PDT for Cultured Cancer Cell by 5ALA-induced Protoporphyrin IX

5ALAより誘導されるプロトポルフィリンIXを用いた 培養癌細胞へのPDT

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Abstract

Photodynamic therapy, PDT, has mainly been done using a laser system and a fluorescence dye which can be absorbed in a targeted cell culture or tumor. Establishing effective protocols of laser irradiation requires a knowledge of the local concentration of the dye in the target.

In this paper, Protoporphyrin IX that can be induced by δ -aminolevulinic acid (ALA) as the precursor of heme was used as the dye and the measurement of concentration of PPIX in cells was assessed in a cancer cell culture. The metabolic precursor ALA was included in the incubation media for 24 hours to induce cellular accumulation of PPIX for the purpose of PDT. The distributions of initial PPIX concentration, C_0 in $\mu\text{g/ml}$, photobleaching light dose, H_0 in J/cm^2 , and photodynamic dose, D_{PDT} which is defined as the number of photons absorbed by PPIX per g of cells, were measured on 124 individual cells using video fluorescence microscopy. The results showed that the H_0 was normally distributed with a mean $\pm$ SD of $135 \pm 56 \text{ J/cm}^2$ using 488 nm light. The C_0 and D_{PDT} were not normally distributed, with mean values (and ranges) of $6 (1-15) \mu\text{g/ml}$ and $5.2 \times 10^{19} (3 \times 10^{18} - 2 \times 10^{20})$ photons absorbed by PPIX per g cells (ph/g), respectively. Individual PDT treatment of 131 cells via the microscope followed by 3-day incubation and assay for colony formation allowed specification of the D_{PDT} of individual cells destined for death or survival. The threshold D_{PDT} to achieve

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cell killing was not shrap, falling between 1×10^{18} - 30×10^{18} ph/g. In conclusion, a measurement system combined with an optical multichannel analyzer and a fluorescence microscope can be considered as a effective method for estimating PPIX concerntratio n in a cell.

Key words : Chemotherapy, photodynamic therapy, photobleaching, breast cancer cells, fluorescence microscopy

邦文要旨

癌の光化学療法 (PDT) は主にレーザと目的の癌細胞や組織に特異的に吸収される蛍光物質を用いて行われる。適切なレーザ照射のためには、療法の対象における蛍光物質の局所濃度を知ることが必須である。本論文では、アミノレブリン酸より細胞内でプロトポルフィリン IX (PPIX) を生合成させ蛍光物質として用い、培養細胞に対して488nmの光源を用いたPDTを実施し、各細胞内のPPIXの初期濃度 C_s 、照射量 H_s 、単位重量当たりの光子吸収個数 D_{PDT} を、124個の細胞につき蛍光顕微鏡法を用いて測定した。結果、 H_s は $135 \pm 56 \text{ J/cm}^2$ (平均値 $\pm$ 標準偏差) であり、 C_s と D_{PDT} は各々6 (1-15の範囲) ($\mu\text{g/ml}$) と 5.2×10^{18} (3×10^{18} - 2×10^{20} の範囲) (photons/g cells) であった。さらに個々の細胞をPDT実施後3日間培養し、コロニー生成によりPDTの効果を確認したところ、細胞を壊死に至らしめる D_{PDT} は 1×10^{18} - 20×10^{18} (photons/g cells) の範囲であった。以上より本稿で提案した蛍光顕微鏡法はPPIXの濃度定量に有効であると考えられた。

INTRODUCTION

Administration of δ -aminolevulinic acid (ALA), the metabolic precursor for the heme synthetic psthway, can induce the accumulation of protoporphyrin IX (PPIX) in cells^{1,2}. Such ALA-induced PPIX has been currently under investigation in many centers for the purpose of photodynamic therapy (PDT) which is a light-activated chemotherapy for cancer and other indications³⁻⁶. PPIX serves as a photosensitizing agent for PDT. Light excitaion of PPIX results in the population of the first singlet state and in its conversion into a triplet state is able to transfer energy to molecular oxygen. This results in the formation of singlet oxygen, which causes cytotoxic reactions. Establishing effective protocols for PDT requires a knowledge of the local concentration of PPIX in a targeted cell culture of tumor. Since PPIX in the cell undergoes photodestruction, called photobleaching, during the irradiation of laser, the photobleaching light dose constant which is the product of the irradiance of the photobleachig light and the photobleaching time con-

