The Distinction of Small Cell and Non-Small Cell Lung Cancer by Growth in Native-State Histoculture


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ABSTRACT

Histological analysis remains the primary method of distinguishing between small cell (SCLC) and non-small cell lung cancer (NSCLC). This distinction has significant impact therapeutically because of their relative difference in chemoresponsiveness (J. D. Minna et al., Principles and Practice of Oncology, pp. 396–474, 1981). Yet for at least 10% of lung tumors, pathologists will disagree upon the classification (A. R. Feinstein et al., Am. Rev. Respir. Dis., 101: 671–684, 1970). Furthermore, current neuroendocrine markers lack specificity for SCLC although the presence of these markers may help predict chemosensitivity (S. L. Graziano et al., J. Clin. Oncol., 7: 1375–1376, 1989; S. B. Baylin, J. Clin. Oncol., 7: 1375–1376, 1989; C. I. Berger et al., J. Clin. Endocrinol. Metab., 53: 422–429, 1981; A. F. Gazdar et al., Cancer Res., 45: 2924–2930, 1985). In vitro growth characteristics may more accurately reflect biological properties of aggressiveness and susceptibility to chemotherapy. In this study, 3-dimensional gel-histoculture was used to retrospectively distinguish between SCLC and NSCLC. Tumor explants from 78 patients with NSCLC and 13 patients with SCLC were grown in gel-supported histocultures with an overall success rate of 92%. These 2 types were distinguishable by their 3-dimensional in vitro tissue architecture. In addition, proliferation rates were measured by the incorporation of tritiated thymidine (Hd Thd). The percentage of cells labeled in the most proliferative active regions of the autoradiographs was termed the growth fraction index (A. F. Gazdar et al., Cancer Res., 45: 2924–2930, 1985; R. A. Vescio et al., Proc. Natl. Acad. Sci. U.S.A., 84: 5029–5033, 1987; R. M. Hoffman et al., Proc. Natl. Acad. Sci. U.S.A., 86: 2013–2017, 1989). The mean growth fraction index for pure small cell lung cancer was 79 ± 10%, differing markedly from that of 35 ± 19% for mixed small cell/large cell tumors, adenocarcinoma (38 ± 16%), large cell undifferentiated carcinoma (40 ± 18%), and squamous cell carcinoma (33 ± 15%) (P < 0.001 in each case). We therefore conclude that 3-dimensional gel-histoculture is a useful means of distinguishing pure SCLC from NSCLC, which may improve treatment decision making.

INTRODUCTION

Lung carcinomas are clinically divided into two major types: SCLC and NSCLC (1). For patients with lung carcinoma, the accurate determination of tumor type significantly influences treatment decision making. In general, SCLC is much more responsive to chemotherapy and consequently this comprises the mainstay of treatment. This is in contrast to NSCLC, which includes adenocarcinomas, squamous cell carcinomas, and large cell undifferentiated carcinomas of the lung. NSCLC is relatively chemoresistant and thus primarily treated with surgical resection for local disease (1, 2). Histological differences between these tumor types can be subtle and interobserver variability in classification occurs relatively frequently (3). Small cell lung tumors have been identified by their neuroendocrine properties, yet significant overlap in the presence of these biological markers exists (4–8). In fact, a recent study demonstrated increased chemoresponsiveness for NSCLC tumors possessing neuroendocrine markers (4). SCLC is further divided into 3 subtypes, “pure” small cell, mixed small cell/large cell, and combined small cell carcinoma (9). Recent studies have shown a poorer response to chemotherapy and shorter survival in patients with the mixed subtype (10). Clearly, biological and histological markers that distinguish characteristics predictive of general chemoresponsiveness would aid treatment decision making.

With a goal of distinguishing biologically between small cell and non-small cell lung carcinoma, we have utilized the native state histoculture system developed by us that successfully cultures human solid tumors at a high success rate (11–14). In the native-state system, tumor explants are grown on 3-dimensional collagen-containing gels in histocultures whereby proliferative cells are labeled with tritiated thymidine (Hd Thd). Autoradiographs are then made from tissue sections to determine cellular proliferation by grain formation due to [3H]Td Thd incorporation. The percentage of labeled cells is then measured to determine the GFI. We have previously shown the correlation of GFI with stage and grade in breast and ovarian carcinomas (13).

