Eradication of Small Cell Lung Cancer Cells from Human Bone Marrow with Immunotoxins

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ABSTRACT

The potential of autologous bone marrow transplantation to improve the treatment results for patients with small cell lung cancer (SCLC) may be limited by the presence of tumor cells in the graft. We constructed immunotoxins (ITs) involving 4 monoclonal antibodies and Pseudomonas exotoxin A and investigated the cytotoxicity of the ITs to H-146 SCLC cells in the presence and absence of normal human bone marrow (BM) cells. The Pseudomonas exotoxin A conjugate with the MOC-1 antibody, which recognizes an NCAM antigen, was inactive, as tested in a reproducible soft agar assay. Conjugates involving the monoclonal antibodies MOC-31, NrLu10, and MLuCl killed about 3.5 log tumor cells at 0.1 μg/ml and >5.0 log at 1 μg/ml. In the absence of BM cells, the combination of the 3 ITs was not superior to each IT used individually. However, when H-146 cells were admixed to nucleated BM cells at the ratio of 1:10, >5 log tumor cell kill was obtained at a concentration as low as 0.1 μg/ml of each IT. Survival of normal BM progenitor cells was only moderately reduced by the IT treatment, even in experiments in which the 3 ITs were used at 2.5 μg/ml each. Freezing and thawing of the BM, as required in a clinical setting, reduced the colony-forming unit, granulocyte-macrophage, and colony-forming unit, granulocyte-erythroid-macrophage-megakaryocyte, by 30–60% in both treated and untreated cultures. We conclude that the use of a mixture of the 3 ITs provides a safe, rapid, and effective method for eradicating SCLC cells from BM used for autologous bone marrow transplantation following high-dose chemotherapy.

INTRODUCTION

Small cell lung carcinoma represents 20–25% of all primary malignancies of the lung. In this disease, a high initial response rate to chemotherapy is associated with a high recurrence rate, and long-term survivors are rare (1). Assuming a dose-response relationship, several groups (2–6) have investigated the effect of high-dose chemotherapy with ABMT.3 Since SCLC frequently metastasizes to the BM (7), however, there is a risk that the transfused BM may be contaminated with tumor cells, even in patients in whom the BM by conventional diagnostic methods is found to be negative (8–12). To avoid the possibility of reintroducing malignant cells into patients undergoing ABMT, measures should be taken to eliminate such cells before reinfusing the marrow.

Immunological, physical, and chemical procedures for purging BM of SCLC cells have previously been reported (13–21). We recently developed an immunomagnetic purging procedure (22), and in the present study we describe evaluation of ITs for their efficacy and specificity in killing SCLC cells in human BM. ITs consist of a tumor-selective ligand, usually a MAb, conjugated to a toxin. Such conjugates have been used successfully in allogenic BM transplantation for preventing graft versus host disease (23–25) and in patients with hematological malignancies for eliminating tumor cells from the BM in conjunction with ABMT and high-dose chemotherapy (26–28).

The ITs used were constructed as conjugates of Pseudomonas exotoxin A, and previously selected (29) anti-SCLC antibodies. This toxin was chosen partly because of its low toxicity to normal hematopoietic cells. Three of the 4 MAB-PE conjugates were highly toxic to H-146 SCLC cells at concentrations which did not significantly affect BM progenitor cells, and the use of these 3 ITs in combination is recommended for use in a clinical setting.

MATERIALS AND METHODS

Bone Marrow. Human BM aspirates were obtained from healthy volunteers or from patients at the Norwegian Radium Hospital with non-SCLC malignancies who were free of tumor cells in their marrow. All samples were obtained with informed consent from the donors. Ten ml of BM were layered on Lymphoprep (Nycomed, Oslo, Norway), and the mononuclear cell fraction obtained was suspended in RPMI 1640 medium (GIBCO, Paisley, United Kingdom) supplemented with 10% FCS.