stant, is also required to predict the photodynamic dose experienced by the target tissue. The photodynamic dose, namely deposited energy per photon density, is defined as the number of photons absorbed by PPIX per g of cells, D_{PDT} (ph/g)⁷. This parameter indicates the number of excited state PPIX molecules generated per unit mass of cells and is proportional to the amount of singlet oxygen tenarated which contributes to oxidative damage leading to cell death. The D_{PDT} parameter provides an importnat dosimetric measure for comparison of the efficiency of different photosensitizers to achieve photodynamic action. Patterson et al⁷, illustrated that D_{PDT} was a constant value independent of the wavelength of irradiation in their study of PDT-induced necrosis in rat livers.

This paper reports the two parameters in a putatively homogeneous population of cancer cells in culture : (A) the initial concentration of PPIX in cells, C_s ($\mu\text{g/cell}$), and (B) the photobleaching light dose constant, H_s (J/cm^2). The measurements involved quantitative video fluorescence microscopy

of single cells during prolonged irradiation. The C_e and H_b were used to predict the photodynamic dose, D_{PDT} , photons absorbed by PPIX/g cells of [ph/g], that would be attained in each cell after complete photobleaching of all PPIX. Furthermore, a colony formation study of cell survival is presented after individual irradiation of single cells via the microscope. The techniques allow specification of the individualized D_{PDT} of cells which were successfully killed and of cells which survived the treatment.

MATERIALS AND METHODS

Cell cultures

The cancer cell line used in the study was MTF7, cloned from a 7, 12-dimethyl-benz[a]-anthracene-induced rat mammary adenocarcinoma, courtesy of Dr. Stephen P. Tomasovic, Department of Tumor Biology, M. D. Anderson Cancer Center⁸. The cells were grown in α -MEM (Alpha-modified, Minimum Essential Eagle, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) in monolayers in petri dishes.

ALA (Sigma Chemical Co., St. Louis, MO) was introduced into the culture media at various concentrations, specified later, and the cells were incubated as monolayers on petri dishes for 24hr. The cell concentration in culture at harvest was $\sim 0.7 \times 10^6$ cells per petri dish which was not confluent.

At the time of the PPIX assay, the cells were loosened from 3 petri dishes by the addition of trypsin to the medium (0.25%) and incubated for 5 min at 37°C. The loosened cells were resuspended in phosphate buffer saline (PBS), centrifuged, and resuspended in 1 ml of PBS, to yield 2×10^6 cells/ml.

Two cell preparations were studied: (Preparation A) Twenty μ l of the cell suspension (40×10^3 cells) were placed on a glass slide with a cover slip for fluorescence microscopy. (Preparation B) Cell digests were prepared by placing 1 ml of PBS containing 2×10^6 cells into 2 ml of Scitigest (Fischer Scientific), a solvent/digestant which yielded a clear solution after 18 hr. The cell digest was placed in a polystyrene cuvette for bulk fluorescence measurements using the OMA.

Fluorescence assay for PPIX

Two fluorescence measurements were conducted. (A) Video fluorescence microscopy ($F_{v,d}$) measured single cells. (B) An optical-fiber fluorescence spectrophotometer ($F_{o,m,d}$) measured cell digests. Comparisons of the two measurements together with calibration experiments using $F_{o,m,d}$ enabled calibration of the video ($F_{v,d}$).

a. Fluorescence microscopy

The level of PPIX in single cells was assayed by the fluorescence microscopy system shown in Fig. 1. Considering the absorption of PPIX, the measurement system used a 488-nm argon ion

Figure 1 Fluorescence microscopy apparatus. An argon ion laser (488 nm) excited cellular PPIX fluorescence and an intensified CCD camera viewed the emission through a 580-nm long-pass filter.

