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Separation of Clonogenic Tumor Cells from Small Cell Lung Cancer Bone Marrow and Small Cell Lung Cancer Cell Lines[1]

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ABSTRACT

One of the major obstacles to successful autologous bone marrow transplantation is tumor contamination of the marrow. We attempted to separate tumor cells from the marrow of patients with small cell lung cancer by layering bone marrow on a discontinuous albumin gradient and then assessing hematopoietic potential (CFU_c) and clonogenic tumor cells ($TCFU_c$) by standard techniques. In the six of seven patients whose bone marrow grew tumor colonies, 75 to 80% of CFU_c could be found in Fraction 3 of the gradient; while 80 to 90% of $TCFU_c$ could be found in light-density Fraction 1 + 2. Furthermore, we observed tumor colony growth in Fraction 1 + 2 in some patients whose unfractionated bone marrow failed to grow tumor colonies. In separate experiments, we layered five cell lines established in patients with small cell lung cancer on the gradient and found that cells from four of the five lines also migrated to Fraction 1 + 2, and $TCFU_c$ from these lines were observed in Fractions 1 + 2 in three of four lines tested. We conclude that gradient fractionation may be one way of removing clonogenic tumor cells from the bone marrow of small cell lung cancer patients prior to autologous transplantation.

INTRODUCTION

Despite recent advances in the chemotherapy of SCLC,[3] only a minority of patients survive beyond 2 years (11). Lack of effective tumor cell kill and emergence of resistant cells are possible explanations for this high relapse rate. Preclinical studies in a variety of tumors have shown that the number of malignant cells which survive after exposure to a drug is a constant fraction of the original tumor load regardless of its size (23, 24). These data suggest that drug dose may be an important factor in cancer chemotherapy and may be particularly important in tumors with high initial response rates, such as SCLC (7). Indeed, clinical studies have shown that complete remission rates and duration of remission in SCLC may be favorably influenced by high-dose therapy (3).

These observations have resulted in a number of attempts to use extremely-high-dose chemoradiotherapy in SCLC followed by autologous bone marrow rescue, thereby averting the major dose-limiting toxicity (9). Unfortunately, this approach is limited by the finding that SCLC frequently involves the bone marrow, making tumor cell contamination a major obstacle to successful autotransplantation (4).

In the current study, we show that bone marrow from SCLC patients can be separated on a gradient into a light-density tumor stem cell population and a heavier hematopoietic stem cell population. Furthermore, we show that established SCLC tumor cell lines can similarly be separated into a light-density fraction that contains the majority of clonogenic tumor cells. These observations may have implications for autologous bone marrow transplantation and may provide one method for reducing tumor cell contamination prior to infusion.

MATERIALS AND METHODS

Bone Marrow Collection and Fractionation. Bone marrow from patients with SCLC and from normal donors was collected after obtaining appropriate consent. Between 2×10^9 and 2×10^8 bone marrow cells were layered onto a discontinuous albumin density gradient as described previously (28). Briefly, bovine albumin solutions of decreasing concentrations are carefully layered on top of one another. The starting albumin solution is 35% (v/w) in Tris buffer. Solutions of 25, 23, 21, and 17% are made by dilution with a sodium chloride-sodium phosphate buffer. The albumin concentrations are adjusted by their refractive index in an Abbe refractometer (Zeiss) at room temperature. Cells suspended in 17% albumin solution are pipeted on top of the 21% albumin layer and centrifuged for 30 min at 10° and $1000 \times g$ at the base of the tube. After centrifugation, the cells are collected at the density interfaces. The cell fraction between the 17 and 21% layers is labeled Fraction 1 + 2, between 21 and 23% it is Fraction 3, and between 23 and 25% it is Fraction 4 (Chart 1). The final fraction consists of cells collected in the bottom of the tube. Fractions are assayed for CFU_c and $TCFU_c$, as described below. In some experiments, bone marrow is layered on a Ficoll-Hypaque gradient, and the interface layer is saved for $TCFU_c$ and CFU_c determination.

Clonogenic Assays. Cells from each fraction (2.5 or 5×10^5) were plated in soft agar for CFU_c, according to the technique described by Pike and Robinson (18), utilizing a blood buffy coat feeder.

