Detection of Small Cell Lung Cancer Bone Marrow Involvement by Discontinuous Gradient Sedimentation

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ABSTRACT

Marrow involvement by small cell lung cancer (SCLC) is detected in 10–23% of patients at initial diagnosis by marrow aspirate and biopsy techniques. To improve the detection and potential monitor marrow involvement by SCLC we have attempted to concentrate malignant cells with clonogenic potential on a discontinuous density gradient (DDG). The bone marrow from 43 patients with SCLC (36 with histologically negative marrow aspirates and biopsies) were separated on a Ficoll-based DDG. Samples were also separated by conventional Ficoll-diatrizoate (FD) centrifugation were isolated and 2.5 x 10^5 cells from each fraction were cultured in 2 ml of 0.3% agar in McCoy's media with 10% fetal calf serum, 2.5 μg transferrin, 1 μg insulin, and 1% penicillin-streptomycin. Colony growth was assessed after 14 days of culture at 37°C and 6% CO₂. Tumor colony growth was seen in eight of 36 (22%) patients with histologically negative marrow as well as in four of seven (57%) patients with known involvement. Mean colony growth per 2.5 x 10^5 cells for all 12 patients was 4.3 colonies for Fx1; 8.8 for Fx2; and 2.7 for Fx3. In contrast mean contrast growth from the Fx3 was 1.8 colonies. Cells clonogenic potential could be demonstrated from Fx2 and Fx3 in seven of 12 and eight of 12 patients, respectively; in Fx3 four of 12 patients had tumor growth. We conclude that separation of marrow samples by DDG concentrates malignant cells with clonogenic potential at least 8-fold compared to FD separation and that the sensitivity of the clonogenic assay in detecting marrow involvement by SCLC is enhanced by DDG sedimentation.

INTRODUCTION

SCLC is a distinct clinicopathological entity which accounts for 20–25% of lung cancer. Some of the features which characterize this disease include its apparent neuroendocrine differentiation, responsiveness to chemotherapy and radiotherapy, and propensity for regional and systemic metastasis. One of the most important predictors for survival in these patients is the extent of tumor dissemination at the time of diagnosis. Less than 1% of patients with disease outside the ipsilateral chest survive beyond 24 months.

Bone marrow involvement by small cell carcinoma of the lung is detected in 10–23% of patients at diagnosis with routine histological studies of bone marrow aspirates and biopsies. With the use of in vitro semisolid culture techniques clonogenic growth of small cell lung cancer has been reported in 8–11% of histologically negative bone marrow specimens. The objective of the present study was to enhance the sensitivity of tumor cell detection by investigating the effectiveness of a DDG in concentrating tumor cells with in vitro clonogenic potential from the bone marrow of patients with small cell carcinoma of the lung. A clonogenic assay known to support the growth of small cell cancer colonies was used to detect malignant involvement. The sensitivity of the DDG technique was compared to a single density Ficoll-diatrizoate separation using identical marrow specimens. These results were correlated with routine histological methods.

MATERIALS AND METHODS

Patient Population. Forty-three consecutive patients with a histological diagnosis of small cell carcinoma of the lung were the subjects of this study. All patients underwent pretherapy staging with a complete history, physical examination, chest roentgenogram, brain computerized tomography, liver-spleen scan or abdominal computerized tomography scan, bilateral bone marrow aspirates and biopsies, bone scan, serum chemistries, liver function tests, and complete blood count. Patients with disease confined to the hemithorax and supraclavicular nodes were considered to have limited disease, all others were considered to have extensive disease. All patients were treated with combination chemotherapy with or without radiation therapy according to ongoing study protocols.

Collection of Bone Marrow. After obtaining informed consent, bilateral bone marrow aspirates and biopsies were done in all patients. Bone marrow aspirates (1–2 ml) were collected in syringes containing preservative-free heparin (100 μl). One half of the aspirate from each side was processed for routine histological study as part of the pretherapy evaluation. The remaining halves of the aspirates from both sides were combined, forced through a 22-gauge needle and diluted with 3–4 ml of RPMI 1640 and processed for culture. After decalcification, the bone marrow biopsies were stained with hematoxylin and eosin. All aspirates and biopsies were examined for the presence of tumor by one investigator (R. F. H.).

Preparation of Discontinuous Density Gradient. The DDG with densities of 1.050, 1.055, 1.060, and 1.065 were prepared by diluting a stock Ficoll solution (30 g Ficoll powder, Sigma, 70 ml of distilled water) with 330 mOsm balanced salt solution until the desired specific density was achieved as judged by refractive index. The balanced salt solution was prepared by mixing 121 parts 0.165 M NaCl, four parts 0.165 M KCl, three parts 0.11 M CaCl₂, one part 0.165 M MgSO₄, and one part 0.165 M KH₂PO₄.

