-cm diameter dish were then incubated in the absence or presence of ALA (100–500 μM) for 16 h before being exposed to light.

2.4. Exposure of the cells to light

The cells were incubated with a specific concentration of ALA for 8–16 h, and 1.0 ml of fresh drug-free medium was then added. Irradiation with visible light was carried out under sterile conditions, using a fluorescence lamp, in a CO_2 incubater. The light was filtered through a glass plate to omit UV light and given from the bottom of the plate to achieve a uniform delivery to the entire plate. The increase of the temperature was confirmed to be less than 2 °C by using a thermo-couple device during exposure to light. The power was calibrated with a power meter, and the period of irradiation was adjusted to obtain fluences of 0.54 and 0.81 J/cm^2. Cell viability was measured by Trypan-Blue exclusion after trypsinization. Each experiment was carried out in triplicate. Controls were as follows: (1) cells exposed to ALA but not exposed to light (dark cytotoxicity), (2) cells untreated with ALA but exposed to light and (3) cells exposed to neither ALA nor light. Cell viability (cell survival) was expressed as a percentage of control cells. Porphyrins were extracted from the cells with 96% ethanol containing 0.5 M HCl, and heme in the cells was converted to protoporphyrin under acidic conditions [18]. The amount of porphyrin was determined by fluorescence spectrophotometry, as previously described [18,19].

2.5. Stable transfection of Balb/3T3 and K562 cells

pEF-human FECH (10 μg) was electroporated into Balb/3T3 cells, as described previously [19]. For selection, G418 (Sigma, St. Louis, MI) at a final concentration of 300 μg/ml was added to

the culture medium. After 7 days, colonies of the G418-resistant cells were trypsinized, seeded in a 24-well tissue culture plate and cultured in medium containing 300 μg/ml of G418. Individual clones were isolated and tested for the expression of mouse ferrochelatase by immunoblotting using anti-ferrochelatase antibodies. Two ferrochelatase-overexpressing clones were obtained, mixed to avoid clonal variation, and maintained in DMEM containing 10% FCS and antibiotics. To obtain K562 cells highly expressing mouse ferrochelatase, pEF-mouse FECH (10 μg) was also electroporated into the cells, as above. For selection, G418 at a final concentration of 500 μg/ml was added to the culture medium. After 7 days, the G418-resistant cells were diluted, seeded in a 96-well tissue culture plate and cultured in medium containing 500 μg/ml of G418. Individual clones were isolated and three ferrochelatase-overexpressing clones were obtained, mixed to avoid clonal variation, and maintained. As control cells (Mock), Balb/3T3 cells or K562 cells were transfected with pEF neo vector, and G418-resistant cells were isolated.

2.6. *Immunoblotting*

The lysates from L929 cells and Balb/3T3 cells were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was done with anti-ferrochelatase, anti-actin and anti-HO-1 antibodies as the primary antibodies [13,14,19].

2.7. *Enzyme assays*

Ferrochelatase activity was measured, using mesoporphyrin and zinc acetate as substrates, as previously described [19]. The protein concentration was estimated by the method of Bradford [20].

2.8. *Uptake of ALA by the cells*

The cells (5×10^5) were incubated in DMEM containing 7% FCS in the presence of 20 μM ALA for a specific period, and the medium was withdrawn. The amount of ALA was determined by using Erhlich's reagent [21].

3. Results

3.1. *The inverse relation between the expression of ferrochelatase and ALA-induced photo-damage in mouse Balb/3T3 cells*

