RECOVERY OF CHINESE HAMSTER CELLS FOLLOWING PHOTOSENSITIZATION BY ZINC TETRAHYDROXYPHTHALOCYANINE

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Summary

The phthalocyanine dyes are attractive sensitizers for photodynamic therapy of cancer. The light fluence response curves for photocytotoxicity of zinc tetrahydroxyphthalocyanine were constructed using the colony-forming ability of Chinese hamster cells as an end-point. The survival curve of cells photosensitized to white light by this dye has a pronounced shoulder followed by an exponential decline. Postillumination hypertonic treatment (0.5 M NaCl for 20 min at 37 °C) enhanced log-phase killing, although to a lesser extent than after exposure to ionizing radiation. While such an enhancement usually indicates that the cells are able to repair potentially lethal damage, delayed trypsinization of photosensitized cells in plateau-phase failed to show a significant increase in cell survival. Thus, the repair of such a damage in plateau-phase is apparently absent. Experiments with split light fluence indicated that log-phase cells can repair sublethal damage during a 24 h interval, as evidenced by the reappearance of the shoulder on the split-dose survival curve.

1. Introduction

Photodynamic therapy (PDT) has shown promise in the treatment of certain malignant tumors [1, 2]. Currently, PDT employs hematoporphyrin

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derivative (HPD) as a photosensitizer, and red light for excitation. Problems inherent to HPD such as poor absorption of the red light and lingering skin photosensitivity have prompted the search for alternative photosensitizers. Phthalocyanines (PC) are a group of photosensitizers [3] which have been proven effective *in vitro* [4 - 7] and *in vivo* [8 - 10].

PC are porphyrin-like synthetic dyes which absorb light intensely in the red range of the spectrum (above 600 nm). The central metal atom is critical for their ability to serve as photosensitizers. Thus, PC containing diamagnetic metals, such as aluminum and zinc, are photobiologically active while no activity is observed with paramagnetic metals such as iron, copper, cobalt or nickel [11]. While the substituents on the peripheral benzene rings affect the solubility of substituted phthalocyanines, no predictable relationship between this parameter and phototoxicity could be defined [12]. A recent review on the photobiology of PC has been published [13].

In order to use rationally these dyes in PDT, an understanding of the factors affecting cellular response to this treatment is required. Thus, using ClAl-PC tetrasulfonate, we have shown a direct relationship between the fluence rate and cell killing, presumably because of the ability of the cells to repair sublethal damage [14]. In the present work it is shown that log-phase cells are able to repair sublethal damage (SLD) photosensitized by ZnPC-$(OH)_4$, while cells in plateau-phase are unable to repair potentially lethal damage (PLD).

2. Material and methods

2.1. Chemicals

$ZnPC(OH)_4$ was synthesized from tetraneopentoxy ZnPC by cleavage of the neopentoxy groups using boron tribromide, as described in ref. 12. The dye obtained after purification contains a mixture of isomers, insoluble in water but highly soluble in ethanol, which are present as non-aggregated units. A stock solution of 1 mM was prepared in ethanol and the appropriate amount was added to growth medium to obtain the final concentration required.

2.2. Cell culture

Chinese hamster cells, line V79-B310H, were grown attached to plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. During the logarithmic phase of growth, the cells double in number every 9 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cell survival was determined using the colony-forming ability as an end-point. For experiments in log-phase, 200 - 20 000 cells were plated in 50 mm Petri dishes. After an ovenight growth with $ZnPC(OH)_4$, the growth medium was removed, phosphate buffered saline (PBS) was added, and the cells were exposed to light. At this time the cells formed microcolonies with an average cellular multiplicity ($\bar{N}$) of 2 - 3. After light exposure, PBS was

removed and DMEM resupplemented. Colonies were stained and counted after 7 - 8 days of additional incubation. For experiments in plateau-phase, 3×10^5 cells were plated in 50 mm Petri dishes. When the culture became confluent (usually within 2 days), the dye was added and the light exposure carried out as above. After light exposure, the cells were suspended by trypsinization, diluted and plated in the appropriate number to yield about 200 colonies per dish. Control experiments in which the yield of trypsinized cells was measured by direct cell counting after photosensitization showed no inhibition of trypsinization by light fluences up to 100 kJ m^{-2}. Results are expressed as the surviving fraction, *i.e.* the number of cells forming colonies in the treated samples divided by the number of untreated controls. Each datum point is the average of triplicate plates. Standard errors of the mean values are shown on the graphs where larger than the symbols. Each experiment was repeated at least twice and variations between experiments were statistically insignificant ($P > 0.1$).

