Elimination of Small Cell Carcinoma of the Lung from Human Bone Marrow by Monoclonal Antibodies and Immunomagnetic Beads

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ABSTRACT

The majority of patients with small cell carcinoma of the lung (SCCL) have bone marrow involvement detected by monoclonal antibodies (mAb). High dose chemotherapy followed by autologous bone marrow transplantation may improve treatment results for patients with SCCL, but the bone marrow may need to be purged of contaminating tumor cells. This study investigates the reactivity of a panel of mAb with two SCCL cell lines and normal bone marrow and the ability of the mAb and immunomagnetic beads to eliminate the SCCL cells from a mixture of 90% normal bone marrow cells and 10% SCCL cells. The mAb and immunomagnetic beads removed 4 to 5 log of SCCL cells in the model system. The immunomagnetic separation did not significantly adversely affect normal hematopoietic progenitor cells, as determined by bone marrow colony-forming units. The results suggest that the mAb and immunomagnetic beads could safely and effectively separate SCCL cells from the bone marrow for autologous bone marrow transplantation following high dose chemotherapy.

INTRODUCTION

SCCL comprises 25–30% of all lung cancers (1). Over 40,000 cases of SCCL were diagnosed and over 35,000 people died of SCCL in the United States last year (2). The majority of patients with SCCL respond to chemotherapy, but the responses are of short duration. The median survival for patients with limited SCCL is 15 months and it is only 9 months for patients with extensive SCCL (3, 4). Many investigators have attempted to improve the treatment results for SCCL by administering intensive chemotherapy followed by ABMT (5–8). However, none of the studies reported any improvements compared to standard chemotherapy. The series are small and many of the patients were poor-risk. Thus, the role of chemotherapy and ABMT for SCCL remains unclear.

Despite a histologically normal bone marrow, mAb can detect contaminating SCCL cells in up to 50% of the patients with limited SCCL and in 80% of the patients with extensive SCCL (9). The bone marrow may have been the source of recurrent tumor in many of the patients who have undergone ABMT, since none of the investigators attempted to remove the possible contaminating SCCL cells from the harvested bone marrow prior to ABMT. We report on our investigations with a panel of mAb reactive with SCCL and the results obtained with the mAb in conjunction with immunomagnetic beads in a model system designed to stimulate the clinical situation, with a low percentage of SCCL cells mixed with normal marrow. One of the difficulties with such a model system is the ability to detect residual tumor cells. One of the SCCL cell lines we used, DMS 273, has a 70–90% plating efficiency in methylcellulose clonogenic cultures, especially when plated at low cell numbers, which allowed us to detect less than 1 log of tumor cells. For example, if we mixed 10^6 DMS 273 cells into normal bone marrow and after the immunomagnetic separation there were 10 DMS 273 cells remaining, we would expect between seven and nine tumor colonies in the cultures.

MATERIALS AND METHODS

Monoclonal Antibodies. The mAb PM-81, SCCL-1, TFS-2, and TFS-4 were purified from ascites fluid on a Protein A-Sepharose column, as previously described (10). The mAb SCCL-124, SCCL-175, AML-1-99, and W6/32 were dilutions of ascites fluid titrated to give maximal binding at the lowest dilution. The mAb P3, Thy-1, and HNK-1 were hybridoma supernatants. The mAb PM-81, AML-1-99, SCCL-1, SCCL-124, and SCCL-175 were developed by one of us (E. D. B.) (11–14). The mAb TFS-2 and TFS-4 were developed by Okabe et al. (15). The mAb HNK-1, P3, Thy-1, and W6/32 were obtained from the American Type Culture Collection.

SCCL-1 is an IgG2a mAb that reacts with the transferrin receptor. The two mAb SCCL-124 and SCCL-175 are of the IgM class and react with most SCCL cells from pathological specimens and SCCL cell lines (12, 16). TFS-2 is an IgG2b mAb that reacts with SCCL as well as many other carcinomas, including breast, gastric, and ovarian carcinomas (17). TFS-4 is an IgG1 mAb that is specific for SCCL and is in Cluster One of the SCCL Antigen System (18). HNK-1 reacts with the CD-57 antigen found on natural killer cells and SCCL cells and is an IgM mAb (19). PM-81 is an IgM mAb and reacts with the CD15 antigen, which is found on myeloid, AML, SCCL, and breast and colon carcinoma cells (20). AML-1-99 also is an IgM mAb, reactive with a M, 124,000 antigen found on hematopoietic progenitor AML and SCCL cells (13, 21). The positive control mAb was W6/32, which is an IgG2a that reacts with histocompatibility leukocyte antigen Class I. The two negative control mAb were P3 and Thy-1, which are IgG1 and IgM mAb, respectively.