In parallel experiments, cells were plated in soft agar as described by Salmon (20). Cells are suspended in an enriched CMRL medium in 0.3% molten agar. One ml of this mixture (5×10^5 cells) is plated in each of 35-mm plastic Petri dishes over a 0.5% agar feeder layer containing various nutrients and growth stimulants. The plates are placed in a humidified CO_2 incubator (5%) at 37° and counted serially in an inverted microscope when a sufficient number of colonies develop. The enriched CMRL contains CMRL 1066, horse serum, $CaCl_2$ (100 mM), insulin (100 units/ml), vitamin C (30 mM), penicillin (100 units/ml), streptomycin (2 mg/ml), and glutamine. To this are added asparagine (6.6 mg/ml), DEAE-dextran (50 mg/ml), and 50 mM freshly prepared 2-mercaptoethanol. The underlayer consists of enriched McCoy's Medium 5A [50% horse serum, 20% fetal calf serum, 2.2% sodium pyruvate, L-serine (21 mg/ml), 200 mM glutamine, and penicillin-streptomycin]. To this are added Trypticase soy broth, asparagine, and DEAE-dextran (50 mg/ml). Plates are scored

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[3] The abbreviations used are: SCLC, small cell lung cancer; CFU_c, colony-forming units, culture; $TCFU_c$, tumor colony-forming units, culture; CMRL, Connaught Medical Research Laboratory; CFU-GEMM, colony-forming units-granulocyte erythroid monocyte macrophage; BFU_e, burst-forming unit, erythroid.

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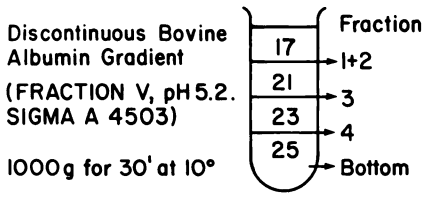

Chart 1. Discontinuous bovine albumin gradient layered with different concentrations of albumin (from 17 to 25%), with each concentration labeled as shown.

at 14 days and examined for the presence of clusters (less than 50 cells) and colonies (greater than 50 cells).

The colonies are picked with a Pasteur pipet and then stained with Wright-Giemsa. The upper layer of some plates are removed, fixed, and stained with Papanicolaou stain (19). Other plates are fixed with glutaraldehyde and 0.01 M sodium cacodylate and then prepared for electron microscopic examination as described previously (12).

SCLC Cell Lines. Cell lines NCI-H69, H250, H60, H128, and N464, established from patients with SCLC, were grown as described previously (8) in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum. The lines were a gift from Drs. Desmond Carney, Adi Gazdar, and John Minna of the National Cancer Institute. Approximately 10^8 to 10^9 cells from each line were layered on the albumin density gradient; then each fraction was plated in soft agar as described above.

RESULTS

Bone Marrow Separation in Patients with SCLC. Bone marrow was obtained from 7 patients with SCLC. As shown in Chart 2, from 40 to 80% of the total number of nucleated cells were found in the bottom fraction. Fraction 3 contained from 10 to 30% nucleated cells, but CFU_c analysis showed (Chart 2) that there was an 8- to 10-fold concentration factor of CFU_c from samples in Fraction 3, with 75 to 80% of the total CFU_cs found in this fraction.

Fractions 1 + 2, 3, 4, and bottom were simultaneously plated for $TCFU_c$. In 6 patients, $TCFU_c$ formed, and separation of CFU_c and $TCFU_c$ could be observed (Chart 2). While CFU_c remained concentrated in Fraction 3, tumor colonies were found predominantly in Fraction 1 + 2. Tumor colonies in these experiments were identified by light microscopy on picked colonies with Wright's stain and on whole-mount specimens with Papanicolaou stain.

In data not shown, we observed that only 3 of 6 patients who had $TCFU_c$ growth *in vitro* had light microscopic evidence of SCLC on bone marrow aspirate or biopsy. Of the 6 patients in whom we could identify tumor colonies in Fraction 1 + 2, only 2 showed tumor colonies when whole, unfractionated marrow was plated, despite the fact that the marrow was aspirated from the same site at the same time and plated the same day.

