Dye-mediated Photosensitization of Murine Neuroblastoma Cells

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ABSTRACT

The purpose of this study was to determine if photosensitization mediated by the fluorescent dye, merocyanine 540, could be used to preferentially kill murine neuroblastoma cells in simulated autologous remission marrow grafts. Simultaneous exposure of Neuro 2a or NB41A3 neuroblastoma cells to merocyanine 540 and white light reduced the concentration of in vitro-clonogenic tumor cells 50,000-fold. By contrast, the same treatment had little effect on the graft's ability to rescue lethally irradiated syngeneic hosts. Lethally irradiated C57BL/6J × A/J F1 mice transplanted with photosensitized mixtures of neuroblastoma cells and normal marrow cells (1:100 or 1:1) survived without developing neuroblastomas. It is conceivable that merocyanine 540-mediated photosensitization will prove useful for the extracorporeal purging of residual neuroblastoma cells from human autologous remission marrow grafts.

INTRODUCTION

Neuroblastoma is the most common solid tumor of early childhood. For patients with advanced (Stage IV) (1) disease, the prognosis is poor. Most of them die within 2 yr of diagnosis (2-4). A response to chemotherapy can be achieved in the majority of cases. However, remissions are usually of short duration, and regrowing tumors frequently fail to respond to additional therapy. In the past few years, several attempts have been made to treat advanced metastatic neuroblastoma with very high (marrow ablative) doses of radio- and chemotherapy followed by a rescue of the patient with an infusion of normal bone marrow (5-13). This approach affords a better chance of eradicating the tumor, because radio- and chemotherapy can be escalated without regard to bone marrow toxicity.

If HLA-identical allogeneic marrow donors are not available, the patient's own cryopreserved remission marrow is sometimes used for grafting, provided there is no evidence of marrow involvement. However, neuroblastomas have a tendency to metastasize to the bone marrow, and there is concern that low numbers of tumor cells may escape detection and inadvertently be reinjected into the patient. Much effort is therefore currently directed at developing methods for the detection and selective destruction of neuroblastoma cells in autologous bone marrow grafts (14-21).

The lipophilic fluorescent dye, MC 540 (22), binds avidly to the plasma membrane of neuronal cells (23-25) and other electrically excitable cells (23). Exposure of MC 540-stained cells to white or green light causes a rapid breakdown of the osmotic properties of the plasma membrane and, eventually, cell death (26). By contrast primitive murine hematopoietic progenitor cells are relatively insensitive to MC 540-mediated photosensitization (27-30), presumably because they have fewer high affinity dye-binding sites.

Since neuroblastoma cells and normal neuronal cells have many plasma membrane properties in common, we speculated that neuroblastoma cells, too, might be highly susceptible to MC 540-mediated photosensitization and thus selectively eliminated from autologous remission marrow grafts. In this paper we present evidence that MC 540-mediated photolysis reduces the concentration of Neuro 2a and NB41A3 neuroblastoma cells 50,000-fold but only minimally diminishes the marrow graft's ability to rescue lethally irradiated syngeneic recipients.

MATERIALS AND METHODS

Animals and Cells. Unless indicated otherwise, female C57BL/6J × A/J F1 (hereafter called B6AF1) mice (6-10 wk old; The Jackson Laboratory, Bar Harbor, ME) were used for marrow transplantation experiments and for the assay of CFU-S2. Female C57BL/6J mice (6-10 wk old) which were used for selected transplantation experiments were obtained from the same supplier. Immunosuppressed animals were housed in sterile polycarbonate cages (≤5 animals per cage) fitted with filter hoods. Autoclaved chow and acidified sterile drinking water were provided ad libitum.

Neuro 2a and NB41A3 neuroblastoma cells (CCL 131 and 147; American Type Culture Collection, Rockville, MD) were cultured in α-medium (KC Biological, Lenexa, KS) supplemented with 10% fetal bovine serum (KC Biological). Both lines were free of Mycoplasma infections. Aliquots of a frozen stock of cells were thawed at regular intervals to prevent the unintentional selection of mutants. Cell cycle parameters have been determined for cultured Neuro 2a cells (33). The generation time is about 10 h; M, G1, S, and G2 phases are 0.5, 2.5, 5, and 2 h, respectively.

