Purging of Small Cell Lung Cancer Cells from Human Bone Marrow Using Ethiofos (WR-2721) and Light-activated Merocyanine 540 Phototreatment

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ABSTRACT

One limitation of autologous bone marrow transplantation for patients with cancer has been the presence of tumor cells in the bone marrow. Methods to eliminate tumor cells while preserving hematopoietic stem cells have been sought. The present study was performed to analyze the in vitro effectiveness of light-activated merocyanine 540 phototreatment (LAMP) and an aminothiol (ethiofos) as a marrow-purging regimen for small cell lung cancer (SCLC). Two human SCLC cell lines (ATCC HTB-119 and HTB-120) were treated with LAMP and exposed to light for varying periods of time up to 120 min. LAMP reduced SCLC cell proliferation and colony formation in a light exposure-dependent manner; colony formation was not totally inhibited until light exposure of 120 min was used. At this light exposure interval, multipotential hematopoietic progenitors, colony-forming units-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), were substantially reduced. In an attempt to diminish hematopoietic toxicity, SCLC cells were incubated with ethiofos (formerly WR-2721) for 1 hour before LAMP. SCLC colony formation was eliminated at light exposure intervals (90 min or less) which had no inhibitory effect on CFU-GEMM. Ethiofos did not protect CFU-GEMM from LAMP inhibition at 120 min. Ethiofos alone had no effect on the SCLC or hematopoietic cells. When normal bone marrow was contaminated with 1 or 5% SCLC cells, ethiofos plus 60 min of LAMP eliminated SCLC cells but had no effect on CFU-GEMM. The results suggest that ethiofos sensitized SCLC cells to LAMP; thus ethiofos-enhanced LAMP may be an effective method for removing metastatic SCLC cells from bone marrow used for autologous marrow transplantation after high dose chemotherapy.

INTRODUCTION

SCLC accounts for approximately 20 to 25% of new lung cancer cases each year (1). Early detection and surgical intervention are of limited value and increased survival in SCLC is directly related to responsiveness of the tumor to intensive combination chemotherapy (1, 2). The dose-limiting feature of this therapy often is hematopoietic toxicity, which can be obviated by autologous bone marrow transplantation; however, SCLC has a high rate of bone marrow metastasis (3) and, in fact, may be hematopoietic in origin (4). Thus ex vivo pharmacological “purging” may be necessary to eliminate residual tumor cells from marrow grafts for autologous bone marrow transplantation.

An effective purging agent must be capable of either totally eliminating or substantially reducing the number of tumor cells yet must spare sufficient hematopoietic stem cells for bone marrow reconstitution. A potentially useful agent is MC-540, an amphipathic fluorescent dye which binds to the lipid domains of cell plasma membranes (5, 6). Phototoxication of MC-540 with green or white light causes deterioration of normal permeability properties and eventual cell death (7–9), and LAMP has been successfully used to remove murine lymphocytic leukemia L1210 cells, human neuroblastoma, and human leukemic cells from bone marrow (10–12).

The toxic effects of LAMP are not limited to tumor cells, and normal hematopoietic progenitors are reduced after phototreatment (13, 14), although LAMP selectivity can be enhanced by preincubation of cells with serum. The present investigation was undertaken to develop a method to reduce this toxicity using the aminothiol ethiofos (formerly WR-2721). This agent was developed to reduce radiation toxicity; Patt (15) and Bacq et al. (16) showed that aminothiol compounds administered to several mammalian species protected normal tissues from whole body irradiation. Subsequent investigations demonstrated that ethiofos protected normal tissues from systemic chemotherapy as well (17–19). In addition to its protective properties, ethiofos appears to have the ability to sensitize tumor cells to a variety of antiproliferative agents (19, 20), a property of potential utility for marrow purging. The present study was performed to analyze the in vitro effectiveness of LAMP as a marrow-purging regimen for SCLC and to determine the effect(s) of ethiofos on LAMP and normal hematopoiesis.