In separate experiments, lymph nodes from 3 patients completely replaced with metastatic SCLC were mechanically minced into single-cell suspensions and then layered on the albumin gradient. All of the cells in each case migrated to Fraction 1 + 2, and in one patient colonies were observed (Fig. 1).

Separation of SCLC Cell Lines. When 10^8 cells from 5 small cell lines (NCI H250, H69, H60, H128, and N464) were layered on the albumin gradient, approximately 80 to 90% of the cells and a similar proportion of tumor colonies migrated to Fraction 1 + 2 (Table 1). In one line (NCI H128), there was more even distribution of clonogenic cells, but the compact nature of the floating aggregates made it difficult to obtain single-cell suspensions with this line. In one line (NCI H60), there were cells in Fractions 1 + 2 and 3, but no clonogenic cells were found in Fraction 3.

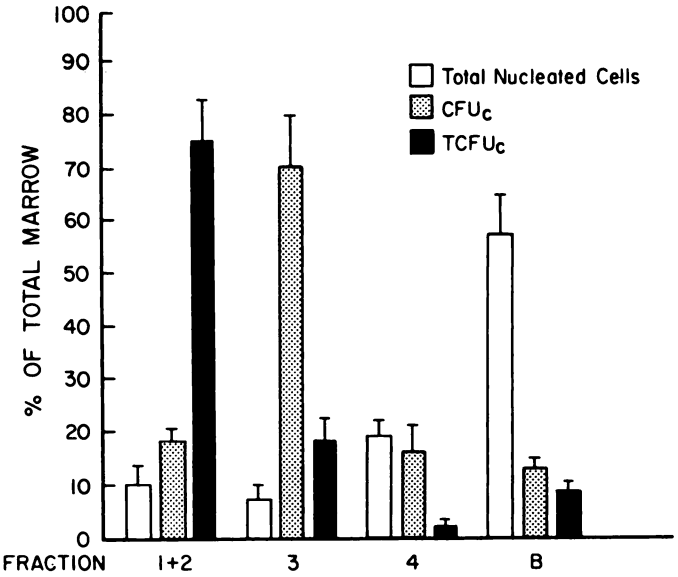

Chart 2. Bone marrow of 6 patients with SCLC plated simultaneously in soft agar for $TCFU_c$ and with methylcellulose for CFU_c after fractionation on the albumin gradient. The bottom fraction contains the majority of nucleated cells, while Fraction 3 contains most of the CFU_c. $TCFU_c$ are identified in Fraction 1 + 2, where only a small proportion of the CFU_c are found. *Bars*, S.D.

Table 1

Gradient fractionation of SCLC cell lines resulting in 80% of cells migrating to Fractions 1 + 2 in 4 lines (NCI N464, H69, H250, and H128)

In addition, tumor colonies ($TCFU_c$) migrate to Fraction 1 + 2 in 3 of 4 lines tested. In NCI H60, although 38% of cells are found in Fraction 3, no $TCFU_c$ are seen, while in NCI H128, although only 13% of cells are in Fraction 3, 50% of $TCFU_c$ are found there.

	% of cells in		$TCFU_c$	
	Fraction 1 + 2	Fraction 3	Fraction 1 + 2	Fraction 3
NCI H60	58	38	1488 ± 38[a]	0
NCI N464	97	0	ND[b]	ND
NCI H69	98		1328 ± 36	0
NCI H250	85	15	1435 ± 38	4
NCI H128	81	13	1555 ± 39	1322 ± 37

[a] Mean ± S.D.
[b] ND, not determined.

Light microscopy using Wright-Giemsa stain on picked colonies, Papanicolaou staining on whole-mount specimens, and electron microscopy on picked colonies demonstrated typical SCLC.

In parallel experiments, 2 of the fractionated cell lines (NCI H250 and H69) were plated in soft agar at varying cell concentrations, and we observed (Chart 3) that the number of colonies was proportional (to the 3/2 power) to the number of cells plated ($r = 0.99$, $p < 0.0001$).