The Neuro 2a and the NB41A3 lines are clonal derivatives of the C-1300 neuroblastoma which had originated spontaneously in a mouse of the A strain (34). For the in vivo experiments described in this paper, we used B6AF1 mice. We did so for the following reason. The A strain is very radiosensitive and therefore poorly suited for bone marrow transplantation experiments which require high doses of total-body irradiation. The C-1300 tumor and its derivatives grow also in F1 hybrids of the A strain. We chose hybrids between the A/J and the C57BL/6J strains because these animals are highly resistant to radiation and therefore particularly well suited for bone marrow transplantation experiments. Hybrid strains with C57BL background are less sensitive to C-1300-derived neuroblastomas than the A strain (35). The minimum number of tumor cells required to kill at least 50% of the hosts is between 102 and 103 in the B6AF1 strain as opposed to approximately 105 in the A strain. Median survival times of tumor-bearing animals are also somewhat longer in the B6AF1 strain. Median survival times of 26.5 and 38.5 days, respectively, have been reported for nonirradiated A strain and B6AF1 mice receiving a s.c. inoculum 104-105 C-1300 neuroblastoma cells (34). We determined median survival times of 39 and 55.5 days, respectively, when nonirradiated animals of the same strains were inoculated with Neuro 2a cells (35). Like other investigators (35), we noticed considerable variability from one batch of tumor cells to another with regard to median survival times.

Bioassays. CFU-S2 were assayed as described by Till and McCulloch (36). In brief, suitable dilutions (the size of the inoculum was adjusted to yield approximately 6 colonies per spleen) of bone marrow cells in HEPES-buffered (pH 7.4) α-medium supplemented with 5% fetal bovine serum were injected via lateral tail vein into B6AF1 mice that had received a lethal dose (10.5 Gy) of total-body irradiation from a 125Cs source (Gammacell 40; Atomic Energy of Canada, Ottawa, Canada).
RESULTS AND DISCUSSION

Both the Neuro 2a and the NB41A3 neuroblastoma cells were highly susceptible to MC 540-mediated photosensitization (Fig. 1). After 90 min of illumination with white light in the presence of MC 540 (15 µg/ml) and fetal bovine serum (15%), the number of colony-forming neuroblastoma cells was reduced approximately 50,000-fold. Exposure to MC 540 in the dark or exposure to light in the absence of dye had no effect (Table 1). An increase in the dye concentration or a decrease in the serum concentration accelerated the photosensitization reaction. However, the basic shape of the dose-response curves and the rank order of sensitivity remained unchanged (results not shown). The routinely used combination of MC 540 (15 µg/ml) and 15% fetal bovine serum was chosen because it made cells only moderately photosensitive. This allowed us to safely manipulate stained cells under low levels of ambient light.

Methylcellulose cultures set up with heavily photosensitized neuroblastoma cells contained few or no colonies, numerous small cell fragments, and some large particles that appeared to be single cells. It was of obvious interest to determine if these single cells were dead or merely arrested in the cell cycle. To distinguish between these 2 possibilities, 8-day-old cultures that had been established with heavily (75%) photosensitized Neuro 2a cells were overlayed with 0.2 ml of a 5-µg/ml solution of fluorescein diacetate in phosphate-buffered saline. A few minutes later, bright fluorescence emanated from cells which were part of a colony. What appeared to be isolated single cells remained dim, indicating that these “cells” were unable to hydrolyze fluorescein diacetate and retain the fluorescent product. MC 540-mediated photosensitization thus eventually killed neuroblastoma cells rather than just arresting them in the cell cycle for prolonged periods of time.
If all cells of a given neuroblastoma line had been equally sensitive to MC 540-mediated photosensitization, semilogarithmic plots of survival curves should (after an initial plateau and shoulder) have followed a straight line. However, Fig. 1 shows that the photosensitization of Neuro 2a and, to a lesser degree, also NB41A3 cells followed a biphasic pattern. Linear extrapolations of the survival curves indicated that the less sensitive subpopulation amounted to about 0.5% of the total population in the Neuro 2a line and to about 10% in the NB41A3 line. Whether these less sensitive subpopulations represented genetically distinct clones, different stages of differentiation, or different stages in the cell cycle remains to be determined. It is also conceivable that cell aggregation or photodegradation of the dye contributed to the nonlinearity of the survival curves. It is, however, worth noting that biphasic survival curves are not a universal finding in experiments of this sort. So far we have observed it in only one of 4 human leukemias and 0 of 3 human neuroblastoma cell lines that have been studied in similar detail.

CFU-S_{12} are thought to be closely related to the pluripotent hematopoietic stem cell. CFU-S_{12} were markedly less sensitive to MC 540-mediated photosensitization than Neuro 2a and NB41A3 cells (Fig. 1). The plateau phase (i.e., the minimal duration of illumination required to cause noticeable damage) was longer, and the slope of the curve to the right of the shoulder was flatter. Linear extrapolations of all 3 survival curves (CFU-S_{12}, Neuro 2a and NB41A3) intersected the y-axis in similar locations (6.4, 5, and 15, respectively). This indicated that highly sensitive neuroblastoma cells and comparatively resistant CFU-S_{12} were killed by similar numbers of cytotoxic events. The marked difference in photosensitivity between neuroblastoma and CFU-S_{12} cells was thus most likely attributable to differences in target size. That is, neuroblastoma cells were probably killed more rapidly than CFU-S_{12}, because they bound more MC 540 than CFU-S_{12}. It is conceivable that differences in the efficacy of endogenous repair or protection mechanisms play a role, too. However, so far we have no evidence that such mechanisms exist in hematopoietic stem cells and Neuro 2a and NB41A3 neuroblastoma cells. Elevated levels of intracellular glutathione afford L1210-L-PAM1 leukemia cells a small measure of protection against MC 540-induced photodynamic damages (38). However, it is difficult to imagine how differences in intracellular glutathione could account for the large difference in photosensitivity that occurs between neuroblastoma cells and CFU-S_{12}.

