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Resistance to Photodynamic Therapy
in Murine Leukemia Cells In VitroHideo KUMASAKA, Tetsuya OKUNAKA, Chimori KONAKA,
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Abstract

A photodynamic therapy (PDT)-resistant variant of the P388 murine leukemia cell line was isolated by repeated PDT with mono-L-aspartyl chlorin e6 (NPe 6) in vitro to the 0.1% survival level, followed by twelve regrowth cycles. The resistant variant was 1.38-fold more resistant to PDT with NPe 6 ($10\sim 15\ \mu\text{g}/\text{mL}$). However, more than $20\ \mu\text{g}/\text{mL}$, there was no difference between cells. The PDT-12 cells had less uptake of NPe 6 after 2h incubation than P388. We examined cross-resistance to adriamycin and the partitioning behavior determinant of the aqueous biphasic system. There was no difference in cytotoxicity and the uptake of adriamycin (ADM) between parental and PDT-12 cells. Partitioning behavior of parental cells and PDT-12 cells was compared. The latter has an inherently higher degree of membrane hydrophobicity. NPe 6 is comparatively hydrophilic, but the P388/ADR cells had reduced accumulation of the drug by extrusion by P-glycoprotein rather than membrane hydrophobicity. Furthermore, NPe 6-PDT cytotoxicity depended on accumulation into cells. Therefore, the PDT-12 cells acquired a hydrophobic membrane by repeating NPe 6-PDT twelve times. The PDT-12 cells were not cross-resistant to adriamycin. NPe 6 penetration into PDT-12 cells was prevented by membrane hydrophobicity.

Key words : photodynamic therapy-resistance, P388 murine leukemia cells, NPe6, membrane hydrophobicity, extrusion by P-glycoprotein

1. Introduction

Photodynamic therapy (PDT), based on the photosensitizer Photofrin, is currently being applied in clinical trials for various

solid tumors¹⁻³⁾. The success of PDT stems from the fact that, by a yet undetermined mechanism, the photosensitizer is selectively retained in tumor rather than normal sur-

rounding tissues, thus enabling differential cytotoxicity^{4, 5)}. The most commonly used sensitizers in PDT are hematoporphyrin derivatives (HpD) and commercially semi-purified Photofrin. However, these compounds have some inherent drawbacks⁶⁾. In recent years, new second generation photosensitizers have been developed, one class of which is mono-L-aspartyl chlorin e6 (NPe 6), while a cheaper diode laser system is commonly used as a light source in pain control, it is difficult to use the diode laser for PDT with commonly available photosensitizers, because its wavelength is too longer and the laser power is low. Katumi et al. developed a new high-power red laser diode system for PDT with NPe 6^{7, 8)}. The laser beam has relatively short wavelength (664 nm) and relatively high power (50-500mW/cm²). Fibrosarcoma in mice was successfully treated with this system⁹⁾. However, the mechanism of tumor destruction in vitro using NPe 6 has yet to be determined. We tried to isolate resistant cells to NPe 6-PDT as a tool in the clarification of PDT mechanisms.

2. Materials and Method

Drugs

NPe6 was obtained from Meiji Seika Kaisha Ltd., (Tokyo, Japan). NPe 6 is an effective photosensitizer with a major absorption band at 664nm, whose potential for PDT has been explored^{7, 8)}. Adriamycin (ADM) was obtained from Kyowa Hakkou Kogyo Co. Ltd. (Tokyo, Japan).

Cells

P388 murine leukemia and P388/ADR, a subline expressing the multi-drug resistance (MDR) phenotype. The P388 cells were used

to isolate PDT-resistant variants and to serve as the parental strain in all experiments. The cells were grown in RPMI-1640 medium supplemented with 8% FCS, antibiotics and 2ME.

Isolation Procedure for PDT-resistant P388 Cell Strain

The P388 cells were subjected to a series of incubations with NPe 6 and light irradiation in order to obtain PDT-resistant strains.

The cells (5×10^4 cells / ml) were plated in 30-mm plastic Petri dishes, and NPe 6 ($25 \mu\text{g/ml}$) was added to the plates. After incubation at 37°C for 4h, the drug medium was removed and the cells were rinsed with fresh growth medium, and then replaced in 30-mm plastic Petri dishes. The dishes were exposed to a light fluence rate of 39 mW/cm² delivered at a fluence of 10J/cm² (664nm wavelength) in vitro to the 0.1% survival level, followed by regrowth for twelve cycles. The surviving strain was designated PDT-12.