DISCUSSION

We have shown that bone marrow of patients with SCLC may be separated into tumor stem cell-containing and tumor stem cell-depleted fractions. Clonogenic tumor cells from bone marrow of SCLC patients are concentrated in Fraction 1 + 2 (light density)

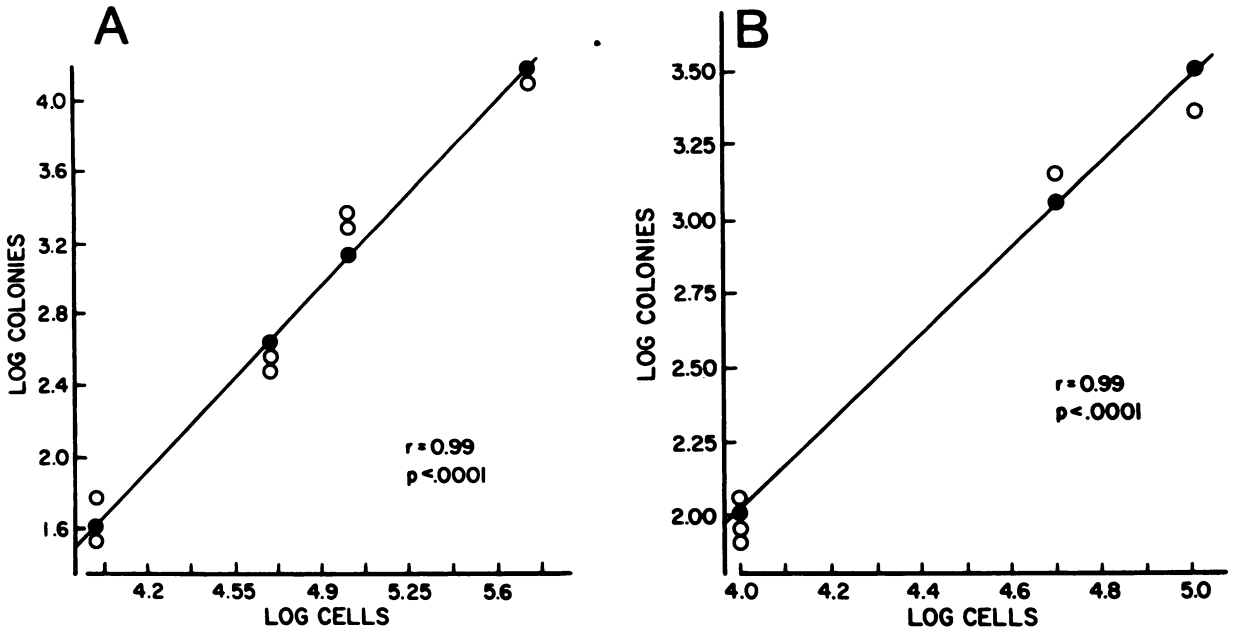

Chart 3. Relationship of ln cells plated *versus* ln colonies in Fraction 1 + 2 after density separation for NCI H250 (*A*) and NCI H69 (*B*). ○, actual data points; ●, "best-fit" data points with the correlation coefficients as noted.

of our gradient, while hematopoietic stem cells (as measured by CFU_c) remain in Fraction 3. Similarly, established cell lines from patients with SCLC can also be fractionated into clonogenic and nonclonogenic populations, with a plating efficiency which decreases with decreasing cell dose. Clonogenic tumor cells from the cell lines migrate to the same light-density fraction as tumor cells from bone marrow of patients with SCLC. These data reemphasize previous observations which note the heterogeneity of tumor cell populations (16) and suggest that some of these properties may be utilized to advantage in order to remove tumor cells from bone marrow.

The albumin gradient used in these studies is not an analytical gradient. Cells are separated on the basis of density, pH, and osmolarity. This procedure was selected because of its empirically determined utility in allogeneic bone marrow transplantation and its ability to handle large volumes.

