Dye-Mediated Photolysis of Human Neuroblastoma Cells: Implications for Autologous Bone Marrow Transplantation

By Fritz Sieber, Sanjay Rao, Scott D. Rowley, and Maya Sieber-Blum

Cells from three different human neuroblastoma cell lines and normal human bone marrow cells were exposed to the lipophilic fluorescent dye, merocyanine 540 (MC 540), and white light. In vitro clonogenic tumor cells were inactivated up to 25,000 times more rapidly than multipotent hematopoietic progenitor cells (CFU-GEMM). It is conceivable that this pronounced difference in sensitivity to MC 540-mediated photolysis can be exploited for the selective killing of residual neuroblastoma cells in autologous remission marrow grafts.

ONE MARROW transplantation allows escalation of antineoplastic therapy without regard to marrow toxicity. It thus affords a greater chance of eradicating tumors.1,2 Autotransplantation of the patient’s own cryopreserved remission marrow virtually eliminates one of the most serious complications of allogeneic marrow transplantation, graft-v-host disease (GVHD). It also obviates the need for HLA-identical allogeneic marrow donors.3,4 However, if the tumor originated in or metastasized to the bone marrow, transplantation of autologous remission marrow carries a significant risk of reinfusing occult tumor cells.5,6

For many tumors, the minimal concentration of neoplastic cells required for their unequivocal detection in bone marrow is ~1%. Thus, in the worst situation, a clinical marrow graft could contain between 10^4 and 10^5 residual tumor cells and still be classified as a remission marrow graft. Much effort is therefore currently directed at developing more sensitive methods for the detection of residual tumor cells and methods for their selective elimination from autologous remission marrow grafts.10,17

Studies with experimental tumors have shown that most physical, pharmacologic, and immunologic purging procedures are capable of reducing the concentration of tumor cells by one to four (occasionally six) orders of magnitude. We may thus have to resort to combination purging protocols to process marrow grafts with high concentrations of residual tumor cells. A combination of methods that are based on different principles may be the most promising strategy.

We have recently shown that Neuro 2a and NB41A3 murine neuroblastoma cells are killed very rapidly when they are exposed to the lipophilic photosensitizer, merocyanine 540 (MC 540) and a strong source of white light.17 By contrast, normal murine pluripotent hematopoietic stem cells are only minimally damaged by the same treatment. Lethally irradiated mice that are transplanted with photosensitized mixtures of tumor cells and normal marrow cells survive and remain free of disease.13 In this article, we present evidence that the same approach may also be applicable to the purging of human marrow grafts contaminated with neuroblastoma cells. MC 540-mediated photolysis reduced the concentration of in vitro clonogenic human neuroblastoma cells 1,000-fold to 50,000-fold, yet preserved 55% of multipotent hematopoietic progenitor cells (CFU-GEMM) from normal bone marrow.

**MATERIALS AND METHODS**

**Cells.** The human neuroblastoma cell lines IMR-32 (CCL 127), SK-N-MC (HTB 10), and SK-N-SH (HTB 11) were obtained from the American Type Culture Collection (Rockville, Md) and maintained in a culture medium consisting of 10% heat-inactivated fetal bovine serum (FBS) (KC Biological, Lenexa, Kan) and 90% α-modified minimal essential medium (α-MEM) (α-medium; KC Biological). Aliquots from a frozen stock of cells were thawed at regular intervals to prevent the unintentional selection of mutants. All three lines were free of mycoplasma infections.

The IMR-32 line originates from an abdominal mass in a 13-month-old child with a tumor diagnosed as neuroblastoma with rare areas of organoid differentiation.18 The line expresses adrenergic traits and consists of two morphologically distinct cell types, small neuroblast-like cells and large hyaline fibroblasts. The SK-N-MC line was isolated from a metastasis to the supraorbital area in a 14-year-old child.19 The cells have epithelial-like morphology and express cholinergic traits. Surface marker patterns are consistent with those of a neuroepithelioma.20 The SK-N-SH line was isolated from a bone marrow metastasis in a 4-year-old child.19 The cells are epithelial-like with adrenergic characteristics. The IMR-32 line was established before treatment was initiated. The two other lines were established after the patients had received radiotherapy and chemotherapy.

Normal human bone marrow cells were obtained from healthy donors for clinical bone marrow transplants after informed consent had been obtained under a protocol approved by the Joint Committee on Human Investigation and in accord with an assurance filed with and approved by the Department of Health and Human Services. A mononuclear cell fraction was prepared by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ).21

