5-Aminolevulinic Acid -Mediated Photodynamic Therapy of Human Glioma Cells In Vitro

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OBJECTIVE To investigate the effect of 5 -aminolevulinic acid (ALA) -mediated photodynamic therapy (PDT) on U251 human glioma cells in vitro.

METHODS U251 human glioma cells were routinely cultured and then treated with ALA, a type of photosensitizer, at various concentrations followed by light irradiation. The PDT-induced phototoxicity of the cells was determined by a MTT assay. In addition, cells were treated with ALA at a fixed concentration and subjected to various doses of light irradiation.

RESULTS With the same light dosage (25.0 J/cm^2), the cell survival rates were 70.16%±5.02%, 50.19%±4.79%, 34.97%±5.34%, 27.04%±4.34%, and 24.26% ±2.76% at ALA concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 mM, respectively (F =279.88, P =0.0000). But the survival rates of the cells incubated with 2.0 mM ALA compared to those with 4.0 mM ALA (27.04%± 4.34% vs 24.26%±2.76%) showed no significant difference (P=0.611). At a single ALA concentration, the cell survival rates were 83.48% ± 6.79% , 68.09%±6.02%, 33.75%± 6.70%, 23.34%± 5.08% and 15.14%± 3.60% for light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2, respectively (F=422.03, P=0.0000). Without exposure to light, however, the cell survival rates were 96.64% ±6.56% , 97.71% ±5.48% , 98.10% ±6.25% , 99.44% ±7.02% , and 95.86% ±7.80% for ALA concentrations at 0.25, 0.5, 1.0, 2.0, and 4.0 mM, respectively (F =0.68, P =0.6085). Without ALA in the medium, the cell survival rates were 98.74% ±6.20% , 96.49% ±7.13% , 97.60% ±5.94% , 95.70%±4.86%, 98.08%±6.26% for light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2, respectively (F=0.6400, P=0.6368) .

CONCLUSION The PDT damage to the U251 cells increased with ALA concentration within a relative lower range, but then plateaued at higher concentrations. PDT damage was proportional to the doses of irradiated light. Without ALA, the light alone caused no photodynamic damage and ALA itself was nontoxic. The ALA-induced PDT appears to be a promising therapy for glioma.

KEYWORDS: glioma, 5 -aminolevulinic acid, ALA, photodynamic therapy, U251.

Primary intracranial neoplasms account for 2-3% of all cancer deaths. Approximately half of these are glioblastoma multiforme (GBM)-the most aggressive variety of glial tumors. [1] Due to the invasive nature of GBM and other glial tumors, complete excision of tumor tissue is extremely difficult. At present there is no satisfactory treatment for these infiltrative neoplasms. The best available treatment

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using surgery, chemotherapy and radiation therapy results in only 15 months of survival for GBM patients. Five -year survival rates are still dismal.

The shortcomings of standard treatment modalities in the management of glioma patients have led to a search for more aggressive focal treatments. Photodynamic therapy (PDT) has been used successfully in the treatment of a wide variety of localized malignancies and may prove useful as an adjuvant therapy in the treatment of resected margins after surgery. [1-5] PDT utilizes a photosensitizing agent that is selectively taken up and/or retained by neoplastic tissue. When absorbing light of an appropriate wavelength, the photosensitizer produces cytotoxic oxygen products causing direct cell death, and/or vascular shutdown.[1,3,4]

Porphyrins, a type of traditional photosensitizer, such as hematoporphyrin derivatives and Photofrin have been used almost exclusively in clinical PDT trials of brain tumor therapy.[1,6] Although favorable results have been reported by a number of clinicians, these photosensitizers have several drawbacks that may limit their applicability in certain situations. For example, the uncommonly long period of cutaneous photosensitization (lasting up to several weeks, even 18 weeks) was utilized in patients following administration of these Photofrin photosensitizers. And furthermore, the relatively poor tumor-to-normal tissue localization observed by several groups may limit the effectiveness of these photosensitizers due to the potential of normal tissue complications.[2-4]