Antibodies and Toxin. MOC-1 (31) and MOC-31 (32) monoclonal antibodies were gifts from Dr. L. de Leij (Groningen, the Netherlands). MOC-1 (IgG1) binds to the neural cell adhesion molecule (CD56/NCAM) (33), and MOC-31 (IgG2a) binds to an epitheial antigen (cluster 2). NrLu10, an IgG2b antibody recognizing a 39-kDa antigen (34), was supplied by NeoRx Corporation (Seattle, WA). MLuCl, kindly provided by Dr. Sylvie Menard (Milan, Italy), is an IgG1 antibody that binds to a saccharide epitope carried by neutral glycolipids, glycoproteins, and mucins (35). PE was obtained from Swiss Serum and Vaccine Institute (Bern, Switzerland).

Preparation of ITs. Each of the antibodies was conjugated to PE via a thioether bond formed with the reagent sulfo-SMCC (Pierce, Rockford, IL) as described previously (36), except that, after incubation of reduced MAb and MCC-PE overnight at 4°C, an equal volume of saturated ammonium sulfate was added, and the preparation was further incubated for 1 h at 4°C. The formed precipitate was collected by centrifugation at 10,000 × g for 10 min and dissolved in 1 ml of PBS and subjected to gel filtration as described earlier (37). Fractions containing purified conjugate as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (38) were pooled and used in the experiments. Protein concentration was determined by a Bio-Rad protein assay using bovine γ-globulin as the standard.

Treatment Procedure. Mechanically dispersed tumor cells (2 × 106), mononuclear BM cells (2 × 106), or a mixture of both (total, 5 × 106) were incubated for 2 h at 37°C with one or more ITs in RPMI/FCS supplemented with 100 units/ml of penicillin and 100 μl/ml of streptomycin. The cells were washed twice in PBS with 1% FCS before seeded out in the appropriate clonogenic assays. In some experiments, treated and untreated cells were frozen under controlled conditions down to −130°C in RPMI containing 20% FCS, 10% dimethyl sulfoxide (CryoServ; Tera Pharmaceuticals Inc., Buena Park, CA), and antibiotics. After 1–2 weeks, the frozen cells were thawed in a 37°C water bath, and the cells were seeded out in the GM and GEMM assays.

Colonizing-Assays For Tumor and BM Progenitor Cells. The number of colony-forming tumor cells was assessed in a soft agar assay (39), as previously described (40). Briefly, soft agar cultures were set up in triplicates in 10-ml tubes by adding 0.2 ml of August rat blood diluted 1:8, 0.2 ml of appropriately diluted BM/tumor cell suspensions, and 0.6 ml of 0.5% agar (DIFCO Laboratories, Detroit, MI). The tubes were incubated at 37°C in 5%
O₂, 5% CO₂, and 90% N₂, and after 21 days of incubation, colonies of >50 cells were counted using a Zeiss stereomicroscope.

The viable clonogenic progenitor cells in the BM after cytotoxic treatment were assessed by a modified version (41) of the method described by Burgess et al. (42). Mononuclear BM cells were suspended to a concentration of 2 × 10⁵ cells/ml of McCoy’s 5A medium (GIBCO) containing 0.3% agar, 15% FCS, 20 ng/ml of GM-CSF (kindly provided by Schering Plough, NJ), and antibiotics. Triplicate 1-ml aliquots were cultured in 35-mm plastic dishes at 37°C in 5% O₂, 5% CO₂, and 90% N₂. After 14 days of incubation, colonies of >40 cells were counted.

In the GEMM assay (43), 2 × 10⁵ mononuclear cells were suspended in 1 ml of Iscove’s modified Dulbecco’s medium (GIBCO) containing 0.8% methylcellulose, 10% FCS, 10% leukocyte-conditioned medium, 30% human plasma, 5 × 10⁻⁴ m 2-mercaptoethanol, and 1 unit of erythropoietin (Cilag Ltd., Schaffhausen, Switzerland). Triplicate 1-ml aliquots were cultured, and colonies were counted as for the GM assay.

RESULTS

Cloning Efficiency of SCLC Cells

When different numbers of H-146 cells were seeded out in soft agar, the number of colonies formed was proportional to the number of cells plated (22). This relationship was close to linear down to 10 cells plated per tube (not shown), and the plating efficiency calculated from the slope of the curve was approximately 40% in 3 independent experiments. Similar curves were established in each experiment, and the curves were used to calculate the tumor cell depletion obtained by the treatment, taking into account the plating efficiency in each experiment. The efficacy of the treatment is given as the logarithm of the number of tumor cells removed.