2.3. Light exposure

Plates containing 3 ml PBS were exposed from above, with the lids on, at room temperature, to a light source composed of a bank of 40 W fluorescent tubular lamps (Sylvania, Daylight) housed in a reflector. The fluence rate was 55 W m^{-2} at the level of the cell monolayer, 15% of which was in the range of $ZnPC(OH)_4$ absorption (600 - 750 nm, $\lambda_{max} = 682$ nm in ethanol).

2.4. Dye uptake

Uptake of $ZnPC(OH)_4$ by the cells was estimated as described previously [12].

3. Results

Figure 1 shows a typical survival curve for Chinese hamster cells following photosensitization by $ZnPC(OH)_4$. As for other PC derivatives, there is an initial shoulder region in which light is relatively inefficient in causing cell killing. Above about 40 kJ m^{-2}, cell survival falls exponentially for cells in log-phase. When cells are treated in plateau-phase, there is about a twofold enhancement in sensitization, presumably because after 16 h cells in plateau-phase took up twice as much dye as cells in log-phase (4.0 and 2.2 nmol per 10^7 cells respectively). Since for the assay of plateau-phase cells trypsinization is always required, inhibition of this process might result in a lower cell count, expressed as an apparent enhanced sensitization. That the enhanced sensitization was not due to an inhibited trypsinization was inferred from the fact that only a slight enhancement of sensitivity occurred when trypsinization followed light exposure of log-phase cells (Fig. 1). Control experiments ruled out this possibility also in the case of plateau-phase cells (see Section 2). This finding is consistent with the absence of a trypsinization

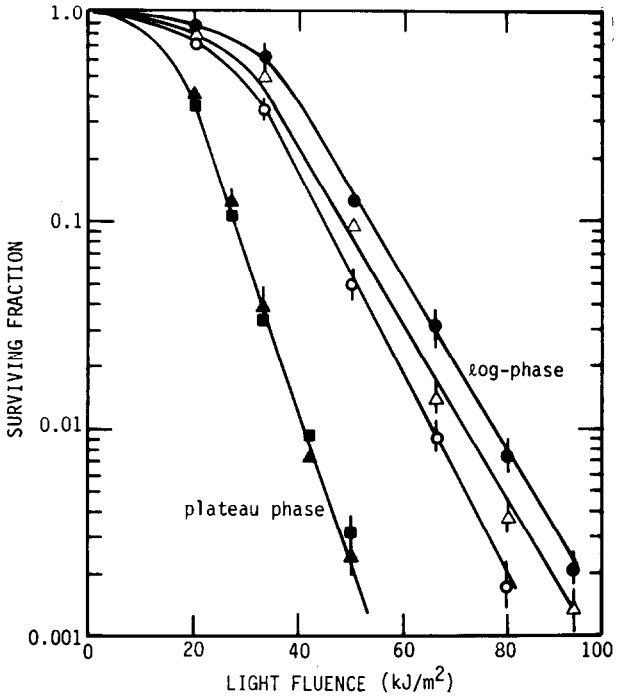

Fig. 1. Survival curves of Chinese hamster cells. Cells in log-phase (●, ○, △) or plateau-phase (▲, ■) after 16 h growth with 6 μM $ZnPC(OH)_4$ were exposed to graded light fluences. The triangles and squares denote cells that were trypsinized and replated after treatment. The open circles denote hypertonic treatment (0.5 M NaCl in PBS for 20 min at 37 °C) after light exposure. The curves for cells that were not trypsinized are corrected for cellular multiplicity (N = 2.4 in the experiment shown). Cells in plateau-phase were either trypsinized immediately after treatment (▲) or incubated for 3 h prior to trypsinization.

effect when photosensitization is induced either by AlPC [15] or AlPC-$(SO_3Na)_4$ [14]. It is noted that a pronounced inhibition of trypsinization occurs after HPD photosensitization [16, 17].