Due to the drawbacks of these traditional photosensitizers, other photosensitizers, such as 5-aminolevulinic acid (5-ALA) are currently being evaluated for use in PDT. [2-4,7-9] ALA itself is not a photosensitizer but it serves as the biological precursor in the heme biosynthetic pathway. In ALA-induced endogenous photosensitization, the heme biosynthetic pathway is used to produce protoporphyrin IX (PpIX)-a potent photosensitizer. Heme is synthesized from glycine and succinyl CoA. The rate-limiting step in the pathway is the conversion of glycine and succinyl CoA to ALA, which is under negative feedback control by heme. Through the introduction of ALA, the regulatory feedback system becomes overloaded causing an accumulation of PpIX, which may cause the photosensitizing effect for PDT following its activation. The combination of excellent tumor-to-normal brain tissue localization, short period of skin photosensitization (24-48 h) and the possibility of oral administration, makes ALA a promising photosensitizer for PDT treatments of glioma patients. Therefore ALA-PDT may have a great potential in the treatment of brain tumors.

The primary aim of this study is to investigate the response of human glioma cells to ALA-mediated PDT in vitro.

MATERIALS AND METHODS

Chemicals

5-Aminolevulinic acid (ALA) obtained from Sigma (St. Louis, MO), was dissolved in PBS at pH 7.0. Stock solutions of 24 mM were prepared and kept in 4℃ before use.

Cell lines

U251 human neuroblastoma cells (U251), obtained from the Cell Bank of the Chinese Academy of Science, were used in the study. Cells were routinely cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Gibco BRL) at 37℃ in a humidified incubator containing 95% air and 5% CO_2. Cells in the exponential growth phase were used in the experiments.

Cell viability assay

The PDT-induced phototoxicity of U251 cells was determined using 3- [4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO) for the assay.[2,7] The MTT assay is based on the activity of mitochondrial dehydrogenases, which can reduce a water soluble tetrazolium salt to a purple insoluble formazan product. The amount of MTT formazan was analyzed spectrophotometrically at an absorbance of 570 nm.

Cell survival (%) = (OD value of treated cells/ OD value of control cells) × 100%.

Photodynamic treatment

The effect of different ALA concentrations on PDT

U251 cells washed 3 times with fresh serum-free medium were added into 96 well flat-bottomed culture plates at 5×10^4 cells per well . After attachment to the surface, the cells in the PDT groups were treated with different ALA concentrations (0.25, 0.5, 1.0, 2.0, and 4.0 mM) in serum-free medium, and incubated for 6 h. The serum-free medium was also used for the cells of control groups. The cells of both PDT and control groups were subsequently irradiated with 635 nm light from an argon ion-pumped dye laser (Coherent, Inc., Santa Clara, CA) with a light dosage of 25.0 J/cm^2. After light exposure the cells were incubated with fresh medium containing 10 % FBS for 24 h before the cell viability was determined by the MTT assay.

The effect of different light doses on PDT

Cells were treated as described above. The ALA concentration in all PDT groups was 1.0 mM, and cells were exposed to various doses of light - 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2.

The effect of different ALA concentrations on U251 cells

Cells were treated as described above, but the cells in the "PDT" groups were exposed to ALA concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 mM and received no irradiation.

The effect of different light doses on U251 cells

Cells were treated as described above, but the cells in the "PDT" groups were irradiated with light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2 in the absence of ALA.

Data analysis and statistics

Data were analyzed with STATA 6.0. Primary data are presented as the mean ± SD. The statistical significance of differences was analyzed using the ANOVA test. A P value <0.05 was accepted as statistically significant.

RESULTS

Fig.1 shows the photocytotoxicity of the U251 cells incubated with different concentrations of ALA. With the same light dose (25.0 J/cm^2), the survival rates of cells were 70.16% ± 5.02%, 50.19% ± 4.79%, 34.97% ± 5.34%, 27.04% ± 4.34%, and 24.26% ± 2.76% for ALA concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 mM, respectively. The results indicate that the cytotoxicity was significantly enhanced as the ALA concentration increased. (F=279.88, P=0.0000)

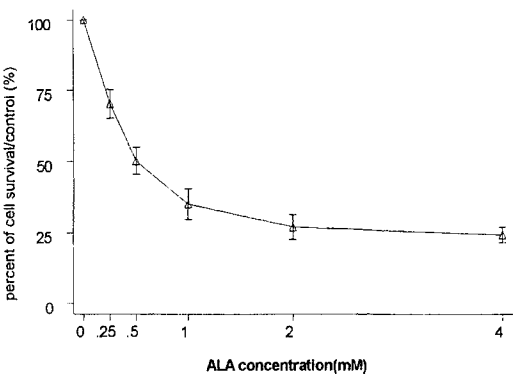

Fig.1. Drug-concentration dependence of cell survival after PDT. Cells were incubated with different concentrations of ALA for 6 h, and then irradiated with the same dose of light (25.0 J/cm^2). Cell survival was measured by the MTT assay. The bars represent SD.