Immunomagnetic Beads. The immunomagnetic beads, Dynabeads (Dynal, Oslo, Norway), are 4.5-μm polystyrene beads with 20% iron in the form γ-Fe₂O₃ and they have a magnetic susceptibility of approximately 10⁻¹² CGS units (22). Dynabeads covalently coated with sheep anti-mouse IgG were used with IgG mAb. Uncoated Dynabeads were incubated with goat anti-mouse IgM (CalTag, San Francisco, CA), at 0.1 mg mAb/ml beads, in Tris- HCl buffer 0.01 M, pH 9.5, for 24 h at 4°C on an Orbitron rotator. The beads were washed 4 times with PBS/0.5% BSA and once with PBS/0.1% BSA and were stored at 4°C. The beads were washed once with PBS/0.1% BSA prior to use with IgM mAb.

SCCL Cell Lines. The SCCL cell line DMS 273 was developed by Pettengill and Sorenson at the Dartmouth-Hitchcock Medical Center (23). The cells have been maintained in continuous sterile tissue culture and are grown in Waymouth's modified medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (Hyclone, Logan, UT), in a humidified incubator with 6.5% CO₂ at 37°C. The SCCL cell line NCI-H69 was developed by Gazdar et al. (24). The cells were obtained from the American Type Culture Collection and have been maintained in

* E. D. Ball, unpublished observations.

7216
continuous sterile tissue culture. The cells are grown in Iscove's modi-
ified Dulbecco's medium (GIBCO) with 10% fetal calf serum (Hyclone), in
a humidified incubator with 6.5% CO2 at 37°C.

Bone Marrow. Bone marrow was obtained from the posterior iliac
crests of healthy paid volunteers, with informed consent. The marrow
cells were separated by Ficoll-Hypaque centrifugation and used the
same day in these experiments. The range of bone marrow mononuclear
cells obtained after Ficoll-Hypaque centrifugation was 1.0 x 10^7 to 5.0
x 10^7/ml of bone marrow aspirated.

Flow Cytometry. The surface antigen phenotypes of the DMS 273
cells and normal bone marrow cells were determined by flow cytometry,
as previously described (12). Briefly, the cells were incubated with the
mAb for 60 min at 0-4°C in the presence of human IgG (Sigma, St.
Louis, MO) to block Fc-mediated binding. The final concentration of
all the mAb was 20 μg/ml. After washing, fluorescein isothiocyanate-
labeled goat anti-mouse IgG and IgM were added, and the mixture was
incubated for 30 min at 0-4°C. The cells were washed again and
resuspended in PBS/BSA/0.1% azide. The percentage of positive cells
and mean fluorescent intensity were determined on the Ortho 50 H
cytotluorograph, and the results were compared to positive and negative
control mAb.

Immunomagnetic Bead Separation. A mixture of 10% SCCL cells and
90% bone marrow cells was incubated with the mAb at a concentration of
20 μg/ml for 60 min at 0-4°C. The cells were then washed 3 times, and
the Dynabeads were added at a ratio of 50 beads:1 SCCL cell. If a
combination of IgG and IgM mAb were used, both sheep anti-mouse
IgG and goat anti-mouse IgM beads were added at a 50:1 ratio. The
mixture of mAb-coated cells and immunomagnetic beads was incubated
for 30 min on an Orbitron rotator at 4°C. The mixture was then placed
in the Dynal magnetic particle concentrator for 2 min; the fluid was
aspirated and called the supernatant. The supernatant was mixed with
the same number of beads and the immunomagnetic separation was
repeated. The log separation was determined by clonogenic methylcellu-
lose cultures for the DMS 273 cells and limiting dilution assays for
the NCI-H69 cells, as described below.