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Dye-mediated photolysis. Single-cell suspensions of neuroblastoma cells were prepared by incubating subconfluent monolayers briefly with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/L Hanks’ balanced saline; GIBCO, Grand Island, NY) and filtering the detached cells through a nylon screen (HD3-15; Tetko, Elmsford, NY) to remove cell aggregates. The filtered cells were washed once with HEPES-buffered (10 mmol/L, pH 7.4) α-medium supplemented with 5% FBS and resuspended at a density of 1 x 10⁶/mL in HEPES-buffered α-medium supplemented with 15% FBS, penicillin (10 U/mL), and streptomycin (10 μg/mL). Cell viability by trypsin blue exclusion was ≥98%. Mononuclear marrow cells were suspended in the same medium at a concentration of 5 x 10⁶/mL. MC 540 (Eastman, Rochester, NY) was added from a 1 mg/mL stock solution in 50% ethanol to a final concentration of 15 μg/mL. Clear polystyrene tubes (15-mm diameter, Corning, Corning, NY) containing the cell suspensions were placed into the center of a circular array (40-cm diameter) of 10 fluorescent light bulbs (Champion F15T8/D, 61 cm long). The cell suspensions were swirled manually every 10 minutes to prevent the cells from settling. Samples were removed at 15-minute intervals to determine the surviving fraction of in vitro clonogenic cells. The photosensitization process was stopped by washing the cells twice with HEPES-buffered α-medium supplemented with 5% FBS and antibiotics. This and all subsequent manipulations were carried out in the dark or under subdued lighting.

Bioassays. CFU-GEMM from normal human marrow were assayed as described by Messner and Fauser. Each 35-mm Petri dish contained 10⁶ nucleated marrow cells and 1 mL of Iscove’s modified Dulbecco’s medium (GIBCO) supplemented with 30% FBS bovine serum, 0.9% methylcellulose, 5 x 10⁻¹ mol/L of 2-mercaptoethanol, 1 U of sheep plasma erythropoietin (step III, Connaught Laboratories, Willowdale, Ontario, Canada) and 5% phytotaxemaglutnin-stimulated leukocyte-conditioned medium. Cultures were incubated for 14 days at 37°C in a humidified atmosphere of 5% CO₂ in air. Clear polystyrene tubes (15-mm diameter, Corning, Corning, NY) containing cell suspensions were placed into the center of a circular array (40-cm diameter) of 10 fluorescent light bulbs (Champion F15T8/D, 61 cm long). The cell suspensions were swirled manually every 10 minutes to prevent the cells from settling. Samples were removed at 15-minute intervals to determine the surviving fraction of in vitro clonogenic cells. The photosensitization process was stopped by washing the cells twice with HEPES-buffered α-medium supplemented with 5% FBS and antibiotics. This and all subsequent manipulations were carried out in the dark or under subdued lighting.

RESULTS

Photosensitization of neuroblastoma and normal bone marrow cells progressively reduced the number but not the size of colonies formed. At the time of evaluation, cultures set up with heavily photosensitized neuroblastoma cells contained few or no cells. However, they contained much cell debris, indicating that MC 540-mediated photosensitization killed and eventually lysed susceptible cells rather than just slowing their growth.

Extensive inhibition of colony formation required exposure to both, dye and light (Table 1). Incubation with dye in the dark had no effect. Exposure to light in the absence of dye reduced the concentration of in vitro clonogenic neuroblastoma cells. However, the reduction was minimal as compared with the reduction achieved by simultaneous exposure to both dye and light (Table 1), indicating that the colony assays were linear down to limiting dilutions, and that colonies were of clonal origin.

MC 540, mercocyanine 540.

Table 1. Effects of MC 540 and Light on In Vitro Colony Formation by Neuroblastoma Cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>SK-N-SH</th>
<th>SK-N-MC</th>
<th>IMR-32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>54.25 ± 2.84</td>
<td>187.25 ± 2.78</td>
<td>135.75 ± 3.66</td>
</tr>
<tr>
<td>Light, 90 min</td>
<td>39.50 ± 3.50</td>
<td>132.00 ± 2.48</td>
<td>44.25 ± 1.75</td>
</tr>
<tr>
<td>(P = .017)</td>
<td>(P &lt; .001)</td>
<td>(P &lt; .001)</td>
<td></td>
</tr>
<tr>
<td>MC 540, dark</td>
<td>53.50 ± 4.66</td>
<td>214.50 ± 11.08</td>
<td>137.25 ± 7.73</td>
</tr>
<tr>
<td>(P = .054)</td>
<td>(P = .867)</td>
<td>(P = .867)</td>
<td></td>
</tr>
<tr>
<td>MC 540, light, 90 min</td>
<td>0.0425 ± 0.0149</td>
<td>0.0100 ± 0.0058</td>
<td>0.0025 ± 0.0025</td>
</tr>
<tr>
<td>(P &lt; .001)</td>
<td>(P &lt; .001)</td>
<td>(P &lt; .001)</td>
<td></td>
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</tbody>
</table>

Data are expressed as mean colony counts of four replicate culture dishes ± SEM. Only simultaneous exposure to both MC 540 and light caused a drastic reduction of colony formation. P values in parentheses: two-tailed Student’s t test.
could be distinguished by their sensitivity to MC 540-mediated photosensitization. A similar degree of sensitivity was evident in both untreated and photosensitized cultures. Colony heterogeneity was therefore not a result of distinct subpopulations of colony-forming cells that could be distinguished by their sensitivity to MC 540-mediated photosensitization.