In this report, we demonstrate that the growth characteristics of small cell lung tumors differ markedly from those of non-small cell lung cancer. The GFI of small cell lung cancer is markedly elevated and can be used along with the histoculture growth patterns to reliably distinguish small cell from non-small cell lung cancer. We also demonstrate the ability to grow lung tumors in vitro from 92% of the samples obtained.

MATERIALS AND METHODS

Tumor Tissue Acquisition. Tumor samples were obtained from various hospitals in the San Diego region as part of a study to determine which in vitro biological parameters correlate with clinical data. In addition, specimens came from Alabama and Los Angeles via overnight delivery systems.

Tumor Histoculture. Tumors were removed surgically and placed into culture medium (Eagle’s minimal essential medium containing Earle’s salts, glutamine, 10% fetal calf serum, nonessential amino acids, and gentamicin) by the pathologist and transported to the laboratory.

The tumors were minced into 1- to 2-mm-diameter pieces and planted on previously hydrated collagen-containing gel matrices (Health Design Industries, Rochester, NY) within 48 h of removal from the patient. Six 1-mm pieces of tissue, each representing a different region of the tumor, were explanted atop one 1 × 2 cm gel to overcome sampling error due to tumor heterogeneity. After allowing tumor growth in culture for 10 days, [3H]Td Thd (4 μCi/ml; 1 Ci = 37 GBq) was added to label replicating cells for 4 additional days.

The cultures were then washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% formalin overnight. The histocultures were consequently dehydrated and rinsed with paraffin. The gel was then placed in an embedding cassette, then embedded so
that all 6 pieces of tumor were at the front of the paraffin block. The block was then sectioned using a microtome so that a continuous ribbon of 5-μm sections was made, each containing 6 representative pieces of tumor. Approximately 10–15 sections of the ribbon were placed on each slide for autoradiographic processing and staining. Two slides were made of each condition. The slides were deparaffinized and then coated with Kodak NTB-2 emulsion in a dark room and allowed to expose for 5 days at 4°C before developing. After rinsing, the slides were stained with hematoxylin and eosin.

Determination of GFI. The slides were then analyzed in a blinded fashion with a microscope epi-illuminated with light polarized by an IGS cube (Nikon). Replicating cells were easily determined by the presence of bright green-reflecting silver grains over the cell nuclei that had incorporated [3H]Thd. Cells were considered labeled if 10 or more grains were present over the cell nucleus. Benign stromal cells were excluded from the measurement by their morphological appearance. When counting, each slide was scanned at ×40 or ×100 power to locate the areas of maximum label. Once these areas were ascertained, the percentage of cells undergoing DNA synthesis was determined in at least 3 of the visual fields for each tumor piece at ×200. The GFI was calculated by dividing the number of labeled tumor cells by the number of total tumor cells.

Acquisition of Pathology Reports. The final pathology report was used to determine stage, grade, and histological class. Tumors were determined unclassifiable if the histological class was equivocal or did not fall into the following types (pure small cell, mixed small cell/large cell, squamous cell, large cell, or adenocarcinoma). Five bronchioalveolar cell tumors were cultured and classified as adenocarcinomas for this report.

Photomicrographs. The slides were reanalyzed at ×200 and ×400 in a blinded fashion. Photographs were taken of at least 3 separate areas showing the greatest cell labeling and then grouped by tumor number. Two observers were shown representative photographs of small cell and non-small cell lung tumors that were taken at an earlier time and not used later. They were then asked to group individual tumors by patterns of cell labeling and tumor invasion into the gel, by reviewing the photographs.

RESULTS

Differentiation of Small versus Non-Small Cell Tumors by GFI. Tumors were obtained from 9 patients with pure small cell lung cancer. All specimens came from the primary tumor and all 9 tumors were successfully cultured in vitro. Large areas of tumor growth into the collagen gel were evident in each case, with a high percentage of cell labeling. The GFI was calculated for each small cell lung tumor specimen and ranged from 67% to 93% with a mean of 79% (SD ± 10%) (Fig. 1).

Four additional tumors were identified as being of the mixed small cell/large cell subtype of SCLC by the criteria of the International Association for the Study of Lung Cancer (9). These tumors were analyzed separately and GFIs ranged from 16% to 60% with a mean of 35% (SD ± 19%).