Figure 1 also shows the enhanced sensitization when the exposure to light of the cells in the log-phase is followed by hypertonic treatment with 0.5 M NaCl. A similar treatment after X-irradiation has a pronounced potentiating effect on cell killing [18].

Repair of SLD was demonstrated by split-dose experiments. In order to avoid artifactual "repair", we first studied the kinetics of disappearance of photosensitivity after removal of $ZnPC(OH)_4$ from the growth medium. Cells incubated in serum-supplemented DMEM lose most photosensitivity within 60 min (Fig. 2). The sensitivity was not affected when incubation was carried out in the absence of serum. Consequently, in initial split-dose experiments, serum-free medium was used during the time intervals between two light exposures to avoid loss of photosensitivity that may be misinterpreted as repair. Unexpectedly, a pronounced sensitization was detected under these conditions. Such a sensitization also occurred, although to a les-

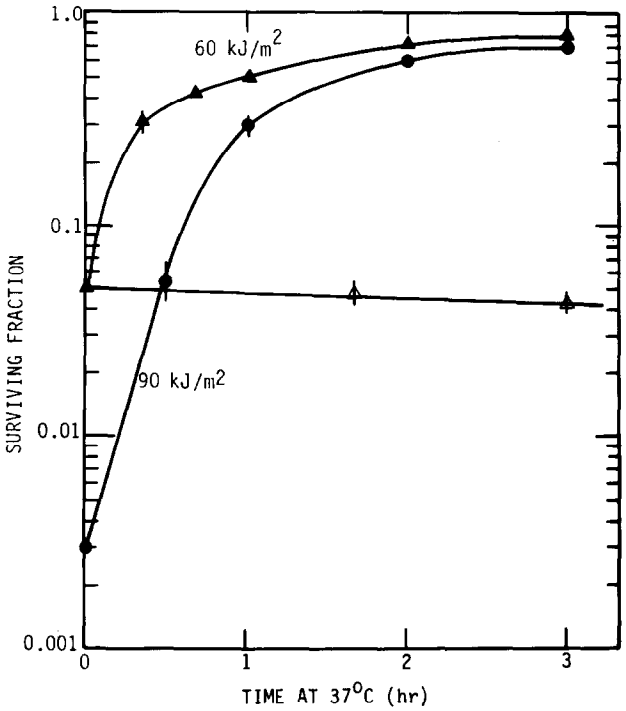

Fig. 2. Kinetics of disappearance of photosensitivity of Chinese hamster cells. Log-phase cells incubated for 16 h with 6 μM $ZnPC(OH)_4$ were incubated in dye-free, serum-supplemented DMEM for various times before exposure to 60 kJ m^{-2} (▲) or 90 kJ m^{-2} (●). △, cells incubated in dye- and serum-free DMEM prior to light exposure.

ser extent, when cells exposed to a single light fluence were incubated in the absence of serum (Fig. 3). In a control test, the plating efficiency of dye-treated cells which were kept in the dark for 5 h at 37 °C in serum-free medium was not reduced. Therefore to observe repair of SLD the incubation between two light exposures was carried out in DMEM - containing serum and, to avoid the loss of sensitivity, the medium was supplemented with dye. Since maximal photosensitivity and dye uptake occur after 4 h incubation with $ZnPC(OH)_4$ [12], further incubation was not expected to affect the response. The results are shown in Fig. 3. Evidently, the sensitivity of the cells increased during the first hour and there is no further change up to 5 h. The increased photosensitivity after 1 h is explained in terms of a 16% increase in the amount of dye per cell at that time. The split-dose survival curve for a 6 h interval in the absence of dye shows again enhanced killing, and also what might be interpreted as the beginning of the shoulder reappearance (Fig. 4). To avoid the enhanced killing, a split-dose survival curve was constructed for a 24 h interval, with dye present only for the last 16 h. Under these conditions, the amount of uptaken dye remained constant at 2.2 ± 0.15 nm per 10^7 cells, the enhanced killing was absent, and a pronounced shoulder reappeared (Fig. 4).