Clonogenic Methylcellulose Cultures. The DMS 273 cells were grown
in methylcellulose cultures as described (25). Standards were performed
in triplicate and contained a known number of DMS 273 cells per dish.
The DMS 273 cells were mixed with 500 μl Waymouth’s modified
medium (GIBCO), 500 μl fetal calf serum (Hyclone), and 1500 μl 2.2%
methylcellulose with modified Eagle’s medium (GIBCO). The ingredi-
ents were mixed thoroughly and 1000 μl were placed in two separate
35-mm Petri dishes (Nunc, Naperville, IL). The dishes were incubated
in a humidified incubator with 6.5% CO2 at 37°C, and the colonies
were counted on day 10 under an inverted microscope. In order to
determine the log separation, the cells remaining in the supernatant
were plated in methylcellulose as above, and the results were compared
to the standards.

Limiting Dilution Assays. The NCI-H69 cells were grown in limiting
dilution assays, with a modification of the method described by Hum-
belt et al. (26). The cells remaining in the supernatant were serially
diluted in wells of microtiter trays (six wells) (Becton Dickinson,
Lincoln Park, NJ) in powers of 10. In order to establish standards for
the assay, the NCI-H69 cells were counted with a hemacytometer and
interleukin 3 and granulocyte/macrophage colony-stimulating factor
(Immunex, Seattle, WA), and 2 units of erythropoietin (Amgen, Thou-
sand Oaks, CA), and the mixture was divided equally into two 35-mm
Petri dishes. After the dishes were incubated in a humidified incubator
with 6.5% CO2 at 37°C, colonies were counted on day 14 under an
inverted microscope.

RESULTS

Flow Cytometry. The isotypes and cell and antigen specificities
of the mAb are shown in Table 1. The percentage of reactivity and mean fluorescent intensity of each mAb with
DMS 273, NCI-H69, and bone marrow cells, as determined by
cytotluorography, are shown in Table 2. The dilutions of ascites
fluid that resulted in maximal reactivity at the lowest dilution
were 1:10 for SCCL-175, 1:100 for SCCL-124 and AML-1-99,
and 1:1000 for W6/32. The same dilutions of ascites fluid were
used for the immunomagnetic bead separations.

Clonogenic Methylcellulose Cultures. DMS 273 cells grew
well in methylcellulose cultures, with a plating efficiency of 40-
90%, depending on the number of cells plated (Fig. 1). The
mAb that produced the greatest percentage of separation with
SCCL cells alone in a vital stain assay (data not shown) were
used with the immunomagnetic beads and a mixture of 10% DMS 273 cells and 90% normal bone marrow cells (1 x 10^6
DMS 273 cells and 9 x 10^6 normal bone marrow cells). The
number of DMS 273 colonies that grew in methylcellulose after
the bead separation is shown in Table 3. If the results are
compared to the standards, the combination of SCCL-175,
HNK-1, and TFS-4 resulted in a 4-5-log separation of DMS
273 cells. Compared to the negative control samples, the com-
bination of SCCL-175, HNK-1, and TFS-4 resulted in a 2-4-
log separation of contaminating tumor cells. Therefore, the
mAb and immunomagnetic bead separation resulted in a non-
specific 0-2-log depletion of the DMS 273 cells. There was no
change in the viability of the DMS 273 cells or bone marrow
cells after immunomagnetic separation, as determined by vital
stain. However, 50-80% of the total cells were lost during the
immunomagnetic separation.

Limiting Dilution Assays. NCI-H69 cells had a low plating
efficiency in methylcellulose cultures, so a limiting dilution
assay was used to determine log separation. When 16 or greater
NCI-H69 cells were plated all the wells had clusters of cells,
and when 8 cells were plated half of the wells had clusters,
as

<table>
<thead>
<tr>
<th>mAb Isotype</th>
<th>Cell reactivity</th>
<th>Antigen reactivity</th>
</tr>
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<tbody>
<tr>
<td>SCCL-1 IgG2a SCCL</td>
<td>erythroid precursors, RBC</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>SCCL-124 IgM SCCL</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SCCL-175 IgM SCCL</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TFS-2 IgG2b SCCL</td>
<td>many carcinomas</td>
<td>M, 39,000 antigen</td>
</tr>
<tr>
<td>TFS-4 IgG1 SCCL</td>
<td></td>
<td>M, 124,000 antigen</td>
</tr>
<tr>
<td>HNK-1 IgM SCCL</td>
<td>Natural killer cells</td>
<td>ND</td>
</tr>
<tr>
<td>PM-41 IgM SCCL</td>
<td>Myeloid precursors</td>
<td>CD-57</td>
</tr>
<tr>
<td>PM-12 AML-1-99 AML cells</td>
<td>PMNs, AML cells</td>
<td>CD-15</td>
</tr>
</tbody>
</table>

ND, not determined.