Tumor Cell Elimination

Efficacy of Individual ITs in Killing SCLC Cells. The conjugates involving the MOC-1 antibody were rather ineffective, with a mean log tumor cell kill of only 0.9 (Table 1). Each of the other 3 conjugates showed, however, high activity against the H-146 tumor cells, with a clear dose-response relationship. Thus, the log tumor cell kill at 0.01 μg/ml of the ITs ranged from 0.6–1.1 and increased to about 3.3 log at 0.1 μg/ml, with up to 5.0 log kill in some experiments. At this concentration, however, the variation in results obtained in individual experiments was quite large. When the IT concentration was increased to 1.0 μg/ml, >5.0 log tumor cell kill was reproducibly obtained with all 3 conjugates.

Efficacy of a Mixture of Three ITs in Killing SCLC Cells. Tumor-associated antigens commonly are heterogeneously expressed on the surface of tumor cells. The activity of a mixture of MOC-31-PE + NrLu10-PE + MLuCl-PE was, therefore, tested, but no significant increase in efficacy was obtained with the combination compared to the effect of the individual conjugates (Table 1).

The experiments described above were performed in the absence of BM cells because of the limited availability of fresh human BM. In the next set of experiments, H-146 cells were mixed with nucleated BM cells at a ratio of 1:10. The binding profile of the respective antibodies to SCLC (29) suggests an advantage in using a panel of conjugates, and, therefore, only the combination of 3 ITs was tested in the mixture of tumor and BM cells. As shown in Table 2, >5.0 log tumor cell kill was obtained with this combination, at a concentration as low as 0.1 μg/ml of each IT. The results show that the presence of human BM does not lower, and possibly may increase, the efficacy of the combination of ITs in killing tumor cells.

Survival of Progenitor BM Cells

Survival of CFU-GM and CFU-GEMM Progenitor BM Cells. Survival of CFU-GM cells was assessed at four different concentrations of the 3 active ITs, in the range of 1.0–10.0 μg/ml. As demonstrated in Table 3, MOC-31-PE and NrLu10-PE showed only minor toxicity to these progenitor cells, except that the highest concentration of MOC-31-PE reduced the fraction of colony-forming units to 28% of that of the control. MLuCl-PE was somewhat more toxic. However, even at a concentration of 5 μg/ml, approximately 50% of the CFUs survived.

The mixture of the 3 immunotoxins was tested at 2 concentrations, both on CFU-GM and CFU-GEMM (Table 4). At 1.0 μg/ml, only a slight reduction in CFU-GM was seen, whereas at 2.5 μg/ml (total IT concentration 7.5 μg/ml), the recovery of colony-forming progenitor cells was 39% (range 23–55%). The combination had almost no effect on the survival of the CFU-GEMM cells, even at the highest concentration tested.

The results demonstrate that the ITs at concentrations that could eradicate the tumor cells from BM had only insignificant toxicity to the normal progenitor cells, thus providing a promising therapeutic window.

Effect of Freezing and Thawing of the Treated BM Cells. In a clinical ABMT procedure, it is necessary to freeze the BM until it is later given back to the patient. To test the effect of this procedure, BM was treated with a mixture of the 3 ITs, each at a concentration of 1.0 μg/ml, and frozen under controlled conditions down to −130°C. After being stored for 1–3 weeks, the nucleated BM cells were thawed and seeded out in the 2 progenitor cell assays. The survival of the CFUs in such treated BM was compared with that of untreated cells directly and after undergoing the freezing and thawing procedure. At the concentrations used, the ITs did not alter the survival of the progenitor cells (Table 5). However, the freezing procedure itself reduced the clonogenic cells to 40–45% of the control values in the CFU-GM assay and to 60–70% in the CFU-GEMM assay. The results suggest that the IT procedure can safely be used in a clinical setting.

