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Elimination of leukemic cells by laser photodynamic therapy

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Summary. We studied the effects of 514-nm laser light-induced merocyanine 540 (MC540)-mediated toxicity on both leukemic and normal bone marrow (BM) cells. Acute promyelocytic leukemia (HL-60) cells were incubated with MC540 (20 μg/ml) and exposed to 93.6 J/cm^2 irradiation at a 514-nm wavelength. Normal bone marrow cells were treated under similar conditions. At this dose, 99.9999% of the leukemic cells were killed while 55% of the BM cell survived. Of the granulocyte-macrophage colony-forming cells (CFU-GM), 27% also survived this treatment. Photosensitization of a mixture of irradiated BM cells mixed with an equal number of nonirradiated HL-60 cell did not interfere with the killing of HL-60 cells. There was no significant reduction in the viability of cells when exposed to the laser light alone. In summary, laser light-induced photosensitization with MC540 has a selective cytotoxicity to leukemic cells; therefore, this procedure may be useful for purging neoplastic cells from autologous BM.

Introduction

Merocyanine 540 (MC540) is one of several voltage-sensitive dyes used for measuring the membrane potential of cells, cell organelles, and membranes [4, 5, 23]. It has been demonstrated that MC540 is selectively taken up by leukemic cells [10]. Recently, MC540-mediated photosensitization of HL-60 and neuroblastoma cells has been reported [1, 2, 17, 19]. In these studies, the source of light used for photosensitization was a 75-W filament light bulb or an array of ten daylight fluorescent light bulbs. It has been reported that when colony-forming cell are exposed to light without any dye, a significant number (25%–65%) of cells are killed; this phenomena has been observed in one human leukemia cell line, murine erythroid progenitor cells, and quail neural crest cells [15, 21]. As much as 80% of the colony-forming lymphoma (Daudi) cells are killed by exposure to white light in the absence of photosensitizers [20]. Ultraviolet (UV) light is emitted by some daylight sources and may play a significant role in this phototoxicity. Wang et al. [24, 25] have shown that fluorescent daylight generates toxic photoproducts in tissue culture medium that can irreversibly damage susceptible cells. Although the exact mechanism of cell death by photosensitization remains unclear, the involvement of reactive oxygen species in the phototoxic reaction has been reported [13, 23]. In this study we used an argon laser as a source of 514-nm radiation to study the effects of MC540-mediated photosensitization of acute promyelocytic leukemia (HL-60) cells, normal bone marrow (BM) cells, and granulocyte-macrophage colony-forming cells (CFU-GM).

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Materials and methods

Reagents. Merocyanine 540 was obtained from Sigma Chemical Co. (St. Louis, Mo.). A stock solution of MC540 was prepared in 50% ethanol-water at 1 mg/ml and stored at −20° C in small aliquots. Final dilutions were made in RPMI-1640 medium to obtain the desired concentrations.

Light source. We used a Spectra Physics Model 171 ion-laser with Model 270 power supply.

Cells. The acute promyelocytic leukemia cell line HL-60 [6, 14] was maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 10 μg/ml gentamycin, and 0.25 m*M* L-glutamine (Gibco, Grand Island, NY) at 37° C in a humidified atmosphere of 5% CO_2 in air. Cells were maintained in log phase with >95% viability. Normal BM cells were obtained from patients participating in marrow transplantation research protocols.

Photosensitization. HL-60 cells and BM cells (3×10^6/ml) in RPMI-1640 supplemented with 10% fetal bovine serum were treated with 20 μg/ml MC540. Treated cells were placed in 35 × 10 mm Falcon petri dishes and allowed to incubate for 1 h at 37° C in a dark environment prior to irradiation with 514-nm light for 7.5 min at 2 W. The cell suspension was swirled at 3-min intervals for uniform exposure. During irradiation, cells were kept on a 1/4-in-thick aluminium plate that acted as an effective heat sink, causing the temperature of the cell suspension to remain at 24° ± 1° C. In a separate experiment, BM cells were irradiated (4500 rad), washed, and mixed with an equal number of HL-60 cells prior to photosensitization. Following irradiation, the cells were washed with RPMI-1640 medium. The washed cells were resuspended in RPMI-1640 and incubated at 37° C. The viability of the cells was determined by the trypan blue dye exclusion method. A 99.9999% kill was defined when there were no viable cell detected after up to 4 weeks long-term culture in a tenfold concentrated suspension in both 4^2 areas of the 0.1-mm Neubauer hema-

cytometer. In all experiments, control cells were incubated with the same volume of solvent (50% ethanol-water) used for dissolving MC540.