PDT Sensitivity

Each cell type (5×10^4 cells/ml) was plated in 24-well plates, and different doses of NPe 6 (0, 10, 15, 20, 25, 30 $\mu\text{g/ml}$) were added to the plates. After incubation at 37°C for 2h in the dark, the drug medium was removed and cells were rinsed with fresh growth medium and then replaced in 96-well plates. The plates were exposed to light fluence rate of 39 mW/cm² delivered at a fluence of 10J/cm² (664 nm wavelength) and returned to a 37°C incubator. After incubation for 24h, the viability was determined by MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide] assay.

NPe 6 Uptake

The cells (5×10^7 cells) were plated in 100-mm plastic Petri dishes, and NPe 6 (10, 15, 20

Figure 1 Survival curves for NPe6-PDT treatment of P388 and PDT-12 cells. Cells were incubated with 10, 15, 20, 25 or 30 $\mu\text{g/ml}$ NPe6 for 2h and exposed to 10J/cm^2 . Each point is the average of triplicate wells and error bars indicate the standard error

$\mu\text{g/ml}$) was added to the dishes and the cells were then incubated at 37°C for 2h. The drug medium was then removed and cells were rinsed with fresh growth medium twice. The samples were frozen/thawed and then lysing fluid, 0.7ml methanol and 0.1ml 0.4% EDTA / 50mM HEPES (pH7.4), was added. The lysates were mixed for 5 min, and centrifuged at 1,200rpm for 5min. The supernatant was analyzed by high performance liquid chromatography (HPLC). The HPLC system consisted of a $5\text{-}\mu\text{m}$ silica column eluted with a mixture of 2 mM-TBA (tetra butyl ammonium)/ methanol (1 : 3, vol / vol). A HITACHI D-7500 integrator was used to analyze the chromatograms.

Adriamycin Sensitivity

The cells (4×10^3 cells/ ml) were plated in 96-well plates and incubated for 24h, and then exposed to adriamycin ($0.001 \sim 0.1 \mu\text{g/ml}$). After 3 days, the sensitivity of cells to adriamycin was determined by MTT assay.

Adriamycin Uptake

The cells (5×10^7 cells) were plated in 100-mm plastic dishes, and adriamycin ($1 \mu\text{g/ml}$) was added and cells were then incubated for

100 or 200 min. The appropriate samples were frozen / thawed and lysing fluid was mixed with 5ml ethylacetate / propanol (4 : 1) and added, and the samples were shaken for 20 min. The cell lysates were centrifuged at 3,500 rpm for 10min., and the supernatant was evaporated. The extracts were dissolved in $250 \mu\text{l}$ H_2O / methanol (1 : 1), and centrifuged at 1,500 rpm for 10min. The upper solution was loaded on an HPLC⁽¹⁰⁾

Partitioning of P388 & PDT-12

Partition coefficients of cells between the phases of aqueous polymer mixtures were measured as follows : 5% (w / v) dextran T500 (Mr 500,000 ; lot 243862 Pharmacia, Uppsala) and 4% poly ethylene glycol (PEG) (Mr 6000 ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were prepared in Dulbecco's phosphate buffered saline (lot No. 320-1885 ; Gibco, Paisley). After thorough mixing, the phases were separated by centrifugation at 3,000 rpm for 20 min. at 22°C . The 2.5ml lower phase and 2.5ml upper phase were mixed with the appropriate concentration of palmitine ($0.00006 \sim 0.0002\%$). This mixture was added to a suspension of 10^6 cells in $100 \mu\text{l}$ isotonic NaCl. Immediately after mixing, a 0.2ml sample was removed for determination of cell concentration. At timed intervals thereafter, 0.2ml portions were taken from the center of the upper phase for cell counting⁽¹¹⁾.