When survival curves were plotted semilogarithmically, the portion of the curve to the right of the plateau (Fig 1) could be fitted by a single straight line, indicating that most colony-forming cells of a given neuroblastoma line were about equally sensitive to dye-mediated photosensitization. Subpopulations that were significantly less sensitive than the majority of cells would have resulted in biphasic or multiphasic survival curves. Small subpopulations of photoresistant cells would have been detected most readily in the IMR-32 line where the survival curve spanned >4 logs.

DISCUSSION

MC 540 is an amphipathic fluorescent dye. In the absence of serum, it binds indiscriminately to the plasma membrane of all cells. However, in the presence of serum which acts like a competitive substrate with binding sites of intermediate affinity, the dye binds preferentially to cells with high-affinity binding sites for dye molecules. High-affinity dye-binding sites abound on electrically excitable cells, leukemic cells, and certain classes of immature blood cells. Some authors have suggested that MC 540 binds preferentially to disordered or cholesterol-free domains in the outer leaflet of the lipid bilayer. Other authors favor the view that a cell’s affinity for MC 540 is a function of the plasma membrane.

The phototoxic reaction is not yet fully understood. It probably involves the formation of reactive oxygen species, and plasma membrane lipids may be their primary target (lipid peroxidation). Cells that adsorb large amounts of dye tend to be photolyzed more rapidly than are cells that bind small amounts of dye, suggesting that a cell’s sensitivity to MC 540-mediated photolysis is primarily a function of the density of high-affinity dye-binding sites.

In this article, we show that three human neuroblastomas were markedly more sensitive to MC 540-mediated photolysis than CFU-GEMM from normal human bone marrow. The fact that the three neuroblastoma lines differed significantly with regard to their sensitivity to MC 540-mediated photolysis is not surprising. The three cell lines were derived from different patients and from primary and secondary tumors from different sites. We do not attach too much importance to the observation that the two lines derived from patients who had undergone chemotherapy and radiotherapy were less photosensitive than the line that had been established from an untreated neuroblastoma. Previous experiments with multiple drug-resistant mutant tumor cell lines have shown that drug-resistant tumor cells were usually not less photosensitive than the corresponding wild type of tumor cell line.

The two cell lines derived from metastatic tumors (SK-N-MC and SK-N-SH) happened to be less photosensitive than the line that originated from a primary tumor (IMR-32). Site-specific alterations in the lipid composition of tumor cells have been described (reviewed in ref. 32). It is conceivable that site-specific differences in photosensitivity occur as well.

Exposure to light in the absence of dye reduced the concentration of in vitro clonogenic neuroblastoma cells by 25% to 65% (Table 1). Similar observations have been made with one human leukemia cell line, murine erythroid progenitor cells, and quail neural crest cells. The mechanism of this phototoxic effect is unclear. We have some suggestive evidence that dye-independent phototoxicity is at least in part...
part attributable to the small amount of ultraviolet (UV) light emitted by some white light sources. It is also known that daylight fluorescent light can generate toxic photo products in tissue culture medium which can rapidly and irreversibly damage susceptible cells.35

Although CFU-GEMM are primitive progenitor cells, they are probably not identical with the pluripotent hematopoietic stem cell. Our data, therefore, do not prove that the human pluripotent hematopoietic stem cell is photosensitive. Such proof could only be derived from the hematologic reconstitution of patients who have received marrow-litho cytoreductive therapy. However, the striking parallels that exist between the photosensitivities of hematopoietic progenitor cells in mice and humans suggest that the pluripotent hematopoietic stem compartment in human is photosensitive. All classes of progenitor cells that have been tested so far follow the same rank order of sensitivity33,36,38 in mice and humans. Late erythroid progenitors are the most sensitive cells, followed in order of decreasing sensitivity by early erythroid progenitors, granulocyte/macrophage progenitors, and day 12 spleen colony-forming cells (CFU-S12) (in the mouse) and CFU-GEMM (in humans), respectively. In the mouse, the pluripotent stem cell compartment appears to be even less sensitive than CFU-S12,17,39 because marrow grafts that are essentially depleted of CFU-S12 are still capable of rescuing lethally irradiated hosts. By inference, we would therefore expect the human pluripotent hematopoietic stem cell to be at least as photosensitive as CFU-GEMM.

If the high photosensitivity of experiment neuroblastomas can be confirmed in spontaneous tumors, our findings have implications for the purging of autologous remission marrow grafts. Despite our limited understanding of the molecular mechanism of MC 540-mediated photosensitization, it seems reasonable to speculate that the parameters that define a cell's sensitivity to MC 540-mediated photosensitization are quite different from those that define affinity for lectins or antibodies, behavior in density gradients or electrical fields, or susceptibility to most antineoplastic drugs. We, therefore, expect to see little cross-resistance if dye-mediated photosensitization is used in combination with other purging methods. This point may be relevant to the treatment of patients with relapsed neuroblastomas which frequently contain drug-resistant clones.

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REFERENCES


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