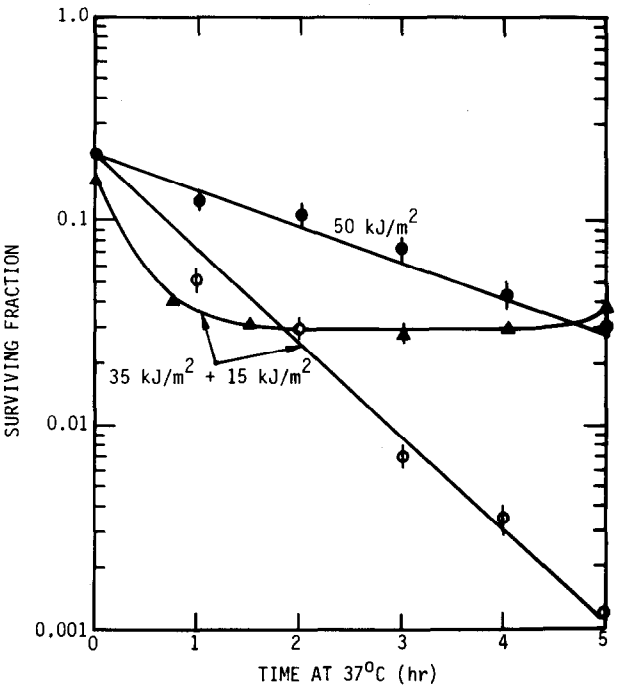

Fig. 3. Single- and split-dose survival kinetics. Chinese hamster cells in log-phase were incubated for 16 h with 6 μM ZnPC(OH)$_4$. The cells were then exposed to light. ●, 50 kJ m^{-2}; following light exposure, the cells were incubated at 37 °C in dye- and serum-free DMEM for various times. ○, 35 kJ m^{-2}; following light exposure, the cells were incubated at 37 °C for various times in dye- and serum-free DMEM, and subsequently re-exposed to 15 kJ m^{-2}. ▲, 35 kJ m^{-2}; following light exposure, the cells were incubated at 37 °C for various times in DMEM supplemented with serum and dye, and then re-exposed to 15 kJ m^{-2}.

A common procedure for the study of repair of radiation-induced PLD is delayed trypsinization and replating of plateau-phase cells [19]. Such an experiment was performed after photosensitization with ZnPC(OH)$_4$ (Fig. 5). Thus, for cells in plateau-phase, only a statistically insignificant (using χ^2 test) 1.5-fold increase in survival occurred within 2 h after light exposure. No increase occurred when trypsinization of log-phase cells was delayed. In contrast to repair of SLD, which entails reformation of the shoulder without a change in the final slope of the survival curve (Fig. 4), repair of PLD is characterized by a reduced slope [19]. Figure 1 shows that 3 h delay in replating of cells in plateau-phase did not affect the survival curve, indicating the absence of PLD repair.

4. Discussion

The present results show that log-phase Chinese hamster cells are able to recover from SLD induced by ZnPC(OH)$_4$ (Fig. 4). No recovery from PLD

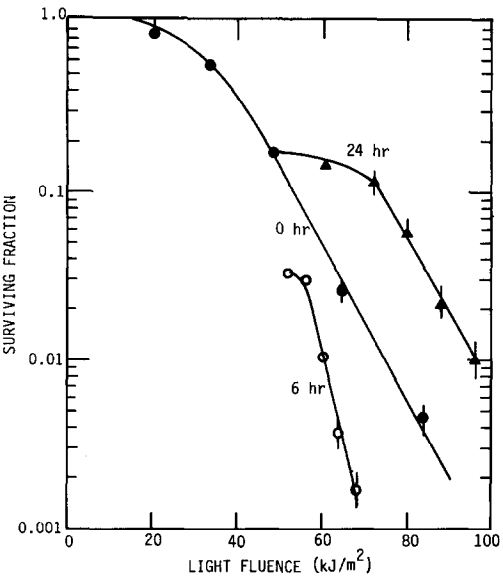

Fig. 4. Split-dose survival curves. Chinese hamster cells in log-phase were incubated for 16 h with 6 μM ZnPC(OH)$_4$. ●, cells were then exposed to graded light fluence, trypsinized and replated for colony formation. ○, cells exposed to 50 kJ m^{-2}, incubated in complete growth medium containing 6 μM ZnPC(OH)$_4$ for 6 h and then exposed to second graded light fluence, trypsinized and replated. ▲, cells exposed to 50 kJ m^{-2}, incubated in complete growth medium for 8 h and then 6 μM ZnPC(OH)$_4$ was added for additional 16 h; the cells were then exposed to second graded light fluence, trypsinized and replated.