Table 1. Efficacy of 4 immunotoxins involving Pseudomonas exotoxin A in killing H-146 human small cell lung cancer cells

<table>
<thead>
<tr>
<th>Immunotoxins</th>
<th>0.01 μg/ml</th>
<th>0.1 μg/ml</th>
<th>1.0 μg/ml</th>
</tr>
</thead>
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<tr>
<td></td>
<td>No. of experiments</td>
<td>Meanᵃ</td>
<td>Range</td>
</tr>
<tr>
<td>MOC-1-PE</td>
<td>ntᵃ</td>
<td>1.1</td>
<td>0.2–2.2</td>
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<tr>
<td>MOC-31-PE</td>
<td>3</td>
<td>0.6</td>
<td>0.6–0.7</td>
</tr>
<tr>
<td>NrLu10-PE</td>
<td>3</td>
<td>1.0</td>
<td>0.4–1.9</td>
</tr>
<tr>
<td>MLuCl-PE</td>
<td>1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>MOC-31-PE + NrLu10-PE</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ MLuCl-PE</td>
<td>4</td>
<td>1.0</td>
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</table>

ᵃ Calculated from observed number of colonies, taking into account the plating efficiency, and determined as the logarithm of the number of cells killed by the treatment.
ᵇ Mean of the results obtained in independent soft-agar experiments, each performed in triplicate.
ᶜ Not tested.
ᵈ Each immunotoxin used at the concentration indicated.
with bead (18) reported 4–5 log cell separation. In a recent study (22) with MOC-31-PE + NrLulO-PE + MLuC1-PE concentration (µg/ml) 1.0 2.5 5.0 10.0 1.0 2.5 5.0 10.0 1.0 2.5 5.0 10.0 1.0 2.5 5.0 10.0

<table>
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<tr>
<th>Concentration (µg/ml)</th>
<th>Mean of the results obtained from 2-4 independent experiments, each performed in triplicate.</th>
<th>%</th>
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<tr>
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<td>64</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
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</tr>
<tr>
<td></td>
<td>10.0</td>
<td>23</td>
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<tr>
<td>MLuC1-PE</td>
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Each immunotoxin used at the concentration indicated.

Fractions of remaining colonies calculated relative to the number of colonies in untreated control cultures.

**DISCUSSION**

Small cell lung cancer is one of the solid tumor types known to be sensitive to chemotherapy. Unfortunately, in most cases the duration of the obtained remissions is short, and only 5–10% of the patients are cured (1). With the aim of obtaining more long-lasting remissions, and of the obtained remissions is short, and only 5–10% of the patients are cured (1). With the aim of obtaining more long-lasting remissions, and of increasing the survival of the patients, high-dose chemotherapy combined with ABMT has been suggested (2–6). In this clinical setting, the toxicology of PE in animals is about 1/10 of that of the toxin ricin, which in a clinical study was shown by others (49) to be advantageous. In addition, PE is particularly useful for preparation of ITs to be used for BM purging.

Several procedures designed to eliminate SCLC cells from BM have been reported (13–18, 21), some of which involve the use of MAbs, either with complement (14, 15, 19) or with immunomagnetic beads (18, 21). With the latter approach, Elias et al. (21) obtained approximately 2.5 log tumor cell removal, whereas Vredenburgh and Ball (18) reported 4–5 log cell separation. In a recent study (22) with 4 MAbs and immunobeads, we reached a maximal purging efficiency in excess of that of the latter group. We have now developed an alternative, very effective immunotoxin approach involving 3 of the 4 MAbs that were used in the immunomagnetic procedure. Individually, each of these immunotoxins produced approximately 3.5 log tumor cell removal at a concentration of 0.1 µg/ml and >5 log at 1.0 µg/ml (Table 3). It should be noted that with immunotoxins, 5 log removal represents the limit of sensitivity of the assay.