Previous studies [8,22] suggested that in cancer cells, protoporphyrin accumulates because of defective heme biosynthesis, based on the increased activity of PBG deaminase and the decrease in ferrochelatase activity. We tried to examine the direct involvement of ferrochelatase in the ALA-induced PDT, and then generated Balb/3T3 cells stably expressing human ferrochelatase. After pEF-human FECH was transfected and G418-resistant cells were selected, two clones stably expressing human ferrochelatase were isolated and mixed. The ferrochelatase activity was examined, and the level of the expression was determined by immunoblotting. As shown in Fig. 1, two specific bands (42.5 and 42 kDa) reacting with anti-ferrochelatase corresponding to human and mouse enzymes were detected in ferrochelatase-transfectants while only mouse ferrochelatase (42 kDa) was detected in Mock DNA transfected control cells. The enzyme activity in the ferrochelatase-tranfectants was about 1.7-fold that in control cells. The amount of heme in the tranfectants and control cells was 0.81 and 0.96 nmol/10^6 cells, respectively. The growth rate of Balb/3T3 cells stably expressing human ferrochelatase was similar to that of control cells. To examine the photosensitivity of the Balb/3T3 cells, they were treated with 100 and 500 μM ALA for 16 h and then exposed to visible light. The death of Mock-DNA transfected control cells treated with 500 μM ALA was dependent on the irradiation (Fig. 2A and B). The cell death occurred as necrosis by the short-period irradiation, and apoptotic cells with DNA fragmentation were not observed. However, ferrochelatase-expressing transfected cells became resistant to the light. Then the level of porphyrin in the cells was examined. When ethanol extracts of the cells were measured fluoro-spectrophotometrically, the fluorescence

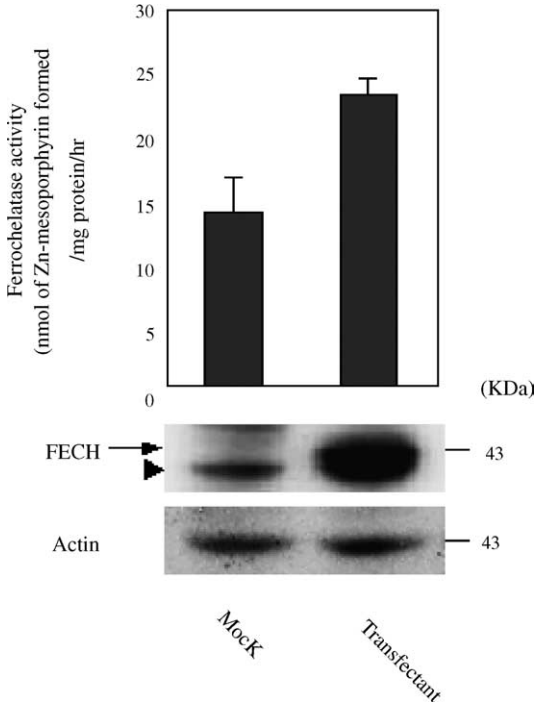

Fig. 1 – The activity and level of ferrochelatase in Balb/3T3 cells stably transfected with ferrochelatase. (Upper panel) Ferrochelatase activity. Mouse Balb/3T3 cells expressing human ferrochelatase and control cells were collected, washed twice with phosphate-buffered saline, and homogenized. The homogenates were centrifuged at 900 × *g* for 10 min. The assay of ferrochelatase activity was performed, using mesoporphyrin and zinc ions as substrates. Data are expressed as the mean ± S.D. of triplicate experiments. (Lower panel) Immunoblot analysis of ferrochelatase. Immunoblotting was performed using anti-ferrochelatase and anti-actin. The positions of human and mouse ferrochelatase are shown by the arrow and arrow-head, respectively.