Tumors from 71 patients with non-small cell lung cancer were planted on collagen gels. Culture success rate was 91%. GFIs were calculated blindly with a mean of 37% (range = 8%–78%, SD ± 16%) for the NSCLCs as a group. For squamous cell tumors the mean GFI was 33% (range = 8%–68%, SD ± 15%) (Fig. 1). The mean GFI for large cell tumors was 40% (range = 17%–74%, SD ± 18%), whereas the mean GFI for adenocarcinomas was 38% (range = 10%–78%, SD ± 16%) (Fig. 1).

The mean GFIs between the 3 classes of non-small cell lung tumors were remarkably similar, ranging from 33% to 40%. This was in marked contrast to the mean GFI for small cell tumors of 79% and was statistically significant (P < 0.001). All nine small cell tumors had a GFI > 66%, whereas only 4 of 71 non-small cell tumors had such a high GFI (Fig. 1). The mixed small cell/large cell tumors had GFIs similar to the NSCLCs with a significantly lower mean than that of the pure SCCs (P < 0.001).

Differentiation of Small versus Non-Small Cell Tumors by Growth Patterns. Fig. 2, a–c, was taken from 3 different small cell tumors and is fairly representative of the growth pattern obtained for this tumor type. The tumors were extremely cellular, frequently containing extensive regions of heavily labeled cells with infiltration into the gel matrix. The pattern of SCC tumor growth was remarkably consistent in contrast to the wider phenotypic range of tumor growth present for the non-small cell lung tumors.

Most NSCLC explants had large areas of central necrosis with peripheral labeling in smaller clusters of cells. Although differentiation between the three non-small cell types was not possible by the pattern of labeling and growth, certain characteristics of each tumor type seemed to be present. Large cell tumors tended to grow into the gel matrix peripherally and had the greatest cellularity of the NSCLC types, with many cells having a high cytoplasm-to-nuclear ratio (Fig. 3, a–c). Adenocarcinomas frequently grew in duct-like patterns on the explant periphery with moderate cellularity within the tumor and moderate gel invasion (Fig. 4, a–c). Finally, squamous cell tumors grew in swirls within the tumor explant, which often contained large areas of necrosis (Fig. 5, a–c). Most of the cell labeling of squamous cell tumors occurred in scattered cells far out in the gel matrix. Some of the five bronchioalveolar cell tumors, interestingly, grew as a monocellular sheet that matched the pattern of growth in vivo (data not shown). The 4 mixed small cell tumors had growth patterns more comparable to the NSCLCs. All 9 pure small cell tumors were correctly identifiable by photomicrographs alone by two separate blinded observers.

DISCUSSION

The ability to reproducibly culture human tumor explants in vitro has been a goal for many reasons. Prognostic information

![Lung Cancer](image-url)
Fig. 2. Representative histological autoradiograms of tumor explants from 3 different patients with small cell lung cancer (a, from patient 526; b, from patient 743; c, from patient 701). Tumor explants of 1 mm² in size were in culture for 11 days followed by 4 days of labeling with [³H]dThd (4 μCi/ml). After fixation, dehydration, and paraffin embedding, the histocultures were sectioned onto slides and coated with emulsion. After a 5-day exposure, the slides were stained with hematoxylin and eosin. Epi-illumination polarization microscopy at × 400 was used to identify replicating cells, depicted by bright green grains over cell nuclei. Small cell tumors were characterized by consistent high cellularity and cell labeling as demonstrated in a, b, and c. See “Materials and Methods” for further methodology.

may be obtainable by measuring in vitro growth characteristics that would influence treatment aggressiveness. Individualized drug therapy may be obtainable by measuring in vitro drug sensitivities. New drug development may progress more rapidly if an in vitro system can be developed that closely mimics in vivo conditions. Finally, patterns of in vitro growth may be able to distinguish different tumor classes.

We have developed an in vitro culturing system for human tumor explants that maintains much of the 3-dimensional in vivo tumor structure, is more rapid than nude mouse heterotransplantation, and is highly successful in a variety of solid human tumor types (11-14). Tumor explants are cultivated on floating collagen-containing gels and then labeled with [³H]-
dThd to quantify cell proliferation by autoradiography.