CFU-GM assays. The granulocyte-macrophage colony-forming cell (CFU-GM) assay was carried out as previously described in detail elsewhere [11]. Briefly, 1×10^5 cells were added to each culture plate containing 1 ml 0.8% methylcellulose (Methocel A4M, Dow Chemical Co., Midland, Mich.), 20% FCS, 100 IU penicillin/100 µg streptomycin, and 100 µl conditioned growth medium GCT mix (Gibco, Grand Island, NY). Triplicate cultures were incubated in a humidified atmosphere of 5% CO^2 in air. Colonies consisting of 30 or more cells were counted on day 10.

Data analysis. All experiments were carried out at least three times, and mean results ±SE are reported. The lower limit of accurate cell detection by hemacytometer count was found to be 3750 cells, as described elsewhere [1]. To increase further the accuracy of cell detection, all cultures were followed for a period of 4 weeks, at the end of which time a tenfold concentrate of the cells was examined with an equal volume of trypan blue for cell viability. The absence of a living cell was considered a 99.9999% cell kill. In a cell dilution experiment, it was found that as few as eight HL-60 cells would regrow to an easily detectable number (i.e., at least 3750 cells). In a separate experiment, 2×10^7 HL-60 cells were photosensitized and viability was monitored for 2 weeks; no living cell were detected at the end of that time.

Results

Dose response of 514-nm laser light on HL-60 cells

HL-60 cells (3×10^6 cells/ml in 35×10 mm petri dishes were irradiated with varying doses of 514-nm light in the presence of MC540. The dose-response curve (Fig. 1) shows that under these conditions, a dose of 93.6 J/cm^2 was required to obtain a 99.9999% cell kill; at this dose of irradiation, no regrowth of cells was observed for a period of 30 days. As a regrowth of cells was observed at all doses of <93.6 J/cm^2, a dose of 93.6 J/cm^2 was used in all subsequent experiments. Approximately 98% of the HL-60 cells were killed by a dose of 31.2 J/cm^2. To kill the last 2%–3% of the cells, a threefold higher dose of irradiation was required. The continuous presence of MC540 during laser irradiation was essential for maximal tumor cell kill.

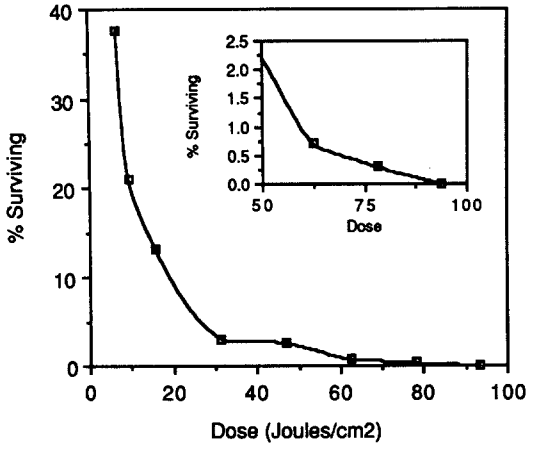

Fig. 1. HL-60 cells (3×10^6 cells/ml) were treated with 20 µg/ml MC540. After incubation for 1 h at 37° in a dark environment, the cells were exposed to 9.3, 15.6, 31.2, 46.8, 62.4, 78.0, and 93.6 J/cm^2 514-nm laser light. Irradiated cells were washed to remove excess MC540 and placed in fresh medium. A representative example is shown. *Insert graph* shows the magnified view of the last three doses on the curve

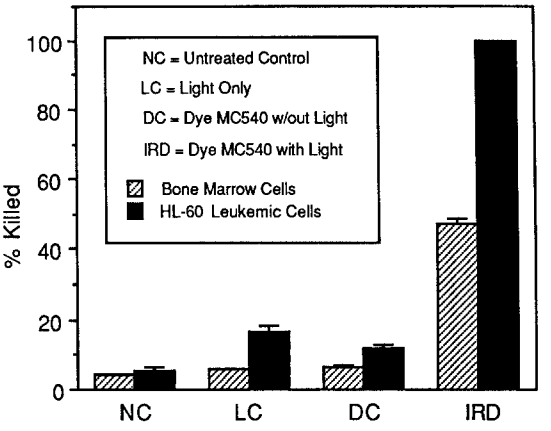

Fig. 2. Bone marrow cell and HL-60 cells (3×10^6 cells/ml) were treated with 20 µg/ml MC540 and irradiated with 93.6 J/cm^2 laser light. Results of six separate experiments are shown (mean ±SE)