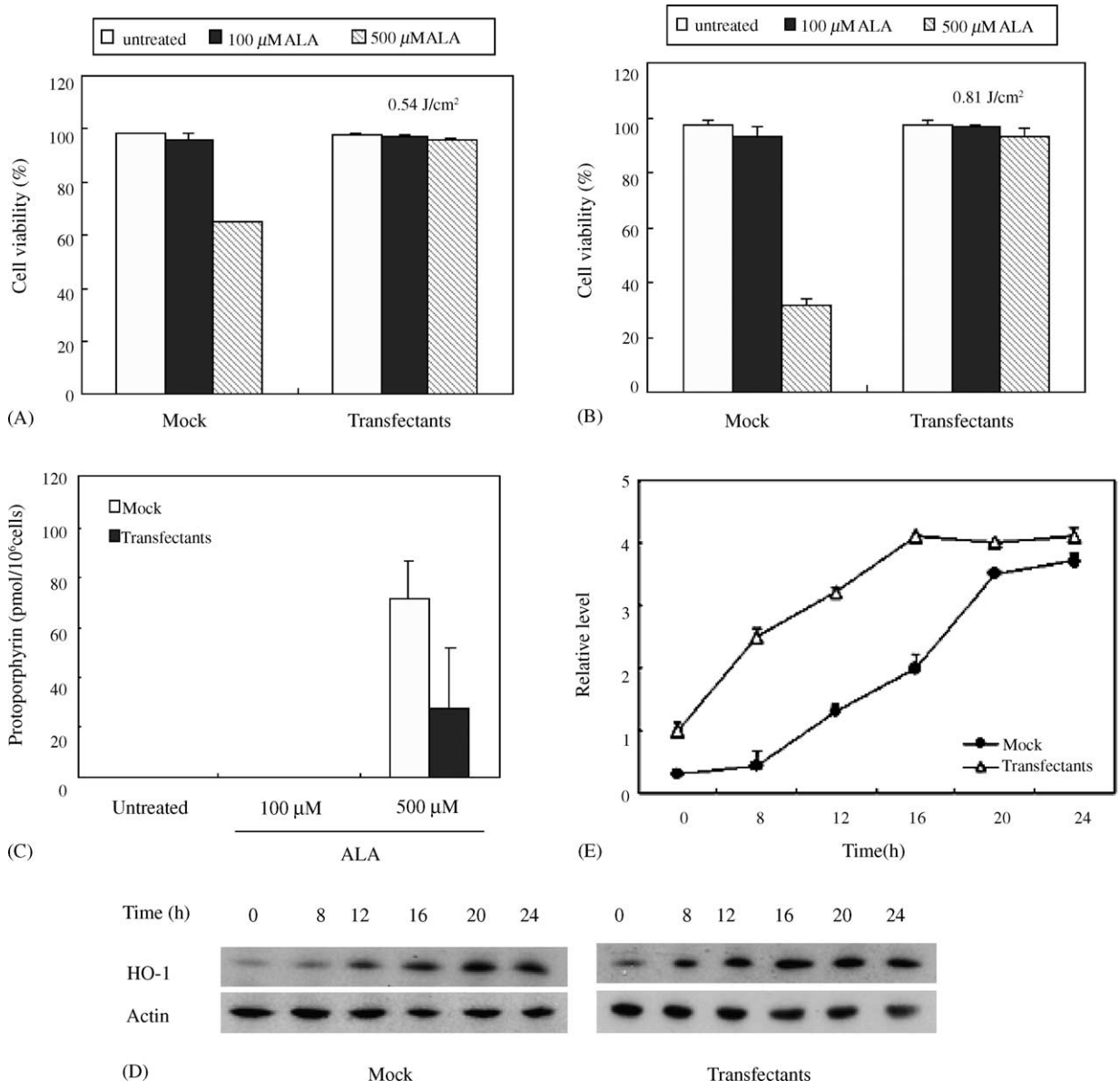

Fig. 2 – The ALA-induced sensitivity to light, the accumulation of protoporphyrin and the formation of heme in Balb/3T3 cells expressing human ferrochelatase. The ferrochelatase-transfectants and Mock-DNA transfected cells were incubated without ALA or with 100 or 500 μM ALA for 16 h, and then cell death was measured at 1 h after exposure to visible light. Light dose: (A) 0.54 J/cm² and (B) 0.81 J/cm². (C) The accumulation of protoporphyrin in ferrochelatase-transfectants and control cells incubated without ALA or with 100 or 500 μM ALA for 16 h was estimated by fluorescence spectrophotometry. Data are expressed as the mean ± S.D. of triplicate experiments. (D) The level of HO-1. The ferrochelatase-transfectants and control Balb/3T3 cells were incubated with 500 μM ALA for the indicated period, and the change in the level of HO-1 in the cells was examined by immunoblotting with anti-HO-1 antibody. (E) Densitometric quantitation of HO-1. Values were obtained by the ratio of intensity of HO-1/actin and are expressed as the mean ± S.D. of triplicate experiments.