The M, 124,000 antigens that TFS-4 and AML-1-99 react with are probably
different antigens, as deduced by quite disparate expression on cells
determined by flow cytometry.

PMNs, polymorphonuclear cells.
ELIMINATION OF SMALL CELL LUNG CANCER WITH ANTIBODIES

Table 2 Reactivities of the monoclonal antibodies with two small cell carcinoma of the lung cell lines, DMS 273 and NCI-H69, and normal bone marrow, determined by cytofluorography

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>DMS 273 cells</th>
<th>NCI-H69 cells</th>
<th>Bone marrow cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% positive</td>
<td>MFI*</td>
<td>% positive</td>
</tr>
<tr>
<td>P3</td>
<td>3.5 ± 1.0*</td>
<td>208.0 ± 24.1</td>
<td>6.5 ± 1.3</td>
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<tr>
<td>THY-1</td>
<td>2.5 ± 1.2</td>
<td>188.7 ± 33.8</td>
<td>ND</td>
</tr>
<tr>
<td>W6/32</td>
<td>99.2 ± 0.7</td>
<td>901.3 ± 67.3</td>
<td>85.1 ± 2.4</td>
</tr>
<tr>
<td>SCCL-1</td>
<td>94.5 ± 5.5</td>
<td>609.5 ± 69.5</td>
<td>97.4</td>
</tr>
<tr>
<td>SCCL-124</td>
<td>74.8 ± 6.6</td>
<td>439.5 ± 29.9</td>
<td>95.7 ± 3.0</td>
</tr>
<tr>
<td>SCCL-175</td>
<td>91.1 ± 4.3</td>
<td>614.2 ± 68.0</td>
<td>87.4 ± 2.8</td>
</tr>
<tr>
<td>TFS-2</td>
<td>3.2 ± 1.2</td>
<td>192.0 ± 1.0</td>
<td>98.0 ± 1.0</td>
</tr>
<tr>
<td>TFS-4</td>
<td>84.8 ± 8.7</td>
<td>586.5 ± 22.1</td>
<td>95.2 ± 2.4</td>
</tr>
<tr>
<td>HNK-1</td>
<td>79.6 ± 2.8</td>
<td>552.0 ± 44.0</td>
<td>90.5 ± 3.9</td>
</tr>
<tr>
<td>PM-81</td>
<td>15.9 ± 9.1</td>
<td>273.3 ± 29.4</td>
<td>98.9 ± 0.4</td>
</tr>
<tr>
<td>AML-1-99</td>
<td>82.9 ± 7.6</td>
<td>524.7 ± 16.9</td>
<td>96.6 ± 1.4</td>
</tr>
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</table>

* MFI, mean fluorescent intensity.
* Mean ± SE.
* ND, not determined.

Table 5 Log separation of NCI-H69 cells from a mixture of normal bone marrow cells and NCI-H69 cells using monoclonal antibodies and immunomagnetic beads

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>n</th>
<th>Dilution*</th>
<th>Growth*</th>
<th>Log separation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCL-175, TFS-4, HNK-1</td>
<td>8</td>
<td>1/10</td>
<td>+</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/100</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/1000</td>
<td>+/-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/10000</td>
<td>+/-</td>
<td>4</td>
</tr>
<tr>
<td>P3</td>
<td>8</td>
<td>1/10</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/100</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/1000</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/100000</td>
<td>+/-</td>
<td>4</td>
</tr>
</tbody>
</table>

* No. of colonies in methylcellulose cultures/300,000 marrow cells. CFU-GM, colony-forming unit-granulocyte/macrophage; BFU-E, burst-forming unit-erythroid.
* Reduction (%) = mean no. of colonies in sample/ mean no. of colonies in control × 100%.

DISCUSSION

We have demonstrated that mAb and immunomagnetic beads can separate SCCL cells from normal bone marrow, with little effect on normal hematopoietic progenitor cells. The use of three mAb, SCCL-175, TFS-4, and HNK-1, resulted in a 4-5-log tumor-cell separation, as determined by clonogenic methylcellulose cultures or limiting dilution assays. Heterogeneity of tumor cell surface antigen phenotypes suggests a cocktail of mAb may be important in eliminating as many tumor cells as possible (28), and our results confirm the need for a combination of mAb. The three mAb, SCCL-175, TFS-4, and HNK-1, shown in Table 4. Therefore, if the wells containing the dilutions from the separation experiments had a cluster of cells, the dilution contained at least 16 NCI-H69 cells. The combination of SCCL-175, HNK-1, and TFS-4 resulted in a 4-log separation of NCI-H69 cells, as shown in Table 5. Compared to the negative control samples, the anti-SCCL mAb resulted in a 3-log separation of contaminating tumor cells.