With the latter approach, Elias et al. (21) obtained approximately 3.5 log tumor cell removal at a concentration of 0.1 µg/ml and >5 log at 1.0 µg/ml. It should be noted that with immunotoxins, 5 log removal represents the limit of sensitivity of the assay. In experiments in which the tumor cells were admixed to normal BM cells, the combination of the 3 ITs eradicated all of the tumor cells at IT concentrations as low as 0.1 µg/ml of each. In comparison, the toxicology to CFU-GM and CFU-GEMM progenitor cells was low, even at 25- to 100-fold higher concentrations (Tables 3 and 4). It is essential that the freezing and thawing of the treated marrow required in a clinical setting should not reveal unacceptable toxicity to normal progenitor cells. When this possibility was examined, we found that the freezing and thawing procedure by itself reduced the number of CFU-GMs and CFU-GEMMs to about 50% of that in control cultures, but the treatment with ITs caused no additional toxicity (Table 5). The presence of active IT remaining in the BM after washing was estimated by determining, in a standard [3H]leucine incorporation assay, the cytotoxic activity in the supernatant of BM cells incubated at 37°C for 1 h. We calculated that approximately 0.75% of the total amount of IT added was still present (not shown). In a clinical setting, this corresponds to 3–5 µg of IT (~1–1.7 µg of free PE) in the BM suspension to be given back to the patient. The toxicity of PE in animals is about 1/10 of that of the toxin ricin, which in a clinical phase 1 trial was well tolerated in doses up to 35 µg/70 kg (47). Clearly, no systemic toxicity would be expected when BM containing <2 µg of PE was reinfused. Altogether, it can be concluded that the immunotoxin approach can safely and effectively be used for purging SCLC cells from human BM.

One advantage of the IT method is its simplicity and rapidity. Thus, after preparation of mononuclear cells, a 2-h incubation and 2 cell washings are the only steps required for purging. In comparison, approximately 5 h are needed for performing the immunomagnetic procedure, which involves an additional 2 cycles of incubation and purging with beads (48). The time saved by using the IT approach is of practical importance, considering the time required for harvesting the BM, preparing mononuclear cells, and freezing of the purged marrow.

Evidently, the activity and specificity of immunotoxins are dependent on properties related to the MAbs used. Other important factors include the choice of toxins and the method by which they are linked to the MAbs (36). The conjugation procedure used here has been shown by others (49) and by us (36) to be advantageous. In addition, PE is particularly useful for preparation of ITs to be used for BM purging. Thus, the toxin itself shows a low toxicity to BM cells (not shown), and together with the specificity mediated by the antibody moiety of the IT, useful therapeutic windows may be obtained for BM-purging purposes.

When choosing MAbs to be used in the clinical setting, it is important to consider the heterogenic expression of tumor-associated antigens on the surface of tumor cells of patients. The antibodies used here were previously selected for their binding to SCLC cell lines and

<table>
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<th>CFU-GM</th>
<th>CFU-GEMM</th>
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<tr>
<td></td>
<td>No. of colonies (mean)</td>
<td>%</td>
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<tr>
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<td>1.0</td>
<td>126</td>
</tr>
<tr>
<td></td>
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<td>MOC-31-PE + NrLulO-PE + MLuC1-PE</td>
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<th>Immunotoxins</th>
<th>CFU-GM</th>
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for their lack of cross-reactivity with normal BM cells (29). Moreover, studies of BM aspirates from SCLC patients had shown that the corresponding antigens were expressed on most tumor cells in nearly all cases studied (8). Conjugates involving MOC-1 proved ineffective, probably because the NCAM antigen to which MOC-1 binds (33) is not adequately internalized from the cell surface. With regard to the antibodies used in the active conjugates, we had indications that the NrLu10 and MOC-31 recognize the same antigen. This was examined in cell-binding studies and by immunoprecipitation of cell membrane lysates, and evidence was obtained that the MAb binds to the same antigen but to different epitopes (not shown). The previously reported binding profiles of these antibodies (29), as well as unpublished data, indicate differential expression of the 2 epitopes, suggesting an advantage of using both MAbs in combination. Since MLuCl binds to a different antigen, we recommend that PE conjugates of all 3 MAbs be used in combination to ensure effective tumor cell kill.

No general agreement exists concerning whether ABMT, with or without purging, can improve the current dismal prognosis of SCLC patients. However, recent results (50) suggest that carefully selected subgroups of such patients may benefit from high-dose therapy and ABMT. Moreover, since there is a risk that tumor cells may be present in the marrow to be transfused, measures should be taken to remove or kill such cells. The rapid, effective, and safe IT-irradiation procedure described here seems well suited for this purpose.

REFERENCES

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