Fig.2 shows the PDT-induced cytotoxicity of the cells incubated with 1 mM ALA and irradiated with various doses of light. The survival rates of cells were 83.48% ± 6.79%, 68.09% ± 6.02%, 33.75% ± 6.70%, 23.34% ± 5.08%, and 15.14% ± 3.60% for light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2, respectively.

As shown in the figure, the survival rate was significantly decreased as the light dose increased (F=422.03, P=0.0000).

The absence of a cytotoxic effect in the cells incubated with different concentrations of ALA without exposure to light is shown in Fig.3. The survival rates of cells were 96.64% ± 6.56%, 97.71% ± 5.48%, 98.10% ± 6.25%, 99.44% ± 7.02%, and 95.86% ± 7.80% for ALA concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 mM, respectively. There was no significant difference in cytotoxicity among the groups (F=0.68, P=0.6085).

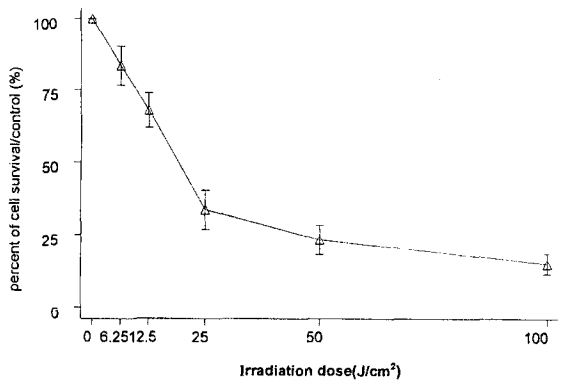

Fig.2. Light-dose dependence of cell survival after PDT. Cells were incubated with ALA (1mM) for 6 h, and then irradiated with different doses. Cells survival was measured by the MTT assay. The bars represent SD.

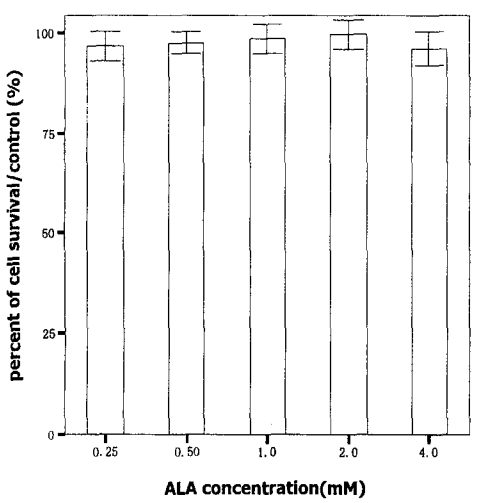

Fig.3. Lack of ALA-induced cytotoxicity. Cells were incubated with different concentrations of ALA for 6 h without exposure to light. Cells survival was measured by the MTT assay. The bars show means ± 1.0 SD.

The data presented in Fig.4 show the lack of photocytotoxicity in the cells irradiated with various doses of light without ALA pre-incubation. The survival rates of cells were 98.74% ± 6.20%, 96.49% ± 7.13%, 97.60% ± 5.94%, 95.70% ± 4.86%, and 98.08% ± 6.26% for light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm^2, respectively.

There were no significant differences compared to the control (F=0.6400, P=0.6368).

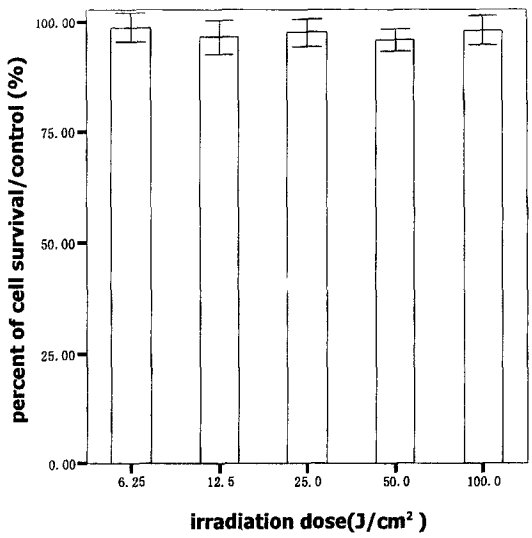

Fig.4. Lack of light-induced cytotoxicity. Cells without ALA incubation were irradiated with various doses of light. Cells survival was measured by the MTT assay. The bars shows the mean ± 1.0 SD.