Physical separation (5), cytotoxic antibodies (25, 29), and the use of *in vitro* drugs (22) have all been reported recently as methods to remove tumor cells from bone marrow. Much of the clinical work on this problem has come from studies in leukemia. Dicke *et al.* (5) reported 28 patients with acute nonlymphocytic leukemia in relapse treated with total-body irradiation and piperazinedione followed by autologous bone marrow transplant. Ten of these patients received marrow that had been separated on an albumin density gradient. Twelve patients (43%) achieved complete remission, with a median duration of 4 months, but there were no obvious differences in patients who received fractionated marrow. Herzig (13) has treated 27 patients with acute nonlymphocytic leukemia with high-dose Cytoxan and total-body irradiation. Sixty-five % achieved complete remission with a median duration of 5 months. The short remission duration may either be due to ineffective therapy or reinfusion of occult tumor cells. There are insufficient data in breast cancer or melanoma to isolate similar trends, although a high relapse rate was not seen in studies of autologous transplantation in non-Hodgkin's lymphoma (10, 14).

A number of studies using pharmacological or immunological methods to isolate tumor cells from bone marrow have recently been reviewed (21). Santos and Kaizer (21) used a congener of cyclophosphamide, 4-hydroperoxycyclophosphamide, for incubation with rat acute myelogenous leukemia tumor cells in marrow-tumor cell suspensions. After incubation, the tumor cells were eliminated while the infused marrow was able to protect lethally irradiated rats from death due to marrow aplasia, although very high cell doses of marrow were used in these studies, making them difficult to apply to humans.

There have been a number of immunological studies in animal systems designed to eliminate tumor cells from bone marrow *in vitro*. Incubation of marrow-tumor cell mixtures with heterologous cytotoxic antiserum to a tumor-associated antigen in the presence of complement resulted in inactivation of clonogenic tumor cells in a C3H mouse lymphoma (6), an AKR mouse leukemia (25), a DBA/2 mouse L1210 leukemia (26), and a WF rat leukemia (1). In all of these instances, bone marrow reconstitution of lethally irradiated animals with treated bone marrow was possible, although again a large overdose of bone marrow was infused. It is therefore difficult to say whether or not there was significant toxicity to normal hemopoietic cells with these methods.

Our data show that separation of SCLC tumor cell lines and tumor cells in bone marrow is feasible and that gradient separation may be a novel and important first step in isolating cell populations of interest. Although most of the tumor colonies in our patients were found in Fraction 1 + 2, in some patients colonies were seen in other fractions as well, so that administration of CFU_c-rich Fraction 3 for bone marrow transplantation may still result in infusion of significant numbers of clonogenic tumor cells. Incubation of Fraction 3 with monoclonal antibodies (with and without complement) directed against SCLC may further eliminate tumor from marrow prior to transplantation. Selective killing of SCLC cells may then be assessed by measurement of $TCFU_c$ and by radioimmunoassay techniques. These studies are

currently under way in our laboratory.

Although our studies document separation of $TCFU_c$ from CFU_c, it is well known (17) that the CFU_c does not represent the true pluripotent hematopoietic stem cell and does not always correlate with hematopoietic reconstitution. Other assays (CFU-GEMM, BFU_e) may detect cells which are closer in lineage to the pluripotent stem cell. However, we have shown previously that restoration of normal hematopoiesis does occur in dogs (28) and in humans (15) following infusion of CFU_c-rich Fraction 3 after marrow-ablative therapy.

Our observation that bone marrow which is negative for tumor cells on aspirate or biopsy may contain tumor colonies is consistent with previous reports by Von Hoff *et al.* (27) for neuroblastoma but differs from data reported by Carney *et al.* (2), who found that in no instance did marrow negative for SCLC grow tumor colonies *in vitro*. Our data, however, are not directly comparable since the only cases where marrow was negative by morphology and positive by the clonogenic assay were in patients whose marrow was fractionated on the albumin gradient. Indeed, our results indicate that in some instances fractionated bone marrow showed tumor colony growth while unfractionated marrow plated simultaneously did not. The reasons for this are not clear, but one may speculate that the fractionation technique in some way concentrates tumor cells enough to increase the plating efficiency to detectable levels, exposing perhaps one of the inherent weaknesses of clonogenic assays. This concept is supported by our observation that NCI H69 and H250 show reduced plating efficiency as tumor cell number decreases, so that small numbers of clonogenic tumor cells may go undetected. At the same time, it is possible that fractionation, by removing cells into Fraction 3, 4, and bottom which would ordinarily be in contact with tumor cells in unfractionated preparations, removes certain humoral or cell-mediated inhibitory growth factors. This concept is currently being assessed in our laboratory. Further studies with other tumors and tumor cell lines are needed to determine the biological and clinical significance of these findings.