pattern showed a maximum peak at 637 nm with excitation at 400 nm, which was consistent with that of standard protoporphyrin. The amount of protoporphyrin in the ferrochelatase-transfectants was much lower than that in control cells (Fig. 2C). To evaluate the production of heme in control and ferrochelatase-transfected cells, the level of HO-1 whose expression is induced by heme was compared. The level of HO-1 in ferrochelatase-transfectants without any treatments was higher than that in the control cells (Fig. 2D and E). Incubation of ferrochelatase-transfectants with 500 μM ALA resulted in the rapid expression of HO-1, as compared with that in control cells, indicating that the strong expression of ferrochelatase stimulates the production of heme and the turnover of heme can be increased. Next, to examine the relation between the expression of ferrochelatase and the accumulation of protoporphyrin in erythroid cells, human erythroleukemia K562 cells stably expressing mouse ferrochelatase were generated. The transfectants showed about

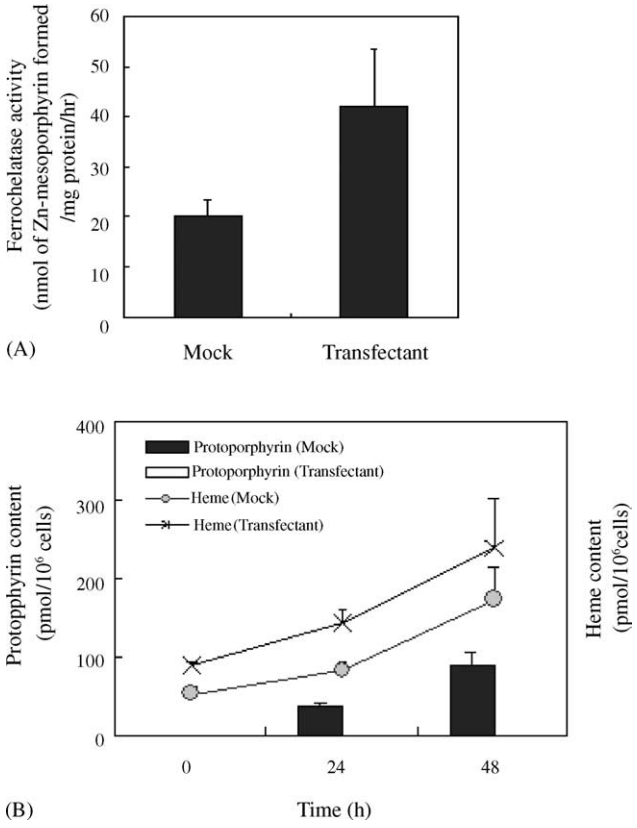

Fig. 3 – The ferrochelatase activity, and the amount of porphyrin and heme in ALA-treated K562 cells stably expressing mouse ferrochelatase. (A) Assays of ferrochelatase activity in control K562 cells and the ferrochelatase-transfectants were done, as described. (B) The transfected and control K562 cells were incubated with 500 μM ALA for 24 and 48 h. The amounts of heme and porphyrin were measured. Data are expressed as the mean ± S.D. of triplicate experiments.

2-fold more ferrochelatase activity than control K562 cells (Fig. 3A). When ferrochelatase-transfectants were incubated with up to 500 μM of ALA, no accumulation of protoporphyrin was observed (Fig. 3B). However, protoporphyrin accumulated markedly in ALA-treated control cells. On the other hand, the formation of heme in ferrochelatase-transfectants was greater than that in control cells, indicating that the production of heme from ALA in erythroid cells was dependent on the expression of ferrochelatase. The subsequent exposure of cells to visible light causes death only of the protoporphyrin-accumulated control K562 cells.