MATERIALS AND METHODS

Tumor Cell Lines. Each of the human SCLC cell lines used in this study (HTB-119, formerly NCI-H69, and HTB-120, formerly NCI-128; American Type Culture Collection, Rockville, MD) originally were established from pleural effusions of two male patients with confirmed histological diagnosis of SCLC. Both lines express biomarkers which are associated with “classical” SCLC cells and form tumors in nude mice (21). Cell lines were maintained in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) that was supplemented with 10% FBS (Sterile Systems, Logan, UT). Aliquots from frozen tumor cell stock were thawed at regular intervals to prevent unintentional selection of mutants and the cell lines were tested periodically for mycoplasma contamination.

Normal Human Bone Marrow. Bone marrow was obtained from iliac crest aspirates of normal volunteers after written informed consent was obtained in accordance with the Institutional Review Board. Cells were collected into preservative-free heparin (1000 units/ml; Gibco, Grand Island, NY) and then immediately diluted with calcium- and magnesium-free Dulbecco’s phosphate-buffered saline. The mononuclear cells were separated on Percoll gradients of 1.090 g/ml density and were washed twice in Dulbecco’s phosphate-buffered saline. The cells were counted and resuspended in MEM-α that contained 10% FBS.

LAMP Treatment. SCLC and bone marrow cells were incubated with MC-540 according to the method of Meagher et al. (14). Cells were diluted in MEM-α that was supplemented with 15% FBS, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Gibco), at a concentration of 5 × 10⁶ cells/ml, and were suspended in clear polystyrene tubes (Corning, Corning, NY). For each experiment, MC-540 (Sigma Chemical Co., St. Louis, MO) was freshly prepared in 50% ethanol.

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3The abbreviations used are: SCLC, small cell lung cancer; LAMP, light-activated merocyanine-540 phototreatment; MC-540, merocyanine 540; FBS, fetal bovine serum; MEM-α, α minimum essential medium; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte; IMDM, Iscove’s modified Dulbecco’s medium; LTCM, long term bone marrow culture.

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and was added to the cell incubation mixture in a final concentration of 15 μg/ml. To induce photolysis, the cells were placed between two liner arrays of two U-shaped 40 W cool white fluorescent lamps (FB40CW16; Westinghouse Co., Philadelphia, PA) placed 40 cm apart. The cells were mixed continuously on a tube rotator during the photo-exposure period.

After LAMP, the cells were washed twice with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-buffered MEM-α medium that was supplemented with 15% FBS and antibiotics. The cell washings and all subsequent manipulations were carried out under subdued lighting.

Controls consisted of untreated cells, cells incubated with MC-540 but not exposed to the light (dark control), or cells incubated in culture medium containing 7.5 μg/ml ethanol and exposed to light (light control).

Ethiofos Treatment. Ethiofos (Ben Venue Laboratories, Bedford, OH) was obtained from the Pharmacy Research Branch of the National Cancer Institute. Ethiofos treatment followed the method of Valeriote et al. (20). Cells were prepared for LAMP treatment as described above. Before addition of MC-540 and exposure to LAMP, cells were incubated at 37°C with 3.0 mg/ml ethiofos, for 60 min in 5% CO₂. Following the incubation, the cells were washed twice in MEM-α that contained 10% FBS, before LAMP was initiated.

SCLC Suspension Cultures. SCLC cells (1 x 10⁵ cells/ml) were plated in 25-cm² flasks in RPMI 1640 medium supplemented with 10% FBS, 1% l-glutamine, and antibiotics. The cultures were incubated at 37°C in a 5% CO₂-humidified air atmosphere for 3 to 7 days. Cell counts and viability, as shown by trypan blue exclusion were assessed for each group of treated and control cultures and the results were expressed as the mean ± 1 SE of at least three separate experiments.

