In Vitro Study of the Biology of Small Cell Carcinoma of the Lung

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We have developed three types of experimental systems for the study of SCCL: (1) serially heterotransplanted tumors in athymic nude mice; (2) continuous, clonable cell cultures; and (3) direct clonogenic assays for tumor specimens. These systems have their own individual advantages, applications, and limitations, but these are interrelated and complementary. The study of these systems has greatly aided our understanding of the biology of SCCL, and its relationship to other lung cancers and the APUD cell system. In addition, new markers for SCCL have been identified, such as a creatine kinase and its BB isoenzyme (CK-BB). These cellular markers may have clinical applications, as serum levels of CK-BB are an indicator of tumor burden. Assays for clonogenic tumor cells may permit selection of optimal drug combinations for the treatment of individual tumors. Variant cultures having the morphology of SCCL, but lacking some or all of the other features, have been identified. While our systems have been used primarily for biological studies, they have clinical applications for both diagnostic and therapeutic purposes.

INTRODUCTION

Small cell carcinoma of the lung (SCCL) is one of the most fascinating of human tumors. It is well established as a morphological, biological, and clinical entity, distinct from other lung cancers. Initially, it was considered an undifferentiated tumor that was associated with a variety of paraneoplastic syndromes caused by "ectopic" hormone secretion. The discovery, in the late sixties, that some cells in most of the tumors contained membrane bound, dense core, neurosecretory-like granules [1,2] led to their inclusion in the amine precursor uptake and decarboxylation (APUD) cell system of Pearse [3]. It was presumed that SCCL and bronchial carcinoids arose from endocrine cells (Kultschitzky) in the normal respiratory mucosa which expressed APUD properties[4]. Pearse postulated that all APUD cells arose from the neural crest, and it was presumed that Kultschitzky cells and SCCL had a different embryological origin from the rest of the endodermally derived respiratory mucosa and non-SCCL lung cancers. However, as we learned more facts about SCCL, it became even more enigmatic. Why did SCCL secrete some APUD cell hormones frequently, and others seldom or not at all? Why did some SCCL tumors contain non-SCCL elements, and how could some SCCL tumors undergo
morphological conversion to other tumor types, especially as the two groups were
supposed to be derived from different germ layers? Do all SCCL cells express APUD
properties, or only the small percentage that have visible neurosecretory granules? Is
SCCL a homogenous group of tumors, or can certain non-SCCL tumors mimic
SCCL morphologically? There are few endocrine cells in the normal adult respiratory
tree, and the premalignant and early neoplastic changes of SCCL are not well
documented. Despite a few reports [5,6], suitable animal models are not generally
available or well studied. To further our understanding of the biology of SCCL, we
undertook the establishment and characterization of tissue culture cell lines from
human SCCL tumors.

THE BASIC APPROACH

Our basic approach was threefold: (1) heterotransplant lung tumors into athymic
nude mice and subpassage them; (2) establish continuous, clonable cultures of lung
cancers free of contamination by stromal cells and prove that these replicating cell
lines were malignant and had biochemical and morphologic features consistent with
SCCL; (3) clone lung cancer specimens directly in semi-solid medium. These
approaches have individual advantages and disadvantages, and are used for different
studies. For these reasons, they are discussed separately. However, as will be shown
later, they are not entirely independent, and some studies require the use of more
than one type of approach.

While our studies concentrated on SCCL, tissue culture lines from non-SCCL lung
cancers were also established, and their properties were compared and contrasted
with those of the SCCL lines. Cell lines were established from primary and metastatic
lung tumors from treated and untreated patients. Most non-SCCL lung cancers were
surgically resected specimens from untreated patients. Because SCCL and mesotheli-
omas are seldom resected, their cell lines were established from metastatic lesions of
treated and untreated patients, and included biopsies of bone, nodes, subcutaneous,
brain, and liver metastases and malignant effusions. Because SCCL are particularly
susceptible to damage by handling procedures, the most successful specimens were
those that required the least mechanical or chemical manipulations. These included
malignant effusions and bone marrow aspirates. The cells in these sites were relatively
viable, and were already disaggregated into single cells or small clusters. In addition,
tumor cells in effusions may be regarded as already having been adapted to “in vivo
culture” conditions, and were more easily adaptable to in vitro culture. The least
successful specimens were those coming from solid lesions such as node or subcutane-
ous metastases. These lesions frequently had extensive areas of necrosis, and required
considerable disaggregation, either mechanically or by trypsinization.