laser (Coherent, Inc., model Innova 90) delivered through an inverted microscope (Zeiss, Inc., model Axiovert 405M) to excite PPIX fluorescence in individual cells. Fluorescence was observed through a 580-nm long-pass filter with an intensified CCD camera (Video Scope International Inc., VS2525 intensifier, CCD200E camera) and recorded by video tape (Panasonic, AG-6300). The laser was delivered through an optical fiber whose termination was imaged through the microscope to yield a uniform-field beam in the image plane. The irradiance of the laser was $5.8\text{W}/\text{cm}^2$ to avoid thermal damage of cells and the beam was $180\text{ }\mu\text{m}$ in diameter. The recorded fluorescence image were sent to a personal computer (Macintosh) for acquisition and analysis using an image processing system (Image, NIH). The measurements were recorded as a porphyrin fluorescence score.

A porphyrin fluorescence score, F_{vid} in laboratory units called [videl units/cell], was defined by the following:

$$F_{vid} = ANF_{camera} \quad (1)$$

where A was the cell cross-sectional area ($\mu\text{m}^2/\text{cell}$), N was the number of pixels per unit area in the image processing system [$2.13\text{ pixels}/\mu\text{m}^2$], and F_{camera} was the fluorescence intensity in laboratory units [video units/pixel].

b. cell digest fluorescence measurements(OMA)

Mesurements of PPIX in cell digests utilized a fluorescence spectrophotometer. The system used a nitrogen-dye laser (Laser Science, Inc., Newton, MA) at 420 nm delivered through an optical fiber to excite cell fluorescence and an optical multichannel analyzer (OMA) (Princeton Instruments, Inc., Trenton, NJ) to detect fluorescence. The same 600- μm -core-diameter fiber both delivered excitation and collected emission. The fiber was placed against the side of a polystyrene cuvette containing a cell digest (preparation B). The porphyrin fluorescence score (F_{oma}) was determined by integrating the area under the fluorescence curve between 600-750 nm and normalizing by a standard reference which was the peak fluorescence of a fluorescein standard solution routinely measured to account for day-to-

day variation in the system. The background F_{oma} for cells grown without PPIX was subtracted from all F_{oma} measurements, and all values of F_{oma} reported here have undergone such a background subtraction.

c. Calibration experiments

Microscope calibration

Cells were incubated for 24 hr in various concentrations of ALA to yield a range of PPIX levels. Individual cells (preparation A) were measured with the video camera (F_{vid}). A cell digest (preparation B) was measured with the fluorescence spectrophotometer (F_{oma}). Figure 2 plots the PPIX fluorescence in the cell digest (F_{oma}) vs the PPIX fluorescence of the same cells measured by fluorescence microscopy (F_{vid}). The relationship was

Figure 2 Calibration curve for PPIX fluorescence in a cell digest (F_{oma}) vs the μg of PPIX added to the cell digest.

Figure 3 Calibration curve relating the PPIX fluorescence measured by spectrometer in a cell digest (F_{oma}) vs the fluorescence measured in cells by video microscopy (F_{vid}).

Figure 4 The brightness of cellular fluorescence as seen by the eye with a microscope through 580-nm long-pass filter, quantified by the video camera as $F_{v,d}/A$. The total fluorescence ($F_{v,d}$) was distributed over the area of the cell (A). Data are plotted versus cell area. Incubation of cells in ALA (+ALA) yielded about 10-fold brighter fluorescence than cells grown without ALA (-ALA).

linear :

$$F_{oma} = A + c_{v,d}^{oma} F_{v,d} \quad (2)$$

where A is the y-intercept (10.5[oma]), and $c_{v,d}^{oma}$ is the slope (0.00044 [oma/(video units/cell)]).

OMA calibration

In a second experiment, the cells not grown in ALA were prepared as a cell digest (preparation B). Known amounts of PPIX [μg] were added to the digest and F_{oma} measurements made. **Figure 3** plots the fluorescence of a cell digest (F_{oma}) vs the amount of added PPIX [μg] yielding a linear relation with a slope, c_{PPIX}^{oma} , equal 249[oma/ μg PPIX].