3.2. *ALA-induced photo-damage and the accumulation of protoporphyrin in mouse fibroblast Balb/3T3 cells, fibroblast-like L929 cells and fibrosarcoma MethA cells*

We next examined ALA-induced photo-damage in mouse firosarcoma MethA cells, as compared with Balb/3T3 and L929 cells. When mouse Balb/3T3 and L929 cells were incubated with 100 and 500 μM ALA, protoporphyrin was accumulated only in 500 μM ALA-treated cells, with the accumulation in L929 cells more than that in Balb/3T3 cells (Fig. 4A). Treatment of MethA cells with 100 μM ALA resulted in the accumulation of protoporphyrin and with 500 μM ALA the accumulation increased, which is about 3-fold higher than that seen in Balb/3T3 cells. The ALA-treated cells were then exposed to visible light. Cell death was observed in the protoporphyrin-accumulated cells (Fig. 4B). MethA cells were more sensitive to the light than L929 or Balb/3T3 cells. To examine whether the expression of ferrochelatase is involved in the photosensitivity of MethA cells, L929 and Balb/3T3 cells, we then compared the level of ferrochelatase among MethA, Balb/3T3 and L929 cells. The expression of ferrochelatase in MethA cells was much lower than that in Balb/3T3 cells or L929 cells (Fig. 4C). The enzyme levels in these cells did not change on treatment with ALA. In separate experiments, we compared the level of PBG deaminase in MethA cells with that in Balb/3T3 cells and L929 cells since it is thought that it may be elevated in tumor cells [22,23]. But the level was similar in both cells. Furthermore, the levels of neither enzyme changed on the treatment with ALA (Fig. 4C), indicating that the accumulation of protoporphyrin can be simply explained by the weak expression of ferrochelatase. To obtain a high degree of photosensitivity among the cells, iron was removed by treatment with an iron-chelator, DFO, which inhibits the ferrochelatase reaction by limiting the availability of iron. When L929 cells were incubated with 50 and 100 μM desferrioxamine plus 100 μM ALA, the accumulation of protoporphyrin was observed, leading to photo-damage, in a dose-dependent manner (Fig. 5). These results indicated that the inhibition of the ferrochelatase reaction augments the ALA-induced photosensitivity of the cells.

3.3. *Uptake of ALA by Balb/3T3, L929 and MethA cells*

Finally, we examined the uptake of ALA by MethA, L929 and Balb/3T3 cells. These cells were incubated with 20 μM ALA and the remaining ALA in the culture medium was measured to estimate the amount of ALA taken up by cells. As shown in Fig. 6, the rate of uptake by MethA cells and L929 cells was greater than that by Balb/3T3 cells. These results indicated that the increased uptake of ALA by MethA cells and L929 cells contributes to the marked accumulation of protoporphyrin. When the uptake of ALA by ferrochelatase-expressing transfected Balb/3T3 cells and Mock-DNA transfected Balb/3T3 cells was examined, both cells took up ALA in the similar extent.

4. Discussion

The present study demonstrated that the reduced function of ferrochelatase and the increased uptake of ALA by tumor cells were associated with the ALA-derived accumulation of protoporphyrin and contributes to ALA-induced PDT. The elevation in the expression of ferrochelatase with the expression of human ferrochelatase in Balb/3T3 cells led to the decrease in the accumulation of ALA and subsequently resistance to photosensitivity. Conversely, mouse fibroblast-like L929 cells became sensitive to light due to the accumula-