Preparation of Cell Suspension. The combined marrows from individual patients were evenly divided with one half of the sample layered over a Ficoll-based discontinuous density gradient and the other half layered over a conventional Ficoll-diatrizoate (Histopaque, density 1.077; Sigma Diagnostics, St. Louis, MO) gradient. After centrifugation at 200 g x 30 min the cellular interphases from three fractions (Fx) corresponding to specific densities 1.050 (Fx1), 1.055 (Fx2), and 1.060 (Fx3) were recovered from the discontinuous density gradient as well a single interphase (FxFD) from the single density Ficoll-diatrizoate centrifugation. After three washes with Hanks' balanced salt solution (GIBCO) the cells from each fraction were resuspended in McCoy's medium (GIBCO) and cell counts and viability determined by trypan blue exclusion. Morphological evaluation of cytocentrifuge preparations of the different fractions were done in the first seven patients in this study. In order to ensure that an adequate number of cells would be available for culture, the cytocentrifuge preparations were frequently acellular of hypocellular making a reliable morphological interpretation difficult. Thus, this phase of the study was not pursued.

Culture Conditions. The clonogenic assays were done with a modifi-
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cation of the Hamburger and Salmon assay (8). Viable nucleated cells at a concentration of 2.5 \times 10^7/ml were cultured in an overlayer of 1 ml of 0.3% agar in McCoy's media supplemented with 10% fetal calf serum, 2.5 \mu g transferrin, 1 \mu g insulin, 1% penicillin-streptomycin and 0.5 mg DEAE-dextran (Pharmacia Fine Chemical, Inc., Piscataway, NJ). The underlayer consisted of 1 ml of 0.5% agar in the above media. All fractions were cultured in triplicate, incubated at 37°C in a 6% CO₂ atmosphere and after 14 days colonies (>30 cells) were scored. In preliminary experiments, normal bone marrow samples were cultured using an identical double-layer agar system. In several of these samples, the growth of normal granulocyte macrophage colony forming units were seen with a range of 0–3 colonies/plate. Thus, at the onset of this study, only specimens in which at least one of the cultured fractions yielded greater than three colonies per plate were considered positive for tumor growth.

Characterization of Colonies. In order to define morphology, in three separate experiments individual colonies were plucked with a pasteur pipet, cytocentrifuged onto a glass slide, and stained with Wright's-Giemsa. Electron micrographs of aspirated individual colonies from three separate specimens were done. Aspirated colonies were fixed in 4% paraformaldehyde, postfixed in 1% Osmium, stained in block with 2% uranyl acetate, dehydrated in graded series of acetone, and embedded in Araldite (Polaron, Hatfield, PA).

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Statistical Analysis. The two-tailed paired t test and the sign test were used to determine statistical significance in all of the experiments.

RESULTS

A total of 86 bone marrow aspirates and biopsies were done in 43 patients with small cell carcinoma of the lung as part of their initial evaluation. In all 43 patients marrow mononuclear cells were separated on a Ficoll-based discontinuous density gradient as well as over a single gradient Ficoll-diatrizoate centrifugation.

Malignant colony formation was seen in 12 out of the 43 patients (Table 1) when samples were separated by DDG as compared to four positive cultures when a single density sedimentation was used. These differences are highly significant by the sign test (P < 0.01). Positive tumor growth was seen in five of 26 (19%) patients who otherwise had disease limited to the chest by routine assessment as compared to seven of 17 (41%) who had extensive disease. In both sets of patients marrow separation by DDG was superior to conventional Ficoll-diatrizoate separation in detecting malignant cells with clonogenic potential.

In Table 2 a comparison of mean colony number and percentage of clonogenic growth per recovered fraction is made. Only specimens in which at least one of the fractions was positive for tumor growth are considered. Malignant clonogenic growth from at least one of the cultured fractions was detected in 12 specimens. Tumor clonogenic growth was more often detected in the cellular fractions F×2 and F×3 isolated from the DDG, with nearly twice the number of positive specimens as compared to F×FD. None of the individual cultured fractions could detect malignant growth in all 12 positive marrow specimens. Malignant cells with clonogenic potential were concentrated in F×2 of the DDG as reflected by the significant increase in the mean number of colonies from 8.8 ± 3.6 compared to 1.0 ± 0.6 colonies in the F×FD fraction (P < 0.02).

A correlation between bone marrow clonogenic growth and sites of disease in all 43 patients is made in Table 3. Clonogenic growth was detected in four of seven patients with histological evidence of marrow involvement and in eight of 36 patients with normal marrow histology. The correlation between marrow histology and positive tumor growth approached but did not reach statistical significance (P < 0.08). Tumor growth was detected in three of six patients with abnormal bone scans and in nine of 37 with normal bone scans. All but one of the patients with histologically positive bone marrow also had a positive bone scan. The proportion of patients with positive tumor growth was similar regardless of the presence or absence of liver or central nervous system metastasis.