HETEROTRANSPLANTATIONS OF SCCL TUMORS

Athymic nude mice are suitable recipients for the heterotransplantation of several,
but not all, human tumors [7,8]. The work of several investigators indicates that nude
mice have some degree of resistance to heterotransplantation, and that such resis-
tance may be due to components of the immune system such as natural killer (NK)
cells [9,10]. We compared and contrasted the subcutaneous (sc) and intracranial (ic)
inoculation of SCCL tumors and cultures [11]. SCCL tumor specimens had a take
rate of 42 percent when inoculated sc, and 89 percent when inoculated ic. The sc
tumors grew as expansile, seldom invasive, non-metastasizing, and non-lethal
tumors. Failure to metastasize was not due to lack of vascular invasion, as tumor
emboli were present in the veins draining the tumors. In contrast to sc tumors, ic
tumors required 10- to 1000-fold fewer tumor cells for successful takes. Ic lesions invariably involved the meninges, with or without involvement and invasion of other areas of the brain. They were invariably fatal, but they failed to metastasize to distant locations. Extradural or solitary intracranial lesions were not observed. The histological appearances of both sc and ic tumors were identical to those of the intermediate subtype of SCCL, with inconspicuous nucleoli and scant cytoplasm, numerous mitoses, extensive areas of necrosis, and DNA staining of elastic fibrils. The tumors could be subpassaged with take rates of 100 percent, and could be established as continuous cultures with take rates approaching 100 percent. Biochemical and ultrastructural studies indicated that their properties were similar to those of fresh and cultured tumors.

Sc tumors are useful for various biological and therapeutic studies, and for growing large quantities of tumor cells. Ic inoculation is a useful tool, especially when small numbers of tumor cells are available, and may provide a model for meningeal carcinomatosis. Both inoculation sites provide further sources for the establishment of continuous cultures.

**PROPERTIES OF CONTINUOUS CELL CULTURES**

We have established more than 35 continuous cultures from 30 patients with SCCL [11,12]. While initial attempts to establish SCCL cultures were seldom successful [12], with experience and development of newer techniques, we have boosted our success rate to approximately 70 percent (provided the specimen contains adequate numbers of viable tumor cells). SCCL cultures consisted of floating cell aggregates which replicated relatively slowly and cloned at relatively low efficiencies. They were aneuploid, had typical SCCL cytology, and induced tumors in nude mice having typical SCCL histology. Non-SCCL lung cancer cultures were also aneuploid and tumorigenic, but, in contrast to SCCL cultures, they demonstrated substrate adherence and relatively short doubling times and high cloning efficiencies. These findings are paradoxical, as the doubling time of SCCL tumors in patients is relatively short. SCCL tumors and cultures have nutritional requirements that are different from those of other forms of lung cancer [13], and our results suggest that routine culture media may lack one or more factors required for the optimal in vitro growth of SCCL. As described below, SCCL cultures have provided major new insights regarding the biology of SCCL and its variants, about its relationship to other lung cancers, and about APUD cells in general.

**CLONOGENIC ASSAYS FOR SCCL TUMORS**

We have adapted methods to grow human tumors directly in a clonogenic assay in semi-solid medium [14,15] and we can successfully clone the majority of lung cancer specimens [13,16]. A single cell suspension (about $10^3$ cells/dish) is plated in a layer of 0.3 percent agarose in growth medium over a base layer of 0.5 percent agarose in growth medium. We have cloned 57/65 (88 percent) of SCCL specimens, and 11/16 (69 percent) of non-SCCL lung cancer specimens. Colonies of SCCL contain 50 to more than 1,000 cells. The colony-forming efficiency is relatively low for almost all human tumors including SCCL (0.003–0.36 percent). However, many cells demonstrate the ability to replicate for a limited number of times, with the formation of clusters consisting of less than 50 cells. These cells are probably also replicating tumor cells. Colonies can be harvested with pasteur pipettes and characterized. SCCL colonies had typical SCCL morphology, including the presence of neurosecretory granules. While only a few individual colonies (20/210) could be successfully
cultured in liquid medium, pooled colonies from 8/10 (80 percent) specimens induced tumors in nude mice after ic inoculation [11], confirming the stem cell origin of the colonies. Cell cultures were established from grossly visible ic tumors, but not from those that were microscopic, thus providing additional sources of continuous cultures.