Although the concentration of CFU-S_{12} was reduced 50-fold by the routinely used photosensitization treatment (MC 540, 15 \mu g/ml; serum, 15%; light, 90 min), the graft's ability to rescue lethally irradiated hosts was only moderately impaired. When lethally irradiated B6AF_{1} mice were transplanted with serial dilutions of photosensitized and untreated marrow cells, nonsaturating numbers of photosensitized marrow cells were about 10-fold less effective at rescuing lethally irradiated hosts than equal numbers of untreated marrow cells (Fig. 2). At the routinely used dose of 5 \times 10^{6} cells per mouse, photosensitized and untreated marrow cells were equivalent (Fig. 2; Ref. 30).

The survival of a lethally irradiated animal may depend not only on the infusion of pluripotent hematopoietic stem cells but to some extent also on the infusion of more mature members of the hematopoietic and lymphopoietic lineages and stromal cells. We know that MC 540-mediated photosensitization all but eliminates erythroid, megakaryocyte, and granulocyte/macrophage progenitor cells (28, 29) and depletes subpopulations of stromal cells that participate in the formation of the adherent layer in long-term marrow cultures.\textsuperscript{4} We also know that circulating (mature) blood cells have a low affinity for MC 540 (26). However, we do not yet fully understand how MC 540-mediated photosensitization affects the survival and function of mature members of the hematopoietic and lymphopoietic lineages. It is therefore not possible to accurately assess damage to the stem cell compartment by comparing serial dilutions of normal and photosensitized marrow cells. However, in view of the potential clinical application of the procedure, the comparison yields quite relevant information, and it is probably correct to conclude that the CFU-S_{12} assay overestimates the photosensitivity of the pluripotent stem cell compartment.

The long-term survival of lethally irradiated recipients of photosensitized marrow grafts was attributable to sustained engraftment by donor cells and not to reconstitution by endogenous hematopoietic stem cells. This was demonstrated by injecting photosensitized marrow cells from B6AF_{1} mice (glucosephosphate isomerase genotype: Gpi-1<sup>P</sup> \times Gpi-1<sup>P</sup>) into lethally irradiated C57BL/6J (Gpi-1<sup>P</sup>) mice. Five mo after transplantation, the glucosephosphate isomerase isoenzyme patterns of peripheral blood hemolysates of donors and transplanted hosts were indistinguishable (Fig. 3). A 10% contribution of the hosts to the erythrocyte mass would have been readily detected by the densitometric evaluation of the chromatograms.

Figs. 1 and 2 suggested that the large differential in photosensitivity that existed between normal hematopoietic stem cells and neuroblastoma cells might provide the basis for a selective

\textsuperscript{4} F. Sieber and R. K. Stuart, unpublished observations.

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Figs. 2 and 3 are not included in this text but are referenced in the main body of the text. The figures are likely to be graphs or images illustrating survival curves and chromatograms, respectively.
elimination of residual neuroblastoma cells from autologous remission marrow grafts. This hypothesis was tested in experiments summarized in Fig. 4. Simulated autologous remission marrow grafts were prepared by mixing $5 \times 10^6$ neuroblastoma cells with $5 \times 10^6$ marrow cells from normal B6AF, mice. If this mixture was injected into lethally irradiated syngeneic recipients, the recipients were reconstituted but died later with obvious signs of neuroblastoma. If the same mixture of tumor cells and normal marrow cells was photosensitized prior to injection, the recipients survived without developing neuroblastomas. We have monitored recipients of photosensitized grafts for up to 11 mo without noticing any signs of tumor growth. When animals were given injections of limiting dilutions of Neuro 2a and NB41A3 cells, the latest tumor-related deaths were recorded 4 mo past transplant.

In one experiment the concentration of Neuro 2a cells in the simulated autologous remission marrow graft was increased 10-fold to $5 \times 10^7$ tumor cells per $5 \times 10^6$ normal marrow cells. None of the surviving recipients developed neuroblastomas. These photosensitized grafts almost certainly contained a few viable tumor cells (Fig. 1), but their number was obviously too low to cause tumors in vivo.