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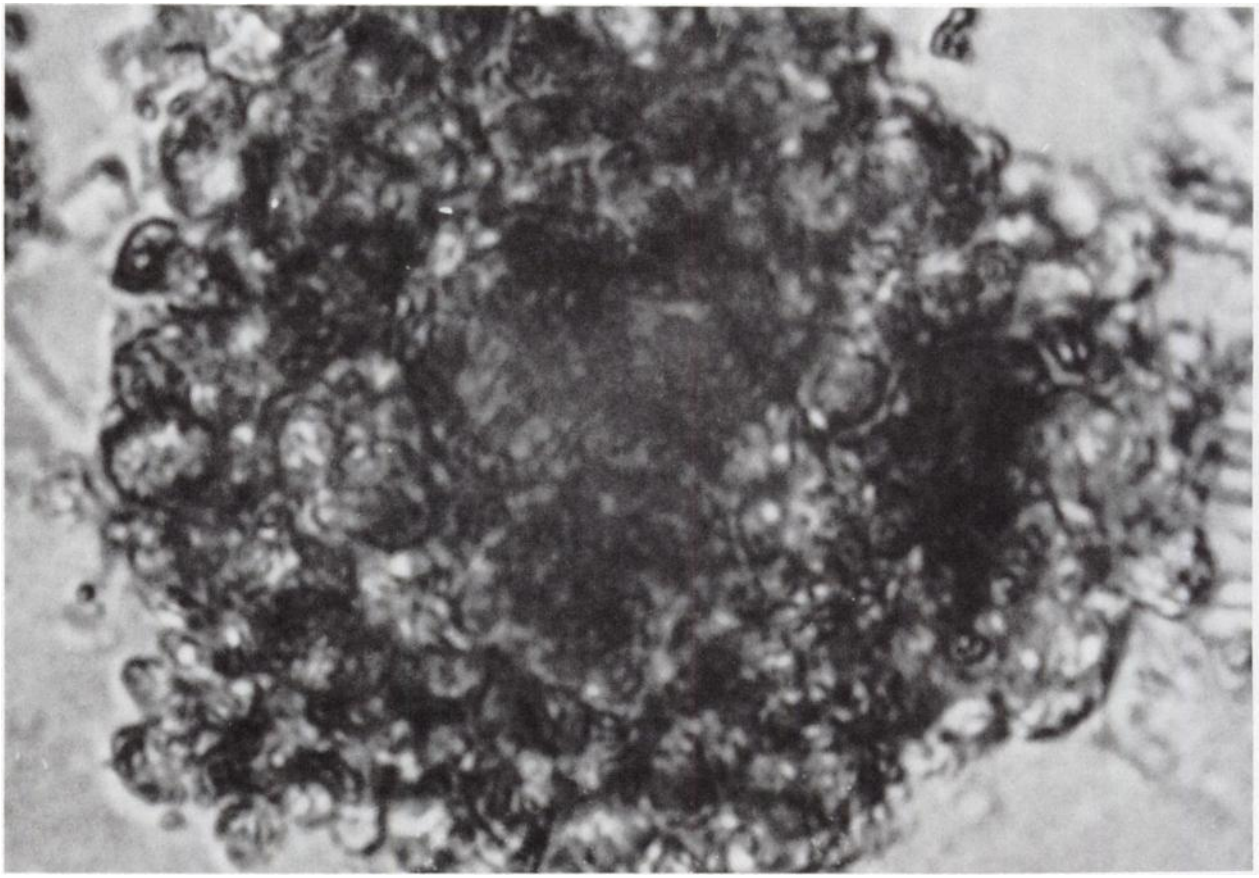

Fig. 1. Colony grown in soft agar from Fraction 1 + 2 derived from a single-cell suspension made from a lymph node replaced with metastatic SCLC.

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