Colonies scored at 12–14 days had a distinct appearance and were easily distinguished from the occasional granulocyte-macrophage colony seen. Tumor colonies were tightly spherical and composed of 30–200 cells. Morphological analysis of individual colonies showed cells consistent with small cell carcinoma as characterized by high nucleocytoplasmic ratio, 1–3 nuclei, and negativity for myeloperoxidase and nonspecific esterase stains. Electron micrographs of all plucked colonies showed single or multiple dense-core granules of neuroendocrine type and approximately 70 nm in diameter confirming the small cell carcinoma nature of the same (Fig. 1).

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DISCUSSION

The prognostic implications of detecting small cell carcinoma beyond the primary and adjacent lymphatic drainage sites are well documented (1). Conventional techniques of bone marrow aspirates and biopsies demonstrate tumor involvement in 10–21% of patients at initial diagnosis and frequently serve as the standard to which other techniques are compared (1, 3).

A recent report by Stahel and colleagues using SM1, a monoclonal antibody which recognizes SCLC cells, suggests that the frequency of marrow involvement by SCLC is substantially higher than that revealed by light microscopy. With the use of indirect immunofluorescence, cells reactive with the monoclonal antibody were detected in 13 of 23 patients with normal bone marrow histology (9). In addition 50% of patients with otherwise disease limited to the chest were found to have bone marrow involvement by this technique. In our study we have attempted to expand and complement these observations by using as our in vitro end-point the detection of malignant cells with replicative potential.

Histological evaluation of bone marrow samples fractionated through a Percoll-based discontinuous gradient from patients with neural crest-derived tumors, has been reported by MacFarlane and coworkers (10). In this study morphological evaluation of the different cell fractions identified malignant cells in only one of 16 patients with negative bone marrow aspirates. The patients in this study were not evaluated with bilateral marrow exams or with bone core biopsies, so it is possible that more aggressive staging procedures would have identified bone marrow malignant involvement in this patient.

Detection of bone marrow involvement by SCLC by the growth of clonogenic tumor cells has been the subject of several studies (4, 7). Carney and coworkers have reported growth of malignant colonies in four of four marrow specimens which were histologically positive for small cell lung cancer (4). In this same study colony growth was not detected in seven histologically negative bone marrow specimens. Pollard and coworkers have reported positive malignant growth in three of 37 (8%) patients with histologically negative bone marrow specimens (5). In our study when bone marrow mononuclear cells were separated over a single density gradient (1.077) results similar to these were seen with tumor growth detected in two of 36 (5.5%) patients with histologically negative specimens. In contrast when identical bone marrow specimens were separated through a discontinuous density gradient, positive tumor growth was detected in eight of 36 (22%) patients. These differences are highly significant ($P < 0.01$) by the sign test. Documentation that small cell cancer was growing in the culture plates was provided by morphology of colonies individually plucked as well as electron micrographs of the colonies from three of these patients showing the dense core granules characteristic of small cell carcinoma.

In our study greater than three colonies per culture from at least one of the cultured fractions was required to call a marrow culture positive for tumor growth. With this definition tumor growth was seen in a total of 12 of 43 patients when cells were
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separated by DDG as compared to four of 36 through Ficoll-diatriozate centrifugation ($P < 0.01$). We believe that the increased sensitivity of DDG in detecting cells with clonogenic potential results from increasing the concentration of malignant cells within the different densities and thus increasing the plating efficiency to detectable levels. Evidence for this conclusion is provided by finding a significant increase in the mean number of tumor colonies in FxF2 as compared to FxFD.

Finally we have detected tumor clonogenic growth in five of 26 patients (19%) in whom extensive pretreatment staging procedures failed to detect disease outside the ipsilateral lung and as such were classified as having limited disease. It is too early to know at this point if this finding will adversely influence the response to antineoplastic chemotherapy or overall survival as compared to patients with limited disease without bone marrow tumor clonogenic growth. Several institutions have initiated autologous bone marrow transplant programs in efforts to increase the curability of patients with limited disease (11). It is thus presumably important that the autologous infused marrow be free of tumor. Further studies oriented at improving the plating efficiency of SCLC in semisolid cultures, through the addition of growth factors such as bombesin, could improve our ability to detect residual disease. Separation of bone marrow mononuclear cells by a discontinuous density gradient could be used to enhance the sensitivity of these assays in detecting disease and help avoid the reinfusion of tumor cells after ablative chemotherapy.

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