Combining the above two calibration experiments, the relationship between a video measurement, $F_{v,d}$, and the cellular PPIX concentration, C_o [$\mu\text{g}/\text{ml}$], was :

$$C_o = \frac{A + F_{v,d} c_{v,d}^{oma}}{c_{PPIX}^{oma} N V_{cell}} = \frac{10.5 + F_{v,d} (0.00044)}{(2e6)(249) V_{cell}} \quad (3)$$

N was the number of cells in the cell digest (2×10^6

cells). V_{cell} was the volume of the particular cell measured [ml/cell]. Each cell's volume (V) was estimated based on the cell's diameter observed in the two-dimensional image acquired by the camera, assuming a spherical shape for the nonadherent cell. Experimentally, the C_o values were specified by Eq. 3 from the $F_{v,d}$ values at the start of irradiation before significant photobleaching could occur. For example, if the $F_{v,d}$ was 50,000 video units/cell, and the V_{cell} was 2.6×10^{-8} ml/cell, then the C_o was $2.5 \mu\text{g}/\text{ml}$ within the cell.

Colony formation

Cells in mid-log phase growth were plated onto petri dishes at a low density, about 200 cells per plate, and incubated in $200 \mu\text{g}/\text{ml}$ ALA for 24 hr. The plates had grids drawn on their bottom surface to facilitate identification of cells at a given position. A sampling of cells was observed with the inverted fluorescence microscope during the individual irradiation of single cells. 36 cells were given $20 \text{ J}/\text{cm}^2$

Figure 5 Histograms of (top) the initial concentration of PPIX, (middle) the photobleaching light dose constant, and (bottom) the photodynamic dose achieved by complete photobleaching of the cellular PPIX.

and 81 cells were given 300 J/cm^2 . Video recordings measured the initial C_o of each cell and the decay due to photobleaching. Because the petri dish imposed a relatively high amount of autofluorescence, only the initial photobleaching could be documented, however the values of H_b obtained were similar to the previous study of cells on glass. In this study, the mean $\pm$ standard deviation for H_b was 102 ± 51 (J/cm^2). Because the cells were adherent to the petri dish, the V_{cell} was calculated by assuming a hemispherical shape. The D_{PDT} of each cell was calculated using Eq. a-3, shown in Appendix, accounting for the radiant exposure utilized (20 or 300 J/cm^2). After exposure, the cells were incubated for 3 days, then scored for signs of successful colony formation.

RESULTS

When one observes a fluorescent cell by eye through the microscope, the total fluorescence is distributed over the cell cross-sectional area, A (μm^2), in the field of view to yield a particular brightness, which can be expressed as F_{cell}/A (pixel units/ μm^2). Figure 4 plots F_{cell}/A versus A . Cells not incubated in ALA ($n=16$) had a mean value of 8.7, and cells incubated in ALA ($n=124$) had a mean value of 107.

Figure 5 shows the histograms of the initial PPIX concentration, C_o ($\mu\text{g/ml}$), the photobleaching light dose, H_b (J/cm^2), and the photodynamic dose, D_{PDT} (ph/g). The mean (and range) of C_o was 6.0 (1-15) $\mu\text{g/ml}$. Control cells not grown in 5ALA showed 0.52 ($\mu\text{g/ml}$). Assuming these C_o values were primarily due to PPIX and that background non-porphyrin auto-fluorescence in the 600-750 nm range was negligible, the amount of PPIX induced by 5 ALA was about ten times the amount of endo-genous PPIX in cells. The H_b was 135 (25-300) J/cm^2 , which was surprisingly variable. The maximum D_{PDT} obtained after complete photobleaching of PPIX was calculated to be 5.2×10^{19} (3×10^{18} - 2×10^{20}) (ph/g).

Figure 6 shows the correlation between D_{PDT} and C_o , and the mean values of 5.2×10^{19} ph/g and 6.0 $\mu\text{g/ml}$, respectively. The slope should be $\epsilon H_b / b\rho$, or 8.7×10^{18} (ph/g)/($\mu\text{g/ml}$), as indicated by the solid line. The noise derives from variations in the experimentally measured H_b and the measured cell size used to estimate V_{cell} for calculation of C_o . The slope overestimates the data at low C_o and underestimates the data at high C_o .

