Positive Correlation between Histological Tumor Involvement and Generation of Tumor Cell Colonies in Agarose in Specimens Taken Directly from Patients with Small-Cell Carcinoma of the Lung

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ABSTRACT

Twenty-seven specimens for in vitro agarose clonogenicity testing were obtained from 25 patients with small-cell carcinoma of the lung (SCCL). The specimens were obtained from bone marrows, pleural effusions, lymph nodes, and liver biopsies. Colony formation was seen in 14 of 15 specimens that were histologically involved with SCCL, but no colony growth was seen in the 12 patient specimens without histocytological evidence of SCCL, including seven bone marrow specimens. Cytological examination of the agarose colonies confirmed their SCCL origin. Colonies reached sizes of 50 to 1000 cells in 7 to 10 days, indicating an in vitro doubling time of less than 24 hr, remarkably shorter than the population doubling times measured in patients. None of the 100 clones picked from these specimens demonstrated the ability to continuously replicate in vitro. These results show an excellent correlation between agarose colony formation and histological tumor involvement and a more rapid in vitro doubling time than that seen in vivo and demonstrate that standard tissue culture conditions do not allow demonstration of a self-renewing stem cell in fresh tumor specimens of SCCL.

INTRODUCTION

Methods to grow human tumors directly from biopsies of patients in a clonogenic assay in semisolid medium should allow study of the biology of human cancer and the identification of specific growth factor requirements of each tumor type and may predict for chemotherapy responses in the patients (4, 6, 7, 11). If therapeutic and biological studies are to be based on the clonogenic assay, it is important to provide cytological examination of individual colonies, correlate the production of colonies with tumor involvement in the patient specimens, and systematically test for the "stem cell" (self-renewing nature) of the tumor cells in vitro.

SCCL accounts for 25% of all lung cancers, and, in spite of initial good responses to chemotherapy in 90% of patients, median survival is short (8- to 14 months), and few patients are cured (3, 9). Screening of in vitro drug sensitivity of this tumor should be useful in the selection of optimal chemotherapy for individual patients. However, the in vitro growth requirements of SCCL have, both in our experience and that of others (5, 10), been stringent, so that, except for single cases, no systematic study has been undertaken on the clonogenicity in agarose of SCCL obtained directly from patients (4, 6).

We report here on the successful cloning in a semisolid medium of tumor cells from specimens of metastatic human SCCL taken directly from patients. We have found that an excellent correlation exists between histological involvement of the specimens and the development of colonies but that the cells do not self renew in vitro to form continuous cell lines.

MATERIALS AND METHODS

Patients and Specimens. Twenty-seven specimens from 25 patients with histologically documented SCCL undergoing approved protocol staging procedures and biopsies were studied. Thirteen patients had not received chemotherapy prior to the collection of cells; the remainder were in relapse following primary chemotherapy or chemoradiotherapy. All patients had extensive-stage disease at the time of study and had been off all therapy for 3 weeks prior to specimen collection. Specimens were obtained from aspiration of pleural effusions, bone marrow biopsy and aspiration, percutaneous needle lymph node biopsies, and liver biopsies obtained under direct vision at peritoneoscopy. Part of each specimen was processed for cytological and histological examination, except for pleural effusions which only were examined cytologically. Single-cell suspensions were prepared by mincing, passing through a 60-gauge stainless steel mesh, and gentle pipetting of the specimens in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Grand Island Biological Co.) and penicillin (50 units/ml) and streptomycin (50 μg/ml). The viability of cell preparation as determined by trypan blue exclusion ranged from 40 to 90%, with the lowest viability in the lymph node specimens.

Culture Assay for SCCL Colony-Forming Cells. Single cells were counted in a hemocytometer, and 1 to 10 × 10^4 viable nucleated cells per ml were suspended in culture medium and 0.3% agarose (Seakem, Rockland, Maine) at 40°. One ml of the mixture was plated in triplicate in 35-mm plastic Petri dishes containing a base layer of 0.5% (v/v) agarose in culture medium that had hardened.

