Elimination of Small Cell Lung Cancer Cells in Vitro from Human Bone Marrow by A Monoclonal Antibody

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ABSTRACT

We report here a useful method for elimination of small cell lung cancer cells in vitro from bone marrow. A monoclonal antibody, TFS-2, which mediates complement lysis and recognizes an antigen present on small cell lung cancer cells but not lymphoid cells or bone marrow cells, was used to clear infiltrated bone marrow. The antibody in the presence of complement effectively killed tumor cells, but it was not cytotoxic to bone marrow cells. When mixed populations consisting of tumor cells and bone marrow cells were treated with antibody and complement, the tumor cells were also effectively killed, except when large numbers of bone marrow cells were present, whereas TFS-2 had no significant effect on bone marrow stem cells, as judged by colony-forming unit assays.

INTRODUCTION

The therapeutic value of many cytotoxic drugs is limited by their toxicity to hematopoietic stem cells in the bone marrow. Much larger doses can be given safely if the marrow is harvested before administration of the cytotoxic agents and then rein infused once the drug has been excreted or metabolized. This technique of autologous bone marrow rescue has been used to allow administration of high doses of melphalan, cyclophosphamide, carmustine, and etoposide in leukemia and lymphoma (2, 7).

SCCL2 is one of the most malignant neoplasms. This tumor, however, has the greatest sensitivity among lung tumors to chemotherapy and irradiation (1). A number of attempts have been made to use high-dose chemoradiotherapy in SCCL followed by autologous bone marrow rescue (8, 9). Although the results of these trials are still controversial, autologous bone marrow rescue is one of the possible approaches to high-dose chemoradiotherapy in SCCL. Unfortunately, this approach is limited by the finding that SCCL frequently involves the bone marrow, making tumor cell contamination (3, 5) a major obstacle to successful autotransplantation. In order to circumvent this problem, we have developed cytotoxic monoclonal antibodies to SCCL (6). This study is designed to see how we could eliminate SCCL tumor cells from bone marrow with the newly developed monoclonal antibodies.

MATERIALS AND METHODS

Antigen Distribution

TFS-2 is a cytotoxic IgG2b antibody prepared by hybridoma techniques against human lung cancer cells as described recently (6). This antibody recognized a Mr 39,000 surface protein which is present on small-cell carcinomas and other cancer cells, as assessed on intact cells by radioimmunoassays and by immunofluorescence of cell suspensions (Table 1). In the bone marrow or spleen, lymphocytes or stem cells did not appear to express the antigens.

Antibody Treatment

Ascites fluids containing high-titered monoclonal antibody were produced by injecting hybrid cells i.p. into BALB/c mice primed with tetramethylpentadecane (Wako Pure Chemical Industries Ltd., Osaka, Japan). A large amount of monoclonal antibody was purified from ascites fluids by ammonium sulfate precipitation and DEAE-cellulose chromatography using DE52-cellulose column (Whatman Chemical Separation, Ltd., Maidstone, Kent, England) (6).

Cells (5 x 10^6) were labeled with 0.1 ml of sodium ^{32}Cr;chromate (1 mCi/ml; New England Nuclear) for 1 h at 37 °C. The cells were washed twice and brought to a final concentration of 6 x 10^6 cells/ml in RPMI Medium 1640 containing 5% fetal bovine serum; 3 x 10^6 radioabeled target cells (50 μl) were incubated with various dilutions of the monoclonal antibody (50 μl) on ice for 30 min followed by addition of rabbit complement (50 μl, 1:3 dilution; Rockland) and further incubation at 37°C in a humidified atmosphere of 5% CO2 in air for 2 h. To elude nonspecific lysis, rabbit complement absorbed 3 times against many cell lines was used. Percentage of cytotoxicity was determined by:

% of cytotoxicity = Experimental release - spontaneous release
Total releasable counts - spontaneous release

To assess the exact percentage of cell lysis, trypan blue dye exclusion tests were also used. Briefly, 3 x 10^4 target cells (50 μl) were incubated with 50 μl of antibody in serial dilutions and 50 μl of rabbit complement as described above, and the surviving cells were counted following the addition of 150 μl of 0.5% trypan blue dye solution.