Clonogenic Tumor Cell Assay. Assay of clonogenic SCLC stem cell progeny was based on a modification of the method of Sieber et al. (11). SCLC cells (5 x 10⁴) were plated into at least 12 wells of a 96-well tissue culture plate, each well containing 0.1 ml of IMDM supplemented with 0.8% methylcellulose, 10 μM monothioglycerol, antibiotics, and 20% FBS. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 7 days, the cultures were terminated using a modification of the method of McMahon and Hankins (22), as follows. Fifty μl of a 1:2 mixture of bovine thrombin (American Dade, Aquada, PR) and citrated bovine plasma (G1BCO) were added to each well tissue culture plate, each well containing 0.1 ml of IMDM supplemented with 0.8% methylcellulose, 10 μM monothioglycerol, antibiotics, and 20% FBS. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 7 days, the cultures were terminated using a modification of the method of McMahon and Hankins (22), as follows. Fifty μl of a 1:2 mixture of bovine thrombin (American Dade, Aquada, PR) and citrated bovine plasma (G1BCO) were added to each microwell and the cultures were incubated overnight. Fibrin clots were removed and flattened on glass slides. They were immediately fixed in 2% glutaraldehyde and stained with hematoxylin. SCLC colonies were identified by characteristic morphology of cell clusters containing at least 20 cells (Fig. 1). The results were expressed as the mean ± SE of four separate experiments.

Hematopoietic Progenitor Cell Assays. Assay of the multipotential hematopoietic progenitors (CFU-GEMM) was based on the method of Fauser and Messner (23). Bone marrow cells (2 x 10⁶) were plated in 96-well tissue culture plates that contained 0.1 ml of IMDM supplemented with 0.8% methylcellulose, 30% FBS, 10 μM monothioglycerol, antibiotics, 3.0 units/ml erythropoietin (TCEpo; AMGen, Thousand Oaks, CA), and 20% conditioned medium from the bladder carcinoma 5637 cell line (provided by Dr. James Loveless, Memorial Sloan-Kettering Cancer Center, Rye, NY). After incubation at 37°C in a 5% CO₂-humidified air atmosphere for 14 days, cultures were terminated as described above for SCLC clonogenic assays, except that colonies were stained with benzidine and hematoxylin (22). CFU-GEMM colonies were identified as large aggregates of >300 cells consisting of a mixture of erythroid, granulocytic, mononcytic/macrophage, and, occasionally, megakaryocytic cells. The results were expressed as the mean ± SE of four separate experiments.

LTMC. Long term marrow cultures were established according to the method of Meagher et al. (24). Mononuclear bone marrow cells (2 x 10⁶) or tumor cell/bone marrow cell mixtures were inoculated into replicate 5-cm² Leighton tubes that contained 2 ml of IMDM supplemented with 5.0 μM hydrocortisone hemisuccinate, 1.0% MEM sodium pyruvate, 1.0% MEM vitamins, 1.0% l-glutamine, 0.8% MEM amino acids, 0.4% MEM nonessential amino acids (GIBCO), antibiotics, and 25% horse serum (Sterile Systems). The cultures were incubated at 33°C in a humidified atmosphere of 5% CO₂. Cultures were fed weekly by removal of 1 ml of growth medium and addition of an equal volume of fresh medium. The cells removed during the weekly feeding were analyzed for the presence of normal hematopoietic progenitors in clonal assays and/or for the presence of contaminating tumor cells in the clonogenic tumor cell assay.

Adherent cell populations were harvested, after removal of the nonadherent cells, by incubation with 2.0 ml of IMDM that contained 0.1% type I collagenase (Sigma) and 20% FBS, according to the method of Coulombel et al. (25). The adherent cell fraction was counted, plated, and analyzed in the clonal assays described for the nonadherent cells.

Statistics. Statistical analysis was performed on total colony formation, comparing the number of colonies from LAMP- and ethiofos/LAMP-treated cultures with colony numbers from untreated controls. Unless otherwise stated, colony counts of 12 replicates from each of four separate experiments (48 determinations) were obtained and the results were expressed as mean ± SE. Statistical significance was determined using Student’s t test and randomized block analysis of variance.