DISCUSSION

In this report we have shown that with the same light dose the overall PDT damage to the U251 cells was proportional to the ALA concentrations, as shown in Fig.1 .The survival rates of cells were from 70.16% ± 5.02% to 24.26% ± 2.76% with ALA concentrations from 0.25 to 4.0 mM (P<0.01). The cell survival with ALA concentrations from 0.25 to 2.0 decreased sharply while no significant change was observed at ALA concentrations from 2.0 to 4.0 mM. In fact, we compared the survival rates at 2.0 and 4.0 mM ALA (27.04% ± 4.34% vs 24.26% ± 2.76%) and found no significant difference (P=0.611). In ALA-PDT, ALA itself is not a photosensitizer. It is a precursor of porphyrins in the biosynthetic pathway for heme.[2-4,7-9] When exogenous ALA is added, the cellular protoporphyrin (PpIX) can be produced. PpIX is a potent photosensitizer, and can lead to photosensitization when being irradiated with light.[2,3,7,8] So our results suggest that the U251-cellular production of PpIX increased with ALA incubation at a region of relative lower concentrations and then became saturated at higher concentrations. Wu [2] also found a saturation by increasing ALA concentrations in

SK-N-SH human neuroblastoma cells and 7903 human hepatoma cells by measuring the fluorescence intensities (635 nm) in each cell sample. Since PpIX is a product in the biosynthetic pathway of heme and the ability of heme, biosynthesis is limited, the saturation of cellular PpIX production at high ALA incubation concentrations is reasonable. This finding is of importance in clinical PDT in order to optimize the dose of ALA.

Photodynamic therapy is based on the concept that certain photosensitizers can be localized in neoplastic tissue, and subsequently, these photosensitizers can be activated with the appropriate energy of light to generate active molecular species, such as free radicals and singlet oxygen ($^1O_2$), which are toxic to cells and tissues.[1,5,7,9] Consequently, if there is not enough energy there will be too few active molecular species and no biological effect.[10] Our findings support this concept as shown in Fig.2. When the U251 cells were incubated at the same level of ALA, the survival rates were 83.48% ± 6.79%, 68.09% ± 6.02%, 33.75% ± 6.70%, 23.34% ± 5.08%, and 15.14% ± 3.60% for light doses of 6.25, 12.5, 25.0, 50.0, and 100 J/cm2, respectively, (F=422. 03, P=0.0000) which illustrates that the survival rate was significantly decreased as the light dose increased. It follows that as the light dose goes up, photosensitizers absorb more energy generating more active molecular species and more cells are killed. In a study of the effect of ALA-PDT on rat C6 glioma, similar results were reported. [1,11] However, other factors, such as self-shielding and photobleaching (self-destruction of the photosensitizer during the PDT), complicate the precise light-dose delivered. [7] If the energy of light delivered in PDT is much higher, photocytotoxicity of cells will not be proportional to the light dose. However, if the dosage of light is too low, the desired photochemistry cannot occur. The resutls of our experiments indicate that the light doses we used were appropriate.

The lack of a cytotoxic effect in the cells incubated with different concentrations of ALA but without exposure to light is shown in Fig.3, indicating that ALA at these concentrations without light exposure shows no cytotoxicity (F=0.68, P=0.6085). ALA is a naturally occurring precursor in the biosynthetic pathway for heme production, so it is reasonable that ALA itself is non-toxic.

Fig.4 shows the lack of a photocytotaxic effect in the U251 cells irradiated with various doses of light but without ALA incubation. The survival rates of the cells showed no significant differences compared to the control (F=0.6400, P=0.6368), indicating that the light we delivered did not cause photodynamic damage.

5-Aminolevulinic acid (ALA) is conceptually different from a traditional photosensitizer. As a precursor itself, it is converted into a photosensitizing heme metabolite, PpIX, within the malignant glioma tissue and then can be rapidly eliminated to prevent prolonged side effects.[2-4,7-9] Our study demonstrates that ALA itself is nontoxic and that the ALA-induced PDT might be considered to be a promising therapy for glioma.

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