In a recent study, the ability to establish cell lines from small cell lung tumors in vitro had a success rate of 31.3% (15). In the native-state histoculture system described here, the culture rate for a 2-week period was greater than 90%. Klein et al. (16) mechanically dissociated small cell and non-small cell lung carcinomas onto feeder layers of a nonmalignant murine C3H10T1/2 cell line. Patterns of growth on the monolayer were used to differentiate between the various small and non-small cell tumor types with a high degree of accuracy. Overall culture success rates matched those in our study. However, the method of Klein et al. (16) is nonquantitative and the tumor cell growth pattern is not as representative of the in vivo state.

As mentioned above, there are frequently difficulties in distinguishing small cell and non-small cell lung tumors. This is

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**Fig. 4.** Representative histological autoradiograms of tumor explants from 3 different patients with adenocarcinoma (a, from patient 159; b, from patient 193; c, from patient 496). Adenocarcinoma tumor explants showed the greatest cellularity and cell labeling in polygonal outgrowths from the tumor edge or bordering ducts within the tumor piece. See Fig. 2 and “Materials and Methods” for methodology.

**Fig. 5.** Representative histological autoradiograms of tumor explants from 3 different patients with squamous cell carcinomas. (a, from patient 1043; b, from patient 369; c, from patient 502). Tumor explants from squamous cell carcinomas frequently had large areas of central necrosis with low cellularity monolayers along the tumor explant edge. Scattered cells deep in the gel matrix were often present and usually labeled. A unique strand-like pattern of growth along the tumor periphery was present in one explant as depicted in c. See Fig. 2 and “Materials and Methods” for methodology.
an important distinction clinically since the respective treatment protocols differ significantly. The presence of neuroendocrine markers is sometimes used to characterize SCLC tissues. Unfortunately, these markers are not yet specific with their expression in as many as 20% of NSCLC tumors (4–17). Indeed, the presence of these markers has been shown to predict greater chemoresponsiveness in patients with NSCLC in *vitro* and *in vivo* (4, 18). These results suggest a common cell lineage between these tumor types (5, 7, 19, 20). Further evidence supporting this theory came after insertion of v-Ha-ras genes in certain SCLC-derived cell lines was shown to induce cell-line characteristics more typical of large cell undifferentiated lung carcinoma (21).

The presence of tumor heterogeneity also suggests a morphological continuum among lung cancer cell types. Many tumors have isolated regions with characteristics of differing histological subtypes. This heterogeneity is further supported by autopsy studies. Five of 40 patients with biopsy-proven small cell carcinoma had evidence of only NSCLC at autopsy (22). These five patients had variable responses to chemotherapy and in general had longer-than-expected survival, suggesting a less aggressive tumor type. Interestingly, 4 of these 5 patients had presented with the intermediate subtype of SCLC, using the older WHO classification scheme (23).

The mixed small cell/large cell subtype of SCLC seems to be less responsive to chemotherapy than the pure small cell subtype with a 16% versus 46% complete response rate in a National Cancer Institute study (10). This also suggests that the mixed subtype of SCLC has diverse biological characteristics that may influence tumor aggressiveness and chemosensitivity. There were 4 cases of small cell lung tumors obtained and cultured in our study that were classifiable as the mixed small cell/large cell subtype. These tumors had a variable GFI ranging from 16% to 60% with a mean of 35%. All four of these tumors primarily had features of large cell carcinoma, and in 3 of the pathology reports the pathologist suggested that treatment be based upon the large cell component. It is hoped that the GFI represents a biological marker that could distinguish between the aggressive and indolent cases of the mixed small cell/large cell subtype of SCLC.

With the native-state histoculture technique used here, tumors classified as SCLC by histological analysis were successfully distinguished by both growth fraction index and growth patterns *in vitro* from NSCLC tumors. This offers the opportunity to study the biology of each tumor type, particularly with regard to regulation of cell proliferation. Since this method of characterization represents a “biological” assay based on cell proliferation, it is hoped that a more accurate prediction of chemoresponsiveness of both SCLC and NSCLC will be obtained. Identification of small cell and non-small cell lung cancers with high GFI s may be a means of distinguishing the most chemoresponsive tumors.

Furthermore, *in vitro* drug sensitivities can be obtained by the addition of chemotherapeutic agents prior to tumor labeling. Thus, this *in vitro* system offers, in addition to the diagnostic distinction demonstrated in this study, the opportunity to evaluate new and standard agents against these tumor types. Further retrospective and prospective analysis will be necessary to further correlate *in vivo* tumor drug responsiveness with the parameters of *in vitro* growth patterns and GFI to determine the full potential of this methodology.

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