Growth Inhibition Tests

To measure growth-inhibitory effects of immune cytolysis, survival of cells in the exponential phase of growth was assayed by the soft-agar cloning technique. Briefly, the cells treated with the antibody and complement were plated in RPMI Medium 1640 containing 20% FCS and 0.3% Bacto-agar on a lower layer of 0.5% agar medium with 20% FCS. Initial cell inoculum was 9 x 10^3/dish. The dishes were incubated at 37°C in a CO2 chamber for 2 weeks, and the number of colonies was counted under an inverted microscope.

Bone Marrow Stem Cell Assays

CFU-E and BFU-E Assays. Mononuclear cells were isolated from human bone marrow by Ficoll-Hypaque centrifugation. The cells (2 x 10^6) were plated in 35-mm Falcon dishes containing a 1.0-ml mixture of Iscove's modified Dulbecco medium (Grand Island Biological Co., Grand Island, NY), 0.8% methylcellulose, 30% FCS in the presence of 5 x 10^-6 M 2-mercaptoethanol, and 1 unit of a Step III preparation of sheep plasma erythropeptin (Connaught Laboratories, Ltd., Willowdale, Ontario, Canada). Dishes were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Colonies were scored with an inverted microscope after 7 (CFU-E) or 14 (BFU-E) days of incubation (10).
REMOVAL OF LUNG CANCER CELLS FROM BONE MARROW

CFU-C Assay. Bone marrow mononuclear cells (1 x 10^5) were plated in 1.0 ml of 0.3% agar culture medium that included McCoy's Medium 5A supplied with additional essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate, and contained 10% FCS. Cultures were incubated at 37°C in air and scored for colonies after 10 days of incubation. Colony morphology was assessed on whole plates as described previously (4).

RESULTS

Cytotoxicity Tests for SCCL Cells. SCCL cells were treated with TFS-2 antibody and complement. As shown in Chart 1, the cells showed a high sensitivity to immune cytolysis. The titer for 50% cytolysis was about IgG at 5 ng/ml. No cytotoxicity was shown to the other cells that did not express the antigen. To assess the exact number of viable cells after exposure to antibody and complement, cell viability was determined by trypan blue dye exclusion tests. It was also determined whether repeated incubations with the antibody plus complement lysed more target cells. SCCL cells were treated one, 2, and 3 times. A single incubation (1 h) with TFS-2 and complement lysed more than 70% of SCCL cells, while 3 incubations with TFS-2 and complement lysed more than 95% of SCCL cells (Chart 2).

Time Response of SCCL Cells to Immune Cytolysis. The incubation time required for killing SCCL cells was examined. Chart 3 shows the relationship between incubation time and immune cytolysis. The antibody concentration of more than 50 ng/ml showed maximal killing of target cells within 30 min. However, with lower doses of antibody, longer treatment was required to achieve maximal killing. Exposure to lower than 0.5 ng/ml of antibody could not attain maximal killing even with 180-min treatment.

Effect of Cell Density on Immune Cytolysis. Cytotoxic titration of antibody was performed against different densities of SCCL cells at a fixed 120-min treatment time in the same manner. Chart 4 shows that the extent of cytolysis depends upon cell density and concentration of antibody, i.e., the more dense the population, the more antibody is needed for immune cytolysis. Even with antibody at 10 ng/ml, cell suspensions containing more than 10^6 cells/well/150 μl were not efficiently treated. The results suggest that a large amount of antibody is necessary to obtain maximal killing in dense cell populations.

Growth Inhibition Tests. SCCL cells (T3M-11) were treated with serially diluted antibody and complement, and they were seeded at the density of 9 x 10^3 cells/dish (60 x 15 mm) in 0.3% agar culture medium. The dishes were incubated at 37°C for 14 days, and survival was determined by counting the colonies developed in the dishes. The colony-forming efficiency was 2.8% in untreated cultures. Chart 5 shows the relationship between