3. Results

PDT Cytotoxicity

Figure 1 shows PDT survival curves for the P388 parental cell line and the PDT-resistant variant cell line following 2h incubation with NPe 6. PDT-12 was approximately 1.38-fold more resistant to PDT as 50% survival frac-

Figure 2 NPe 6 uptake per 10^7 cells in P388 and PDT-12 cells at 2h incubation determined by HPLC. Three independent experiments were conducted with each cell type and the error bars indicate the resulting standard error

tion ($P < 0.01$). However, at more than $20 \mu\text{g}/\text{ml}$, there was no difference between the cell lines. In order to test the reproducibility of these results, PDT survival experiments were carried out with P388 & PDT-12 cells run under identical conditions three times.

NPe 6 Uptake

Comparisons of relative NPe 6 uptake between P388 and PDT-12 are shown in **Fig. 2**. The uptake by PDT-12 was less than that of parental P388 at various concentrations. A significant difference was observed between P388 and PDT-12; NPe 6 uptake ratio, P388 / PDT-12 were 1.46 ± 0.018 at $10 \mu\text{g}/\text{ml}$, 2.11 ± 0.079 at $15 \mu\text{g}/\text{ml}$ and 1.60 ± 0.099 at $20 \mu\text{g}/\text{ml}$ of the accumulation of NPe 6.

Adriamycin Sensitivity

The survival curves of PDT with NPe 6 for P388, P388/ADR and PDT-12 are shown in **Fig. 3**. The P388/ADR cells showed a high degree of resistance to adriamycin, as ex-

Figure 3 Survival curves for adriamycin sensitivity of P388, P388 / ADR and PDT-12 cells. Cells were incubated with $0.001 \sim 0.1 \mu\text{g}/\text{ml}$ adriamycin. Each point is the average of triplicate wells and error bars indicate the standard error

pected. There was, however, no significant difference in adriamycin sensitivity between P388 and PDT-12 cells.

Adriamycin Uptake

The uptake of the various adriamycin fractions in each cell line is shown in **Fig. 4**. The adriamycin uptake of P388/ADR was less than that of the other cell lines at various concentrations. However, no significant difference was observed between P388 and PDT-12.

Partitioning of P388 & PDT-12

In the absence of palmitine, cells rapidly dropped to the interface of the complete partitioning system, and addition of palmitine promoted the partitioning of cells into the upper phase as shown in **Table 1**. With P388 cells, a partition ratio of 50 was achieved after 20 min., using 0.0001% (w / v) palmitine. The PDT-12 cells needed 0.0002% of palmitine. With each cell line, more than 90

Figure 4 Adriamycin uptake per 5×10^7 cells in P388, P388/ADR and PDT-12 for 100 or 200 min. incubation determined by HPLC. Three independent experiments were conducted with each cell type and the error bars indicate the resulting standard error

% of the cells could be brought into the upper phase by addition of sufficient palmitine. The PDT-12 cells required approximately 2-fold more of either ester than did P388 to achieve this result.

4. Discussion

The primary goal of this study was to derive NPe 6-PDT-resistant P388 cells for future use as a tool in the study of PDT mechanisms of action in vitro and in vivo. Isolation of drug-resistant cell lines has produced models for subsequent biochemical and molecular studies relating to drug action, drug resistance, and cross-resistance¹²⁾. This has been achieved, but the degree of resistance is not high, so work on further enhancing the resistance is in progress. PDT is a particular technique which utilizes a photosensitizer and laser. Few systematic studies of cellular resistance to photodynamic therapy have been reported to date. The mechanisms of resistance to PDT can be divided into two categories: intercellular

Table 1 Partitioning behavior of P388 and PDT-12 cells

Palmitine %	P388	PDT-12
0.00006	45	28
0.0001	52	40
0.0002	78	67

Cells were partitioned for 20 min. Numbers represent percentage of total cells bound in upper phase. The partition ratio of 50% was achieved, the P388 cells needed 0.0001% palmitine and PDT-12 cells needed 0.0002% palmitine