Hematopoietic Progenitor Cell Assay. The results of the colony-forming unit assays are shown in Table 6. Compared to an equal number of marrow cells not treated with the mAb and magnetic beads, the immunomagnetic separations decreased the colony-forming unit-granulocyte/macrophage by 25–42% and the burst-forming unit-erythroid by 19–46%.
react with >90% of SCCL tumors and cell lines, and the mAb react with different antigens (12, 15, 19).

The use of mAb has demonstrated that 60–80% of patients with SCCL have bone marrow involvement, despite only 10–20% of bone marrow samples being histologically positive (9, 29–31). Stahel et al. (9) used a mAb specific for SCCL, SM-1, and by indirect immunofluorescence demonstrated that 69% of the patients had marrow involvement, although only 16% of the marrows were positive for tumor involvement by histochemical stains (9). Another mAb reactive with SCCL, MOC-1, detected SCCL cells in 19 of 30 patients (63%), and only 6 of the 30 (20%) were positive histologically (29). A panel of mAb detected marrow involvement in eight patients with a histologically normal marrow undergoing intensive chemotherapy and radiotherapy followed by ABMT. The detection of marrow involvement with the mAb predicted later relapse in six of the eight patients, compared to no relapses in the patients without marrow involvement (30).

In an attempt to improve the treatment results of SCCL, many investigators have treated patients with intensive chemotherapy followed by ABMT (5–8). Spitzer et al. (5) treated 32 limited-disease SCCL patients with intensive chemotherapy and ABMT following induction chemotherapy. There were 22 complete responses (69%); 6 remain disease-free, 4 for 4 years or longer (5). Of the 22 patients who achieved a complete response, 13 relapsed, and only 2 of these were in the lung only; another 4 were both local and distant relapses. Two other series treated predominantly extensive-disease SCCL patients, one with total body irradiation and chemotherapy and the other with intensive chemotherapy alone, but only 1 of 39 patients survived for more than 2 years if the two series are combined (6, 7). None of the series of intensive chemotherapy followed by ABMT have reported an improvement in survival for SCCL patients, compared to patients treated with standard chemotherapy.

It is impossible to discern whether the transfused bone marrow or inadequate ablative therapy is the source of recurrent SCCL after the intensive therapy and ABMT. Since the majority of patients with SCCL have bone marrow involvement, as detected by mAb, bone marrow purging is probably necessary if intensive chemotherapy followed by ABMT is going to improve the treatment results of SCCL. An important conclusion from the previous studies of intensive therapy and ABMT is that only patients with a complete response, or very good partial response to induction chemotherapy, benefit from intensification and ABMT (5–8).

In model systems, mAb and complement effectively killed 99% of contaminating SCCL cells, without adversely affecting hematopoietic progenitor cells (17, 25). Our immunomagnetic bead separation produced more effective tumor cell separation and avoided the potential complications of transfecting a biological product with antigenic capability and of the product variability associated with complement. Meagher et al. (32) reported that ethiophos and light-activated mercycyanine 540 phototreatment eliminated all detectable SCCL clonogenic cells, without affecting the hematopoietic progenitor cells. The low plating efficiency, 0.2%, of the two SCCL cell lines, NCI-H69 and NCI-H128, limits the conclusions that can be drawn about the potential of the mercycyanine 540 purging process. In vitro chemotherapy can successfully purge the marrow of contaminating SCCL cells, as demonstrated by Benard et al. (33). They propose using cisplatin in vitro to eradicate the SCCL cells from the bone marrow. Since the SCCL cell lines used by Benard et al. were derived from patients prior to any treatment and may not represent tumor cells in vivo because induction chemotherapy prior to intensive chemotherapy and ABMT may induce some resistance to chemotherapy, a different approach such as immunomagnetic separation may be preferable. There are no reports of intensive chemotherapy followed by ABMT of marrow purged in vitro for SCCL. Our results suggest that the mAb and immunomagnetic beads could safely and effectively separate SCCL cells from the bone marrow for ABMT following high dose chemotherapy.

REFERENCES

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