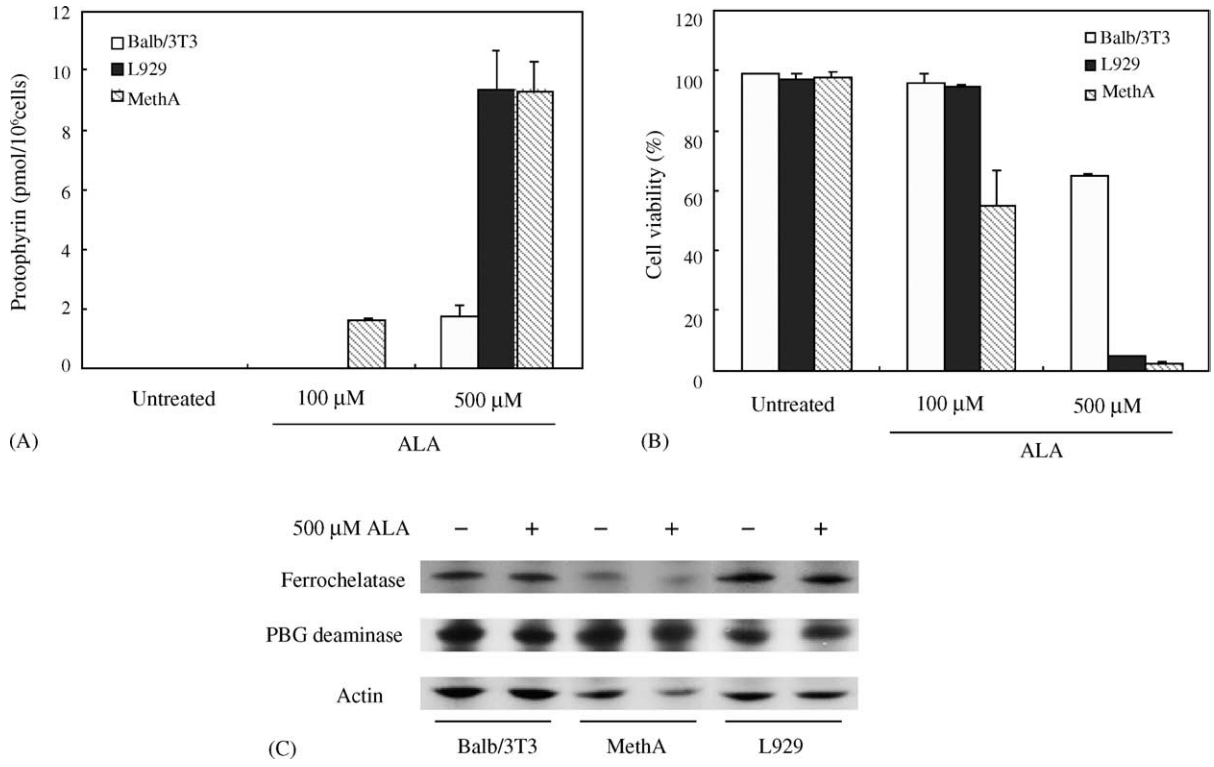

Fig. 4 – The ALA-induced photosensitivity and the accumulation of protoporphyrin in Balb/3T3 cells, L929 cells and MethA cells. (A) The cells were incubated without ALA or with 100 or 500 μM ALA for 16 h, and the accumulation of protoporphyrin in the cells was determined. (B) Cell death treated without ALA or with 100 or 500 μM ALA for 16 h was measured, as described in the legend to Fig. 2. Data are expressed as the mean ± S.D. of triplicate experiments. (C) Immunoblot analysis of ferrochelatase and PBG deaminase. The cellular proteins in Balb/3T3 cells, MethA cells and L929 cells treated without ALA or with 500 μM ALA were analyzed by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted, using anti-ferrochelatase, anti-PBG deaminase and anti-actin.

tion of protoporphyrin when the reaction of ferrochelatase was decreased by treatment of the cells with desferrioxamine. With erythroleukemia K562 cells, the ferrochelatase-transfectants did not accumulate protoporphyrin, and became resistant to light. Thus, the expression of ferrochelatase was inversely related to the ALA-induced accumulation of protoporphyrin, followed by photo-damage.