accumulation of photosensitizers, and others. Singh et al. isolated Photofrin-PDT-resistant cells (RIF-8), which were obtained by repeated photofrin incubation and light treatment of mouse radiation-induced fibrosarcoma (RIF) cells⁹⁾. RIF-8 cells did not show reduced Photofrin uptake^{9, 13)}, and were thus classified in the latter category. Mitchell et al. also observed a degree of porphyrin-PDT resistance and impaired porphyrin accumulation in CHO-MDR cells¹⁴⁾. The resistance to PDT of CHO-MDR and human breast carcinoma cells (MCF-7) was associated with decreased intracellular concentration of Photofrin^{14, 15)}. These cell lines thus seem to belong to the former category. PDT-12, obtained by repeated NPe 6-PDT and regrowth for twelve cycles reduced NPe 6 uptake (Fig. 2). NPe 6 uptake ratio between P388 and PDT-12 was 2.11 ± 0.079 at $15 \mu\text{g} / \text{mL}$. Further, NPe 6-PDT sensitivity was dependent on the accumulation of NPe 6 in our preliminary work. Therefore, PDT-12 cells seem to belong to the former category. This implies that the involved mechanisms differed from those in Photofrin-PDT resistant RIF-8, but are similar to those in CHO-MDR and human breast carcinoma cells (MCF-7). The observation was consistent with the finding that P-glycoprotein is at least partly

effective in altering NPe 6 transportation. As regards chemotoxic cross-resistance in PDT-resistance cells, Singh et al. reported that RIF-8 cells were not cross-resistant to adriamycin and found no difference in the uptake of adriamycin between parental cells and RIF-8 cells⁹⁾. Roger et al. reported that RIF-8 cells were cross-resistant to cisplatin¹⁶⁾. In contrast, in multi-drug-resistant (MDR) cell lines, Giannotti et al. reported no difference in sensitivity to PDT with sulfonated aluminum phthalocyanine between parental and MDR human erythroleukemic cells¹⁷⁾, while Marchesini et al. found 1.4-fold higher Photofrin resistance under specific treatment conditions in adriamycin-resistant human MCF-7 cells¹⁵⁾. P388/ADR, a subline of P388 murine leukemia selected for anthracycline resistance, exhibits a broad cross-resistance pattern and an enhanced outward drug transport system characteristic of the MDR phenotype¹⁸⁻²⁰⁾. If there had been P-glycoprotein amplification in the PDT-12 cells, one would expect PDT-12 cells to be less adriamycin sensitized than P388 cells (Figs. 3 and 4). However, we found no difference in adriamycin uptake and cytotoxicity. We speculated that PDT-12 cells differ in the expression of P-glycoprotein from parental cells. The possibility that reduced photosensitizer is the cause of membrane hydrophobicity has been examined by comparing the partitioning behavior of P388 and PDT-12, as a complementary approach to the study of NPe 6-PDT resistance. Gerson summarized data relating partitioning behavior in uncharged systems to membrane hydrophobicity, and provided information on the relationship between surface energy of cells and partitioning behavior²¹⁾. Kessel com-

pared the partitioning behavior of P388 to that of P388/ADR¹¹⁾. P388/ADR had an inherently higher degree of hydrophobicity due to membrane glycosylation. An enhanced level of cell-surface glycoprotein is associated with development of resistance to natural product¹⁰⁾. We also found that NPe6-PDT-resistant cells inherently had a 2-fold higher degree of hydrophobicity (Table 1). NPe6 is comparatively hydrophilic. PDT-12 cells showed reduced accumulation of NPe6, because of the barrier of membrane hydrophobicity other than P-glycoprotein, and enhanced outward transport. These findings suggest the possibility of membrane changes that decreased the cellular concentration of NPe 6. Marchesini et al. found 1.4-fold higher Photofrin resistance under specific treatment conditions in MCF-7 cells¹⁵⁾. Similarly, PDT-12 showed approximately 1.38-fold higher resistance to PDT than parental cells at 10 to 15 $\mu\text{g}/\text{mL}$ NPe6 (Fig. 1). However, the degree of resistance was not high, and at more than 20 $\mu\text{g}/\text{mL}$, there was no difference between the cell lines, because the intracellular concentration reached a fatal dose under fixed fluence of 10 J/cm².

In conclusion, we have demonstrated that resistance to NPe 6-PDT can be achieved in vitro. PDT-12 cells acquired a hydrophobic membrane by repeating NPe 6-PDT twelve times. No researches has isolated PDT-resistant cells showing reduced intercellular accumulation of photosensitizers. The PDT-12 cells were not cross-resistant to the chemotherapeutic agent adriamycin. Furthermore, NPe 6 did not penetrate into PDT-12 cells due to the barrier of membrane hydrophobicity, rather than the extrusion by P-glycoprotein.

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