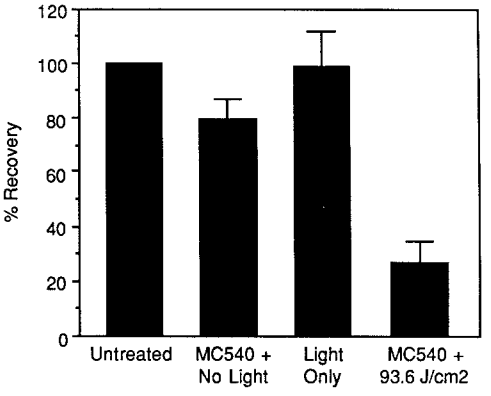

Fig. 3. Bone marrow cell and HL-60 cells (3×10^6 cells/ml) were treated with 20 µg/ml MC540 and irradiated with 93.6 J/cm^2 laser light. After irradiation, the cells were washed and resuspended in fresh medium. 1×10^5 cells were then plated in triplicate for CFU-GM assays as described in *Materials and methods.* Colonies were counted on day 10. Results of four separate experiments are shown (mean ±SE). Absolute numbers of colonies per 1×10^5 were: nontreated (93.4±8.23), dye alone (74.1±7.6), light alone (92.1±13.4), dye + light (24.9±7.4)

Comparison of laser light-induced photolysis of HL-60 and BM cells

Since a dose of 93.6 J/cm^2 514-nm irradiation in the presence of MC540 resulted in a 99.9999% kill of HL-60 cells, the same dose was used to treat normal BM cells. Normal BM cells or HL-60 cells (3×10^6 cells/ml) were treated with 20 µg/ml MC540 as described in *Materials and methods.* The results indicated (Fig. 2) that 55% of the normal BM cells survived the treatment that killed 99.9999% of the HL-60 cells. The cytotoxic effect of laser light alone in the

absence of MC540 was <6% for BM cells and <18% for HL-60 cells; this cell kill was not attributable to hyperthermia, since the temperature of the cell suspension remained at 24° ± 1° C. In simulated BM experiments, a regrowth of HL-60 cells was not observed for a period of 30 days. These results are similar to those obtained in the photosensitization of HL-60 cells alone, indicating that the presence of BM cells does not interfere with the killing of Hl-60 cells; they suggest that normal BM cells are less sensitive to MC540-mediated photosensitization than HL-60 cells.

The CFU-GM cell were found to be less sensitive to MC540-mediated cytotoxicity than HL-60 cells. The CFU-GM data is shown in Fig. 3. At a concentration of 20 μg/ml MC540 and an irradiation dose of 93.6 J/cm^2, 26.7% ± 7.4% of the normal hematopoietic progenitor cells survived, whereas at the same dose 99.9999% of the leukemic cells were killed. There was no significant reduction in CFU-GM cells when they were exposed to laser light alone.

Discussion

The precise mechanism of MC540-mediated cytotoxicity is not known, although involvement of the formation of free radicals has been suggested [13, 23]. The disordered or cholesterol-free domains of the lipids in the membrane appear to be the binding site for MC540 [12, 26]. The preferential uptake of MC540 by the neoplastic plasma membrane may be the function of its electrical property [10, 22]. It has been shown that the uptake of MC540 by leukemic cells is four to five times greater than that of normal cells and that the cells that take up more MC540 are more photosensitive than the cells that take up small amounts of dye [22]. In this paper we demonstrated that 514-nm light-induced, MC540-mediated cytotoxicity is effective against leukemic cells and is significantly less cytotoxic to normal BM cells. These results are in agreement with previously reported findings [17–20]. We also showed that there was no significant (<10%) reduction in the viability of CFU-GM cells after exposure to the light alone, which was probably due to the use of monochromatic light as a source of irradiation. The deleterious effects of UV-B radiation on the accessory function of human blood-adherent mononuclear cell and resident epidermal cells have recently been reported [7, 8, 16]. Our results on CFU-GM cells also showed greater survival rates of 27% compared to the 18% reported earlier [1]. Since exposure to daylight alone is known to reduce the viability of colony-forming cells, the actual percentage of surviving CFU-GM in the previously reported study [1] may have been lower when compared with that of nontreated cells. We have investigated the deleterious effects of laser light on cell migration, lymphokine production, and the responsiveness of cells to lymphokines, particularly human migration inhibition factor, which plays an important role in confining the macrophages at the site of inflammation in vivo [3, 9]. In this study, no cytotoxic effects were observed with 514-nm laser light (unpublished results). These data suggest that a monochromatic light source is desirable to minimize the cell damage by nonspecific wavelengths of light and to increase the number of surviving normal cells by using highly tumoricidal doses. Further work is needed to characterize the conditions (e.g. the effect of human serum proteins) for laser photodynamic therapy for clinical ex vivo purging of autologous BM grafts.

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