Previous investigators compared the ability to produce heme in tumor cells with that in isolated resting tissue cells, and showed high levels of heme in tumor cells [24,25]. They suggested that the activity of the heme-biosynthetic pathway, except for the last step, could be greater in tumor cells than normal cells. This study showed that MethA and L929 cells took up much more ALA than Balb/3T3 cells, and the potency of photo-damage dependent on the accumulation of protoporphyrin was also related to the amount of ALA taken up by the cells (Fig. 4). Thus, the increased uptake of ALA contributes to the specific accumulation of protoporphyrin in cancerous tissues in vivo. Similarly, tumor tissues preferentially accumulate the hematoporphyrin derivative Photofrin and this may be dependent on the activity of cells, since metabolically active tissues such as the liver and kidney also accumulate exogenously added Photofin [26,27]. It is possible that active neoplastic cells positively take up small molecules including ALA and Photofin.

A previous study showed that the ferrochelatase activity in Morris hepatoma cells was much weaker than that in hepatocytes [28]. The low level of ferrochelatase in hepatoma cells was exceptional since normal liver cells require much heme to maintain their functions, and the amount of hemoproteins such as cytochrome P-450 and catalase needed in hepatoma cells was less than that in hepatocytes [29]. A comparison of the ferrochelatase activity among tumors of extra-hepatic tissues has yet to be made. The present study showed that the expression of ferrochelatase in MethA cells was less than that in fibroblasts. Otherwise, the level of PBG deaminase was similar in all cell lines (Fig. 4C). This result is inconsistent with previous observations [23] that the increase in the level of PBG deaminase in cancer cells contributes to the accumulation of protoporphyrin. It is difficult to draw a conclusion on different levels of heme-biosynthetic enzymes based on a comparison of ferrochelatase and PBG deaminase between cell lines of different origins. In this connection, drug-induced tumor cells of the rat stomach showed about two-thirds of the ferrochelatase activity found in non-transformed cells of the rat stomach (O. Shimokawa, S. Taketani and H. Matsui, unpublished observations), suggesting that a small difference in the expression of ferrochelatase between normal and tumor cells may contribute to the selective accumulation of protoporphyrin in tumors. Furthermore, much information

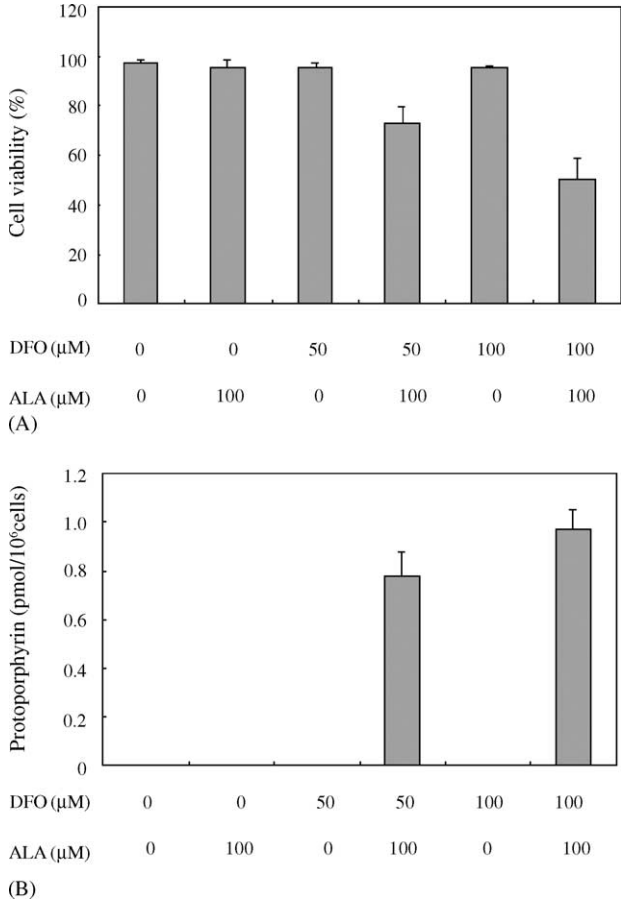

Fig. 5 – Effect of DFO on the ALA-induced accumulation of protoporphyrin and photo-damage in L929 cells. L929 cells were incubated with or without 100 μM ALA plus the indicated concentration of DFO for 16 h, and then exposed to visible light. Light dose = 0.54 J/cm^2. (A) After trypsinization, the vital cells were counted. (B) Before exposure to light, the cells were collected and the amount of protoporphyrin was measured. Data are expressed as the mean ± S.D. of triplicate experiments.