Cultures were incubated at 37° in a humidified atmosphere of 5% CO₂ - 95% air. Plates, examined with an inverted phase microscope, were initially checked to confirm that only single cells had been plated and, thereafter, checked on alternate days for growth. Colonies (≥50 cells) were counted 7 to 14 days after plating when colony size and viability were optimal. Individual colonies were picked with a Pasteur pipet and either suspended in Saccamano’s fixative for 30 to 60 min or transferred to liquid culture medium in individual 0.2-ml microwells of a 96-well microtiter II plate (Falcon Plastics, Oxnard, Calif.). These cultures were fed weekly and observed for continuous...
replication for 8 weeks. Cells in fixative were placed on glass slides directly or by cytocentrifugation and allowed to dry. The slides were then stained with hematoxylin and eosin, and the cytology of the cells was compared with the tumor cells in the original cell suspension and in the biopsy specimen. For comparison, 2 permanent SCCL cell lines (NCI H60 and NCI N231) established at this laboratory were handled in an identical fashion. NCI H60 was established in vitro from a cytologically established at this laboratory were handled in an identical fashion. NCI H60 was established in vitro from a cytologically positive pleural effusion of a patient with SCCL in relapse from primary chemotheraphy. NCI N231 was established from a SCCL tumor heterotransplanted into athymic nude mice. Both cell lines grow as floating cell aggregates and have been maintained in continuous culture for greater than 12 months. They have human enzyme markers and express features of amine precursor uptake and decarboxylation cells, including high dopa decarboxylase activity (1), formaldehyde-induced fluorescence, and neurosecretory granules. Both cell lines are typical SCCL histology.

RESULTS

Growth and Identification of Colonies. Colony formation was found in 14 of 15 specimens histocytologically involved in SCCL. However, no colonies or "clusters" (cell aggregates of less than 50 cells) were observed in the histocytologically negative specimens (Table 1). The numbers of colonies per plate ranged from 20 to 250, yielding a CFE per plated nucleated cell of 0.02 to 0.25%. The approximate percentage of the nucleated cells that cytologically were SCCL was determined by examination of the stained original cell suspensions, and it was calculated that the CFE per plated SCCL was 0.05 to 1.5%. This closely correlated with the CFE of established SCCL cell lines of 1 to 5% (Table 1). The number of colonies per plate for each positive specimen was within ±20% (S.D.) of the mean of triplicate samples, and the number of colonies observed formed a direct linear correlation with the number of cells plated. Cell doubling was observed 24 to 48 hr after plating. Although initial growth was brisk, cell death usually occurred 14 to 21 days after plating. Cultures were not refed, and no attempt was made to serially subculture the clones in agarose. Colonies consisted of approximately 100 to 1000 tightly packed cells forming tight spherical aggregates (Fig. 1). The colonies reached their peak size in 7 to 10 days, indicating an in vitro doubling time of less than 24 hr. The morphology of the colonies did not vary from one positive specimen to another, although colony size in each specimen showed a wide range. Only colonies of greater than 50 cells were scored, although clusters were observed in many cytologically positive specimens and were seen in the single positive pleural effusion which failed to yield colonies. The SCCL cell lines cloned well in agarose (Table 1), but, in contrast to fresh tumor specimens, the initial growth of these cells in agarose was slow, and colonies reached their peak size in 14 to 18 days. In addition, colony size was more uniform in the cell lines when compared to fresh tumor specimens.

Cytological examination of multiple colonies from each positive specimen revealed only cells with the typical cytological appearance of SCCL (Fig. 1). Granulocyte colonies were not seen. In addition, the cells had the same cytological characteristics as the tumor cells in the original biopsy specimen. A total of 100 colonies were successfully picked from 12 of the 15 positive specimens and transferred into liquid culture medium. Microscopic examination of the microwells confirmed the presence of cells in each. Clones were transferred 5 to 10 days after plating when active growth was observed. None of these transferred colonies demonstrated continuous replication in vitro. In contrast, 16 of 18 colonies picked from control agarose cultures of the 2 SCCL cell lines replicated continuously after transfer to microwell culture (Table 1).

DISCUSSION

These studies have demonstrated that specimens containing histologically identifiable metastatic tumor from patients with SCCL can form colonies in agarose with an efficiency of approximately 1% per SCCL cells plated and 0.02 to 0.25% of the nucleated cells plated. This compares favorably to other CFE for fresh tumor cell samples (4, 7, 11). Cytological examination of colonies demonstrated that these colonies contained tumor cells, and in all instances the cells in the colonies were cytologically identical with the tumor cells in the original clinical specimens. In contrast, the absence of colony formation in the cytologically negative specimens (including 7 bone marrow specimens) suggests that the system is not permissive for granulocyte or other nonmalignant cell colony formation. Examination of multiple colonies revealed only cells with a morphology consistent with SCCL. These data indicate that colony growth was due to proliferation of SCCL cells in vitro. However, inasmuch as heterogeneity of SCCL has been demonstrated in vivo (1), it is possible that the cloning cells may represent a selected subpopulation of SCCL cells in the tumor specimens. If approximately 1% of the cytologically identifiable SCCL cells can form colonies in this assay, one can estimate that cytologically negative specimens contained less than 0.1 to 0.01% SCCL cells. It will be of great importance to correlate the presence or absence of clonogenic cells with the ability to form tumors. This would have great implications for autologous bone marrow transplantation protocols in progress at our own