Clonogenic assays were developed to allow for eventual selection of therapy for the individual patient. They can also be used to screen new drugs. Our preliminary observations using fresh specimens and established cell lines [DNC et al, unpublished data], with drugs clinically useful in the treatment of SCCL (adriamycin, vincristine, VP16, vindesine, and BCNU), indicate that tumors from untreated patients are sensitive, while those from relapsed patients are resistant. Thus, our findings confirm the clinical observation that relapsed SCCL tumors tend to be resistant to further chemotherapy.

SELECTIVE GROWTH OF SCCL IN A DEFINED MEDIUM

We have developed a hormone supplemented, serum free, medium for the growth of SCCL [17]. The medium consists of five components: selenium, hydrocortisone, insulin, transferrin, and 17 B-estradiol (HITES). SCCL cell lines have been maintained in HITES for more than 12 months, with retention of their characteristic morphological, ultrastructural, and biochemical features. Because HITES medium is selective for SCCL, 14/15 (92 percent) of fresh SCCL tumor specimens propagated successfully in it for periods of 3–15 weeks, and 10 of these specimens were established as continuous cultures [13]. In contrast, the vast majority of non-SCCL lung cancers and stromal cells do not replicate in HITES medium. Thus, use of this medium is a highly selective method for the propagation of SCCL cells, and we have used it for the rapid augmentation of tumor cells in specimens not otherwise adequate for drug testing by clonogenic assays. In addition, HITES medium is useful for the purification and identification of peptides and other SCCL cell products because, in contrast to serum supplemented medium, it contains relatively few complex molecules. We are currently exploring its use for the selective growth of the precursor cells of SCCL present in the normal respiratory tree. In addition, we would like to develop selective growth formulas for the other histologic types of lung cancer.

SCCL AND THE APUD CELL SYSTEM

The identification of neurosecretory granules and serotonin in some SCCL tumors led Pearse to include them in his APUD cell system [3]. APUD cells are a diffuse collection of cells scattered in many organs and having amine handling properties. Pearse originally proposed that all APUD cells were derived from the neural crest, but has recently modified his views [18] in the face of overwhelming evidence to the contrary. We have summarized the evidence that SCCL and their presumptive precursor cells are, like the rest of the respiratory tree, derived from endoderm [19]. This evidence includes failure to demonstrate the neuroectodermal origin of the respiratory and GI endocrine cells, the relatively frequent presence of tumors of mixed SCCL and other histologies, the in vivo and in vitro conversion of SCCL to other tumor types, and the occasional expression of APUD cell properties by other histological types of lung cancer.

Despite the continuing controversy regarding the APUD cell concept, SCCL cells express the full range of APUD cell properties. While studies of clinical and autopsy specimens [20,21] indicate a wide range of expression, SCCL cultures express them uniformly [12]. Thus, all cultures have easily identifiable neurosecretory granules and
very high levels of the key APUD cell enzyme L-dopa decarboxylase (DDC, EC 4.1.1.28). They fluoresce after exposure to formaldehyde vapor (indicating amine precursor uptake and, perhaps, decarboxylation), and most secrete one or more polypeptide hormones [12,22,23]. Analysis of more than 130 clones of seven SCCL cultures indicated that high levels of DDC were retained in all cultures [AFG et al, unpublished data]. In contrast, the clones had multiple patterns of polypeptide secretion [24].