in plateau-phase cells was observed (Figs. 1 and 5). Since both PLD and SLD are recovery processes defined operationally in radiation biology [20], in photosensitization these terms must be used with caution since factors other than repair could be involved, such as redistribution of the dye and increase in dye uptake by the cells [21]. However, the potentiation of cytotoxicity by hypertonic treatment suggests that in log-phase cells the capacity to repair PLD does exist, although it is smaller than that after exposure to ionizing radiation [18]. The reasons for this difference probably reflect the different lesions induced by the two agents. Thus, while the DNA is the main cellular target to ionizing radiation, PC-induced photosensitized damage of DNA is of minor significance [15]. It is noted that PLD is expressed in log-phase V79 cells following HPD photosensitization by incubation at 4 °C [22]. The absence of PLD repair after ZnPC(OH)$_4$ photosensitization of cells in plateau-phase but not in log-phase cells may contribute to the higher sensitivity of the former (Fig. 1). Another contributing factor may be the observed increased uptake of the dye in plateau-phase cells.

The demonstration of repair of SLD (Fig. 4) was hampered by two factors. First, incubation of the cells in serum-free DMEM after light exposure caused further enhancement of cytotoxicity, presumably because under these conditions PLD was expressed. Interestingly, this was more

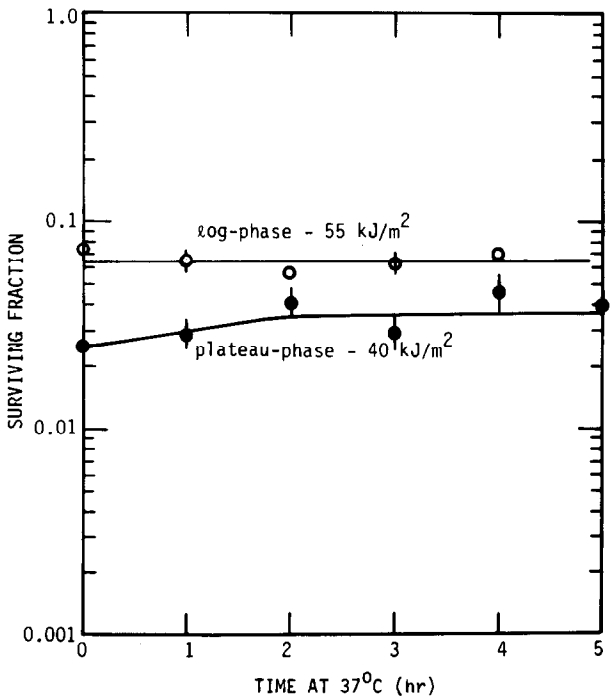

Fig. 5. Repair of potentially lethal damage. Chinese hamster cells in log-phase were exposed to 55 kJ m^{-2} (○) and cells in plateau-phase were exposed to 40 kJ m^{-2} (●) following 16 h incubation with 6 μM $ZnPC(OH)_4$. At different times after light exposure the cells were trypsinized and replated.

pronounced when followed by a second light exposure (Fig. 3). A similar effect of dose fractionation was observed for HPD photosensitization [21]. This implies that the expression of PLD induced by the first exposure makes the cells more sensitive to a second exposure. A possible reason is a redistribution of the dye to more sensitive cellular sites prior to the second exposure.

As previously [14, 15], we now report the absence of any inhibition of detachment by trypsinization of V79 cells by PC photosensitization. Using the same cell line and sulfonated GaPC, Hunting *et al.* [23] reported that light exposure after 1 h incubation with the dye inhibited detachment. We believe that this apparent discrepancy is due to the different incubation times (16 and 1 h respectively), rather than to the different dyes used. It is noted that HPD-induced inhibition of detachment was also reduced when the incubation time with the dye was increased [16].

The tumor vasculature is a primary target in the photodynamic effect of PC [10]. Direct cytotoxicity to the tumor cells is relatively small immediately after PDT and increases with time after treatment [24]. Therefore, endothelial cells are probably the clinically relevant cell population. Whether

such cells can recover after PDT may determine if occlusion of blood vessels will be temporary or permanent. Studies of fluence response curves for endothelial cells as well as tumor cells are in progress.

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