<table>
<thead>
<tr>
<th>Source</th>
<th>SCCL</th>
<th>SCCL*</th>
<th>No. of colonies with clonal growth</th>
<th>No. of colonies/10^6 nucleated cells plated</th>
<th>% of CFE for SCCL plated</th>
<th>No. of colonies demonstrating continuous replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Specimen Pleural effusion</td>
<td>0/4</td>
<td>4/5</td>
<td>100-200</td>
<td>0.4-1.5</td>
<td>0/10</td>
<td></td>
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<tr>
<td>Bone marrow</td>
<td>0/7</td>
<td>4/4</td>
<td>30-100</td>
<td>0.3-1.0</td>
<td>0/30</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0/1</td>
<td>2/2</td>
<td>68-250</td>
<td>0.09-1.0</td>
<td>0/25</td>
<td></td>
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<tr>
<td>Lymph node</td>
<td>0/0</td>
<td>4/4</td>
<td>20-48</td>
<td>0.05-0.1</td>
<td>0/35</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0/12</td>
<td>14/15</td>
<td>20-250</td>
<td>0.05-1.5</td>
<td>0/100</td>
<td></td>
</tr>
<tr>
<td>Established SCCL Lines</td>
<td>NCI H60</td>
<td>1/1</td>
<td>1000</td>
<td>1.0</td>
<td>8/8</td>
<td></td>
</tr>
<tr>
<td>NCI N231</td>
<td>1/1</td>
<td>5600</td>
<td>5.6</td>
<td>8/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2/2</td>
<td>1000-5600</td>
<td>1.0-5.6</td>
<td>16/18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Of the 27 clinical specimens from 25 patients, 15 were histologically positive (SCCL+) and 12 were negative (SCCL -). Seven of 15 positive specimens and 6 of 12 negative specimens were obtained from patients who had not received prior therapy for SCCL.

CFE = (number of colonies x 100)/(number of SCCL cells plated).
and other institutions because of the known propensity of SCCL to involve the bone marrow (8).

The in vitro doubling time of SCCL tumor cells in the clonogenic assay is considerably more rapid than the population doubling times found in patients (2) and also appears to be more rapid than that in established SCCL cultures. Whether this represents cell death in vivo or exit of cells from the proliferative cycle remains to be determined.

The ability of primary tumor cells forming colonies to continuously replicate has not been vigorously tested in other systems (1—4). We have shown that a large number of SCCL colonies failed to continuously replicate after transfer to microcultures under conditions where established SCCL lines clone and exhibit continuous replication. This observation suggests either that: (a) the cells generating these colonies do not have unlimited self-renewal capacity; or (b) the cells require specific growth factors or conditions for continued growth that are not supplied in our test system. Identification of such factors or conditions would be of potential therapeutic interest.

In conclusion, we have demonstrated that: (a) SCCL cells obtained from metastatic deposits form colonies in agarose with a CFE of 0.02 to 0.25% of the nucleated cells plated and up to 1.5% of the SCCL cells plated; (b) there is an excellent correlation between colony formation and histocytopathological involvement of specimens with SCCL; (c) the tumor cell origin of these colonies has been confirmed by cytological examination; and (d) the colonies do not yield continuously replicating cell lines. These studies should form the basis for testing chemotherapeutic sensitivity of SCCL in vitro and determining the growth factor requirement of SCCL.

REFERENCES

Fig. 1. Morphological appearance of SCCL colonies. A, colonies in soft agarose 7 days after seeding a bone marrow sample histocytologically positive for SCCL. The SCCL cells are in tightly packed spherical aggregates. Phase contrast, × 32. B, a single colony, picked from the soft agarose plate illustrated in A, containing several hundred cells, fixed in Saccomanno’s fluid, and stained with hematoxylin and eosin. H & E, × 100. C, liver aspirate demonstrating 3 hepatic cells with granular cytoplasm and a group of metastatic SCCL cells. H & E, × 400. D, dispersed cells from a single colony from the patient illustrated in C 10 days after seeding in agarose. The cells have the typical cytological appearances of SCCL cells, scant cytoplasm, nuclear molding, finely granular chromatin, and inconspicuous nucleoli. H & E, × 400.
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