The fact that the above experiments were performed with mixtures of bone marrow cells from healthy mice and of freshly trypsinized cultured neuroblastoma cells raises 3 questions. (a) Do pluripotent hematopoietic stem cells in tumor-bearing animals retain their resistance to MC 540-mediated photosensitization or is it reduced, perhaps by a tumor-derived humoral factor? (b) Does trypsinization make cells more photosensitive? (c) Since the composition of cellular lipids is in part under local control (e.g., a function of the lipid composition of the culture medium) and since MC 540 is a lipophilic dye, can the high photosensitivity of neuroblastoma cells be explained by the fact that the tumor cells had been maintained in culture medium?

To answer the first question we inoculated healthy B6AF, mice with $10^6$ Neuro 2a cells. Three wk later, the tumor-related deaths would have occurred), the animals were used as marrow donors. Photosensitized marrow from these tumor-bearing mice was as effective at rescuing lethally irradiated hosts as photosensitized marrow from normal donors. Twenty mice were transplanted with photosensitized marrow from tumor-bearing animals ($5 \times 10^6$ cells per animal). All 20 animals survived and remained free of disease. Mice that were transplanted with nonphotosensitized grafts from tumor-bearing animals did not develop neuroblastomas. At the time of the marrow harvest, the Neuro 2a tumor cells had obviously not yet metastasized to the marrow in large enough numbers to cause tumors in recipient animals. This experiment therefore could not rule out short-range interactions between tumor cells and normal hematopoietic stem cells. However, with L1210 leukemia cells we were able to obtain higher marrow concentrations of tumor cells (30), and in this situation, the photosensitization of the pluripotent stem cell was not affected by its close association with tumor cells.

To answer the second question, we subjected marrow cells to the same trypsinization procedure as neuroblastoma cells prior to the photosensitization step (Table 2). Photosensitized trypsinized marrow grafts were as effective at rescuing lethally irradiated recipients as nontrypsinized grafts. A direct comparison of the photosensitivities of trypsinized and nontrypsinized neuroblastoma cells was not possible because digestion with proteolytic enzymes was essential for the preparation of a single cell suspension from neuroblastoma monolayers.

It is unlikely that the pronounced photosensitivity of neuroblastoma cells was solely a function of their having been maintained in culture. We have shown previously that cultured and freshly explanted L1210 leukemia cells are equally sensitive to MC 540-mediated photosensitization (30) and that cultured melanocytes are impervious to MC 540-mediated photosensitization (25).

The 2 neuroblastoma lines (Neuro 2a and NB41A3) used for this study are 2 of the many clonal derivatives of the C-1300 tumor which has "impeccable credentials" (35) as a neuroblastoma. The many similarities between clinical tumors and C-1300-derived tumors include the morphological and functional heterogeneity that is so typical of clinical neuroblastomas.

It is unclear why Neuro 2a and NB41A3 neuroblastomas are more sensitive to MC 540-mediated photosensitization than normal pluripotent hematopoietic stem cells. It has been hypothesized that the binding of MC 540 is regulated by the electrical properties of the plasma membrane (23, 26) or by the composition or distribution of plasma membrane lipids (39, 40). Our data are compatible with either model.

Table 2 Photosensitivity of trypsinized and untreated marrow cells

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Photosensitization</th>
<th>Survival of lethally irradiated hosts</th>
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<tbody>
<tr>
<td>Saline</td>
<td>None</td>
<td>10/10</td>
</tr>
<tr>
<td>Saline</td>
<td>MC 540, 15 µg/ml, 90 min</td>
<td>10/10</td>
</tr>
<tr>
<td>Tryptsin:EDTA</td>
<td>None</td>
<td>10/10</td>
</tr>
<tr>
<td>Tryptsin:EDTA</td>
<td>MC 540, 15 µg/ml, 90 min</td>
<td>10/10</td>
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Unlike antibodies and lectins which react with proteins and carbohydrates, MC 540 appears to react primarily with the lipid portion of the plasma membrane, and, unlike many drugs, MC 540-mediated photosensitization expresses little if any cell-cycle specificity (30). MC 540-mediated photosensitization is also effective against mutant tumor cell lines that have become multiple-drug resistant (38). Merocyanine 540-mediated photosensitization therefore appears to be a potentially useful addition to combination purging protocols that include antibodies, lectins, drugs, and physical separation methods.

ACKNOWLEDGMENTS

We thank Dr. George W. Santos and Dr. Lyle L. Sensenbrenner for stimulating discussions and Mary Ann Isaacs and Evelyn Connor for technical assistance. Part of this work was presented at the 1985 Meeting of the American Federation for Clinical Research in Washington, DC, May 3–6, 1985, and published in abstract form (41).

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