Figure 7 shows bleb formation, an indicator for photodynamic damage to cell membranes, in a cell after 350 J/cm^2 of laser exposure which was sufficient to cause nearly complete photobleaching of PPIX. Before the cell was exposed to the laser (Fig. 7a), there was no bleb. After exposure (Fig. 7b), the cell displayed a bleb at the 10 o'clock position. The bleb itself had no PPIX fluorescence.

The results of the colony formation study are shown in Fig. 8. The calculated D_{PDT} for each cell is specified by the y-axis. In the study, 36 cells re-

Figure 6 Correlation of the maximum photodynamic dose (D_{PDT}) and the PPIX concentration in cells (C_o), with mean values 5.2×10^{19} ph/g and $6 \mu\text{g/ml}$, respectively. The slope should be $\epsilon H_{ps}/b\rho$, as described in Eq. a-4.

Figure 7 Bleb formation in cell exposed to laser radiation that completely photobleaches the cellular PPIX. (a) Cell before exposure. (b) Cell after exposure.

ceived 20 J/cm^2 and 81 cells received 300 J/cm^2 radiant exposure. After 3 days incubation, the occurrence of cell colonies was scored. The cells killed by PDT and those that survived are compared. The D_{PDT} obtained with 300 J/cm^2 was higher than with 20 J/cm^2 , of course, and the rate of colony formation was lower at the high exposure than the low exposure, $3/81 (3.7\%)$ vs $8/36 (22\%)$, respectively. However, there was not a sharp threshold value of D_{PDT} which demarcated death from survival. Some dosages were as low as 1×10^{18} but achieved cell kill. Other dosages were as high as 3×10^{19} and failed to kill. The threshold D_{PDT} for killing falls in the range of 1×10^{18} – 3×10^{19} . Within that range, most cells were killed but a significant fraction survived. This work continues to explore cell killing vs survival at low D_{PDT} exposures.

DISCUSSIONS

PDT is a form of light-activated chemotherapy for the treatment of malignant neoplasia. Relatively non-toxic photoactive compounds will preferentially accumulate in malignant tissues and yield a phototoxic effect when stimulated by the absorption of photon. A major advantage of ALA induced PPIX is its rapid conversion into subsequent metabolites along the heme synthetic pathway. The elevated PPIX concentration is transient only 24 hours in

Figure 8 Colony formation study. Cells individually received light exposure while video recordings documented C_a and H_{pb} , allowing the D_{PDT} of individual cells to be calculated. Cells received either 20 or 300 J/cm² at 488 nm wavelength. The D_{PDT} values of cells that died and cells that survived to form colonies are shown. The lowest dose that killed was about 1e18 ph/g and the highest dose that failed to kill was 3e19 ph/g.

vitro. Therefore, the side effect of prolonged photosensitization which is a consideration with the use of administered porphyrin, is avoided. This study of ALA induced PPIX aimed to investigate the variation of PDT dose, deposited energy per photon density, in culture cells for estimating the efficacy of PDT for tissue by using the fluorescence microscopy. The fluorescence microscopy developed in this work can measure the initial concentration and the photobleaching time constant simultaneously. In addition to this measurement method, two calibration methods were developed to assay the concentration of PPIX cell by cell. The result of the total fluorescence distributed over the cell varied widely depending on the cross-sectional area. The area of the cell varied but the normalized value, $F_{c,d}/A$, showed the difference between the cells with ALA and cells without ALA. This result showed that the measurement of each cell cross sectional area was essential for estimating the fluorescence intensity from a cell. The distribution of the initial