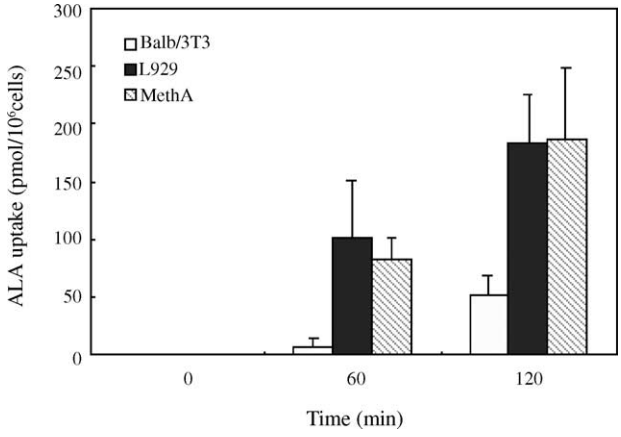

Fig. 6 – The uptake of ALA by Balb/3T3 cells, MethA cells and L929 cells. The cells (5×10^5) were incubated with 20 μM ALA for the period indicated, and an aliquot of the medium was withdrawn. The amount of ALA in the medium was determined, as described. Data are expressed as the mean ± S.D. of five experiments.

has already been obtained about the mechanism and biodistribution of ALA and the porphyrin precursor in the heme biosynthetic pathway. A previous study suggested that the uptake of ALA inversed with a more rapid proliferation of tumor cells [9]. The present study directly showed that the uptake of ALA by L929 and MethA cells was greater than that by Balb/3T3 cells and led to a marked accumulation of protoporphyrin. Together with the low level of ferrochelatase in tumor cells, this favors porphyrin accumulation by tumor cells, thus providing a biological relation for the clinical use of ALA-based diagnosis and PDT. Clinical applications of topical ALA-induced PDT have already archieved promising results, indicating that this modality is an effective and practical method for the treatment of superficial benign and malignant diseases of the skin and internal hollow organs.

Tumor cells may have a special need for iron, making them more susceptible to the effects of iron chelation [30]. A high dose of iron-chelators caused toxicity, due to a decrease in iron- or heme-containing enzymes for respiratory chain and DNA synthesis [31]. Furthermore, the protein level of ferrochelatase in cells was decreased by the treatment with DFO [13]. The prolonged treatment of tumor cells led to apoptosis [31,32]. These properties have been explored in relation to the treatment of carcinoma in vitro and in an animal model. The present data showed the close relation between the accumulation of protoporphyrin and the photodamage in DFO-treated cells. Namely, the incubation of Balb/3T3 and L929 cells with 100 μM ALA did not cause the accumulation of protoporphyrin, but in the case of treatment with 100 μM ALA, DFO induced the accumulation of porphyrin, followed by light-induced cell death. Conversely, photodamage was directly triggered by the light-dependent reaction of protoporphyrin.

Our study shows that ferrochelatase is important for ALA-induced PDT. Both erythroid and non-erythroid cells highly expressing ferrochelatase displayed resistance to light. The difference in the expression of ferrochelatase between normal and tumor cells is not remarkable, but the slight reduction of activity in tumor cells may contribute to the accumulation of protoporphyrin. Furthermore, the uptake of ALA by the tumor cells is somewhat greater than that by normal cells, indicating that a slight increase in the uptake is effective in terms of photosensitivity. Many other factors involved in the specific accumulation of protoporphyrin and determining the outcome of PDT may be present. Demonstration of the additional factors as well as roles of heme-biosynthetic enzymes will facilitate the improvement of ALA-induced PDT.

Acknowledgments

We thank Dr. Shigeru Sassa, Rockefeller University, for kindly providing the antibody for PBG deaminase. This study was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, The Naito Foundation and The Shimizu Foundation Research Grant for 2003.

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