RESULTS

Effect of LAMP on SCLC Cells. A 2–3 log, light-dependent, reduction in the number of LAMP-treated SCLC cells recovered from 7-day suspension cultures was observed when the length of light exposure was increased to 90 min (Table 1). Incubation with MC-540 in the dark had no effect on SCLC growth.

SCLC colony formation also was reduced by LAMP (Table 1). Sixty min of light exposure reduced colony formation by 50% and after 90 min fewer than 5% of control colonies from either cell line were detected; however, total elimination of SCLC colonies required 120 min of light exposure.

Table 1 Effect of LAMP on SCLC

Values represent mean ± SE of four separate experiments, each consisting of 12 replicates.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Day 7 cell counts (X 10⁴)</th>
<th>Cells plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>40 ± 1</td>
<td>109 ± 4</td>
</tr>
<tr>
<td>Dark, 120 min</td>
<td>38 ± 7</td>
<td>129 ± 14</td>
</tr>
<tr>
<td>Light, 120 min</td>
<td>37 ± 8</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>LAMP, 60 min</td>
<td>3 ± 1*</td>
<td>48 ± 3*</td>
</tr>
<tr>
<td>LAMP, 90 min</td>
<td>4 ± 0.03*</td>
<td>0.5 ± 0.2*</td>
</tr>
<tr>
<td>LAMP, 120 min</td>
<td>0*</td>
<td>0*</td>
</tr>
</tbody>
</table>

* Significant difference from control groups, P < 0.001.

a Significant difference from control groups, P < 0.01.
Effect of Ethiofos Pretreatment on LAMP-exposed SCLC Cells. When SCLC cells were incubated with ethiofos followed by LAMP for 90 min, cell proliferation in the 7-day suspension cultures and colony formation in methylcellulose were eliminated (Table 2). Ethiofos treatment alone had no effects on SCLC cell proliferation.

Effect of LAMP on Hematopoietic Progenitors. LAMP had no significant effect on CFU-GEMM at light exposure times up to 90 min; the number of CFU-GEMM were significantly reduced at 120 min (Table 3). The addition of ethiofos to LAMP had no effect on CFU-GEMM at any interval tested (Table 4).

Effect of ethiofos and LAMP on SCLC-contaminated Bone Marrow Colony Formation. When the combined effect of ethiofos and LAMP for 60 min was examined on SCLC-contaminated bone marrow, SCLC colony formation was completely abolished and no toxic effect on bone marrow CFU-GEMM was observed (Table 5). Similar results were obtained when 1% SCLC cells were used instead of 5% SCLC cells (data not shown). Treatment of the SCLC-contaminated bone marrow cells with either ethiofos alone, MC-540 without light activation, or light alone had no effect on either SCLC colony formation or CFU-GEMM progenitors (Table 5).

Effect of LAMP on Normal Hematopoietic and SCLC Cell Proliferation in LTMC. LTMC were established from two bone marrow specimens contaminated with 5% SCLC cells, after LAMP and 90 min of light exposure (Table 6). Nucleated cells containing CFU-GEMM were recovered from the nonadherent portion of LTMC during the entire period of culture. The steep declines observed in the recoverable nonadherent cell numbers from the drug-treated LTMC are similar to results obtained for untreated LTMC. When the LTMC were terminated at week 4, CFU-GEMM were also recovered from the adherent fraction. Clonogenic SCLC cells also were detected after the first week of LTMC and increased markedly by week 2. Analysis was not performed beyond week 2 because LAMP treatment alone did not completely eliminate the SCLC cells from LTMC (Table 6).

Combined Effect of Ethiofos and LAMP on Hematopoietic and SCLC Cell Proliferation in LTMC. When the SCLC-contaminated marrow specimens were treated with ethiofos and LAMP, no significant difference in hematopoietic progenitors was observed in LTMC when compared with LAMP alone; however, in contrast to LAMP, no SCLC progeny were detected in the nonadherent or adherent LTMC fractions during weekly examinations up to 4 weeks (Table 6). In addition, no SCLC were present in LTMC adherent fractions at the fourth week of culture.