PPIX concentration, C_a ($\mu\text{g/ml}$), and the photobleaching light dose, H_b (J/cm²), showed that both values also widely varied depending on each cell. This means that C_a and H_b should be measured cell by cell to avoid errors caused by the difference of PPIX synthesis or cell cycle in each cell before PDT. Next, as shown in Fig. 6, the apparently homogeneous population of cells is behaving so heterogeneously with respect to PPIX accumulation and photobleaching. The cell cycle may effect this result but further investigation is needed. The concept of a threshold photodynamic dose was studied using another photosensitizer, aluminum chlorosulphonated phthalocyanine (AISPC)⁷. They reported a threshold D_{PDT} of 3.8×10^{18} for achieving necrosis in rat liver. There authors also estimated a threshold D_{PDT} of 8.6×10^{17} for the photosensitizer Photofrin II to achieve necrosis in human tumors, based on the work of Potter³. Recently, Chen et al¹⁰, have reported a threshold D_{PDT} of 4.0×10^{18} for Photofrin II to achieve necrosis in dog prostate. The

estimated range for a threshold D_{PDT} of 1×10^{18} - 3×10^{19} reported for PPIX in this paper is consistent with the values for Photofrin II. PPIX appears relatively efficient in achieving photodynamic killing of cells. ALA induced PPIX synthesis directly within the target cancer cells. In contrast, administration of pre-synthesized porphyrin relies on transport by diffusion from the vascular space into the cancer cells. The difference of the pathway may cause the relatively high efficiency of PPIX.

CONCLUSION

The amount of PPIX attained by the cells ranged between 1-15 $\mu\text{g/g}$ cells, with a mean value of 6.0 $\mu\text{g/g}$ cells. The photobleaching dose constant was $135 \pm 56 \text{ J/cm}^2$. The photodynamic dose obtainable after complete photobleaching ranged between 2×10^{18} - 5×10^{19} with a mean value of 5.2×10^{19} [photons absorbed by PPIX per g cells]. A colony formation study indicated that the threshold D_{PDT} required for cell killing fell in the range of 1×10^{18} - 3×10^{19} .

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APPENDIX

Photobleaching and fluorescence

Photobleaching of PPIX in a cell is expected to follow first-order kinetics with respect to the radiant exposure, $H(\text{J/cm}^2)$, of the single cell. Assume that the initial distribution of PPIX is uniform in a cell at a concentration $C_0(\mu\text{g/ml})$. The PPIX in the

cell undergoes photodestruction, called photobleaching, and C_o decays as :

$$C(t) = C_o \exp(-t/\tau) \quad (a-1)$$

where

$$\tau = \frac{H_b}{E} \quad (a-2)$$

The photobleaching time constant, τ [s], depends on the irradiance of the photobleaching light, E [W/cm²], and the photobleaching dose constant, H_b [J/cm²]. In our experiments, we measured τ at a specified E and deduced H_b .

The photodynamic dose

The photodynamic dose, D_{PDT} , depends on the irradiance, E , and the concentration of PPIX, C_o , but this product must be integrated over the exposure time, T , since photobleaching depletes the PPIX according to Eq. a-1. The integral is :

$$D_{PDT} = \frac{\epsilon}{b\rho} \int_0^T C_o \exp(-t/\tau) E dt \quad (a-3)$$

$$= \frac{\epsilon C_o E \tau}{b\rho} (1 - \exp(-t/\tau)) = \frac{\epsilon C_o H_b}{b\rho} (1 - \exp(-H/H_b))$$

where ϵ is the extinction coefficient of PPIX at 488 nm [0.023 cm⁻¹/(μg/ml)], b is the energy per photon at 488nm [4.07 × 10⁻¹⁹ J/photon], ρ is the density of a cell [~1.0 g/ml], $C(t)$ is the PPIX concentration as it photobleaches, E is the irradiance [W/cm²], T is the exposure time [s], H is the radiant exposure equal to ET [J/cm²], and H_b [J/cm²] is the photobleaching exposure that reduces C (t) by a factor $1/e$.

If the PPIX is completely photobleached ($t \gg \tau$, or $H \gg H_b$), the maximum D_{PDT} obtained is proportional to the product $C_o H_b$.

$$D_{PDT} \text{ for complete photobleaching} = \frac{\epsilon C_o H_b}{b\rho} \quad (a-4)$$

This study measured both C_o and H_b for individual cells. The maximum D_{PDT} obtainable in individual cells was calculated using Eq. a-4. In the colony formation study in this paper, cells received a radiant exposure of either 20 or 300 J/cm² and Eq. a-3 was used to calculate the true D_{PDT} of each cell.