Table 1

<table>
<thead>
<tr>
<th>Antigen present</th>
<th>Lung cancer (various cell types)</th>
<th>Stomach carcinoma</th>
<th>Colon carcinoma</th>
<th>Pancreas carcinoma</th>
<th>Choriocarcinoma</th>
<th>Cervical carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen absent</td>
<td>Hematopoietic cell lines</td>
<td>Lymphoblastoid cell lines</td>
<td>Melanoma</td>
<td>Neuroblastoma</td>
<td>Skin fibroblasts</td>
<td>Lung fibroblasts</td>
</tr>
</tbody>
</table>


**Cr-labeled target cells were incubated with serially diluted TFS-2 (O), or normal mouse IgG (A) on ice for 30 min followed by further incubation with rabbit complement at 37°C for 2 h.** SCCL cells (TNSC-1, TNSC-2, T3M-11), lymphoblastoid cells (Namalva), myeloid cells (K-562), peripheral blood mononuclear cells (PBMC). Each point is the mean of 2 determinations.

Chart 2. Repeated treatments of target cells with TFS-2. Target cells were incubated with TFS-2 (3.3 μg/ml) on ice for 30 min followed by further incubation with rabbit complement for 1 h. The procedure was repeated 2 or 3 times. Surviving cells were counted by trypan blue dye exclusion tests. O, TNSC-1; ●, T3M-11; △, NCI-H69. Each point is the average value of 2 determinations.
Removal of Lung Cancer Cells from Bone Marrow

Chart 3. Time response of T3M-11 SCCL cells exposed to various concentrations of TFS-2. O, normal mouse IgG; □, TFS-2. The results are the average of duplicate determinations.

Chart 4. Cytotoxic titration of TFS-2 against different densities of T3M-11 SCCL cells. O, 0.5 ng TFS-2/ml; x, 50 ng/ml; △, 500 ng/ml. Each point is the mean value of 2 determinations.

Chart 5. Inhibition of colony formation in T3M-11 cells. Each point is the average value of 2 determinations.

Chart 6. Cytotoxic effect of TFS-2 in the presence of bone marrow cells. 51Cr-labeled T3M-11 cells were treated with TFS-2 (3.3 μg/ml) plus complement in the presence of 1 x 10^5 (△) or 1 x 10^6 bone marrow cells/well (15 μl) (□). □, control.

The results suggest that SCCL cells which may infiltrate into bone marrow are effectively eliminated when the bone marrow cells are treated at the lower density.

Effect on Bone Marrow Stem Cells. It is important to ascertain whether the therapy which killed tumor cells spared the bone marrow cells, thereby enabling potential in vivo recovery. Bone marrow cells did not show reactivity with the antibody (Chart 7). Bone marrow mononuclear cells were treated with the antibody and complement ("Materials and Methods"). After antibody treatment, bone marrow stem cells were assayed by the standard CFU-C, CFU-E, and BFU-E methods, which measure progenitor cells committed to the granulocyte-macrophage, and...
poses that the antigenic specificity of the antibody spares bone marrow stem cells that will reconstitute the patient. Despite extensive studies on high-dose chemotherapy of SCCL with autologous marrow rescue (8, 9), recurrence of the cancer is common, which may be due to reinfusion of tumor cells that had infiltrated into marrow before treatment, or due to residual tumor cells. Until now, there have been no cytotoxic monoclonal antibodies to SCCL (6). In this study, we have demonstrated that TFS-2 mediates complement-mediated lysis of SCCL cells in bone marrow both effectively and safely. Treatment of SCCL cells with antibody plus complement lysed the target cells but failed to affect colony growth of bone marrow-derived CFU-C, CFU-E, or BFU-E (Table 2). It has been shown that the target cells were effectively killed at the density of lower than $2 \times 10^6$ cells/ml with the antibody. At much higher densities, they were not efficiently killed. It has been shown that the coexistence of large number of bone marrow cells (more than $6.7 \times 10^7$ cells/ml) also significantly affects the lysis of target cells. It is therefore recommended that bone marrow cells, which may be infiltrated with SCCL cells, should be treated at the density of less than $2 \times 10^6$ cells/ml, in order to achieve maximal killing of the tumor cells. In these conditions, TFS-2 at only 50 ng/ml was enough to achieve maximal lysis.

This antibody will serve as a powerful potential tool for the in vitro elimination of SCCL cells from bone marrow, thus facilitating high-dose chemotherapy of SCCL with autologous bone marrow rescue.

REFERENCES

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