DISCUSSION

Addition of ethiofos to LAMP successfully eliminated all detectable SCLC clonogenic cells at light exposure intervals which had no apparent effect on hematopoietic progenitors. No detectable SCLC colonies were found during 4 weeks of LTMC and normal stem cells were preserved, as determined by CFU-GEMM assays. Thus, ethiofos appears to sensitive SCLC cells to cytotoxic therapy, as shown for leukemia (20); in addition to
sensitizing malignant cells to cytotoxic drugs and radiation (17, 18), the aminothiol can augment photolysis as well.

The finding that SCLC cells are susceptible to LAMP extends the number of tumor types which are sensitive to this treatment. The effect was light exposure and time dependent, with complete elimination of tumor cells occurring at 120 min. At this exposure, no differential advantage of tumor cell kill compared to normal hematopoietic cells was apparent, inasmuch as CFU-GEMM were substantially reduced. Light exposure intervals up to 90 min were not associated with detectable hematopoietic toxicity; however, SCLC cells and colonies were still present. Small numbers of tumor cells have been shown to effectively repopulate in a host animal previously cured of the primary tumor (10, 26); these findings suggest that purging agents probably need to totally eliminate tumor cells from marrow grafts to be useful. The proliferative potential of the few residual colonies was apparent in the LTMC experiments, in which SCLC colonies increased from 20 to 210 colonies/5 × 10⁶ cells within 2 weeks of culture (Table 6).

No evidence that ethiofos conferred protection from LAMP on normal hematopoietic cells was obtained. This may be due to failure of ethiofos to be metabolized in culture to a form which has protective activity, except during prolonged incubation (27–29), or may be related to a proposed mechanism of aminothiol protection, repair of DNA damage (27, 30, 31), which should be of no relevance to the LAMP mechanism of membrane damage. LAMP toxicity has been demonstrated to be independent of cell cycle status (32) and is postulated to be due to generation of toxic free radical products (33, 34). The mechanism for ethiofos potentiation of LAMP is unknown. Ethiofos may increase MC-540 binding or uptake by the tumor cells, thus enhancing tumor cell photolysis.

To test the utility of LAMP for eliminating contaminating SCLC cells, an in vitro bone marrow model and well characterized SCLC cell lines were used. Although this represents a simplification of patient bone marrow, where accurate detection of occult tumor cells may be difficult and the effects of prior treatment hard to assess, it was useful for demonstrating that clonogenic tumor cells can be removed from normal bone marrow without the simultaneous elimination of hematopoietic precursors.

Currently a human equivalent to the murine spleen colony assay described by Till and McCulloch (35) is unavailable to measure human pluripotent stem cells. However, the ability to grow CFU-GEMM, which are thought to be closely related developmentally to the pluripotent stem cell, in clonal assays and the ability to recover these marrow precursors from LTMC permit correlations to be made concerning the effects of different purging methods on the preservation of hematopoietic stem cells. Although the number of CFU-GEMM may not accurately represent potential for in vivo bone marrow engraftment, data from Roodman et al. (36) showed a good correlation of CFU-GEMM with myeloid marrow reconstitution. Given that methods to test in vivo potency are not available for human bone marrow and that engraftment delays have been observed after reduction in committed myeloid progenitors (37, 38), methods to prevent clonogenic marrow cell loss seem advisable.

The reductions in weekly recoverable nonadherent cell numbers which occurs in all LTMC probably reflect cell death due to unsatisfactory culture conditions. The observed cell losses could be explained by lack of correct growth factors or inadequate amounts of these factors present in LTMC. However, the fact that relatively stable numbers of CFU-GEMM are still recoverable from LTMC suggests that the cells lost are not critical to maintenance of the cultures.

In conclusion, the ability of intensive combination chemotherapy to improve response rates in SCLC suggests that application of autologous bone marrow transplantation using purged marrow may be useful in treating SCLC. Ethiofos-assisted LAMP fulfills the requirements of a successful purging method by completely eliminating SCLC colony-forming cells and sparing hematopoietic cells associated with marrow reconstitution.

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