**CREATINE KINASE—A NEW MARKER FOR SCCL**

SCCL tumors and cultures are characterized by very high levels of creatine kinase (CK, EC 2.7.3.2) predominantly in the form of its BB isoenzyme (CK-BB) [25]. Electrophoretic analyses of CK isoenzymes indicated that CK levels in SCCL were quantitatively but not qualitatively different from those in normal lung and other lung cancers. Because CK-BB is measured by a sensitive radioimmunoassay, while total CK activity is measured enzymatically, we determined serum levels of CK-BB in 67 untreated patients having SCCL. Elevated levels were present in 16/41 (39 percent) with extensive-stage disease, but in none of 26 (0 percent) of patients with limited-stage disease. Our preliminary findings [DNC et al, unpublished observations] indicate that elevated serum levels reflect tumor bulk, and are present in only a small percentage of patients having metastases in one site, while 100 percent of patients with metastases to four or more sites have elevated levels. Serum levels may also be used to monitor response to therapy, and relapse of tumor after therapy induced remission.

**SCCL TUMOR VARIANTS**

While we have indicated above that SCCL cultures uniformly express APUD cell properties and high levels of CK-BB, certain cultures, established from patients having classical SCCL by light microscopic criteria, lacked these properties. Some of the variants retained SCCL morphology and growth characteristics in culture, while others did not. Ultrastructure of these tumors indicated that they consisted of primitive cells lacking neurosecretory granules, but they had numerous desmosomes and prominent bundles of filaments. By conventional ultrastructural criteria they would be classified as poorly differentiated squamous cell carcinomas. Because they have many of the features of the basal cells in the respiratory mucosa, the possibility that they represent true undifferentiated tumors or perhaps “stem” cell tumors must be considered. While these tumors are treated as SCCL, we do not as yet know whether their response to cytotoxic therapy is similar to that of SCCL.

**MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN SCCL CELL LINES**

SCCL tumors frequently undergo changes in morphology after therapy [26,27], especially to large cell carcinoma, although virtually any form of lung cancer may be represented. The change may be partial or complete. Morphological change is accompanied by loss of the biochemical characteristics of SCCL. Similar changes have been observed in our cell lines, including long-term cultures and heterotransplants passaged many times [19,28]. In vitro changes are accompanied by loss of all APUD markers, but high levels of CK-BB are retained, indicating discordant regulation of the two enzyme markers CK and DDC [25]. Altered morphology in vitro is accompanied by rapid growth, high cloning efficiencies, loss of typical SCCL morphology in in vitro culture and, sometimes, by an increase in DNA content. Our interpretation of these changes is discussed below.
Our lung cancer cell lines, and similar ones established by others [22,23], have greatly aided our understanding of a complex tumor. Because of the constant biochemical and ultrastructural characteristics of SCCL, we regard it as a differentiated tumor, closely related to other polypeptide secreting APUD tumors. We presume it arises either directly from endocrine cells in the respiratory tree, or, more likely, from common precursor cells committed to differentiation along endocrine lines [19]. While the function of the Kulitschitzky cells are not known, at least two polypeptides, bombesin and calcitonin [29,30], are present in them. We presume that their function is the elaboration of multiple polypeptides that have nutritional or differentional effects on neighboring mucosal cells. Whether one type of endocrine cell exists in the lung or whether multiple functional forms occur is a moot point. We further presume that elaboration of hormones by SCCL is an extension of the function of their normal counterparts, and should not be regarded as "ectopic." Altered morphology to large cell carcinoma with loss of biochemical and ultrastructural characteristics should be regarded as loss of differentiation. The presence of other non-SCCL elements, both before therapy and, more frequently, after therapy, suggests a close interrelationship between all forms of bronchogenic carcinoma. Their presence cannot be explained on the basis of two separate embryological origins for lung cancers. These changes may have clinical significance, as SCCL tumors are therapy sensitive, while other forms of lung cancer are resistant. Human tumor cell lines have greatly increased our knowledge and understanding of SCCL. They also have clinical applications, both for diagnostic and therapeutic purposes.

REFERENCES

1. Hattori S, Matsuda M, Tateishi R, et al: Oat-cell carcinoma of the lung containing serotonin granules. Gann 59:123-129, 1968
2. Bensch K, Corrin B, Pariente R, et al: Oat cell carcinoma of the lung: its origin and relationship to bronchial carcinoids. Cancer 22:1163-1177, 1976
3. Pearse AG: The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J Histochem Cytochem 17:303-313, 1969
4. Bonikos DS, Bensch KG: Endocrine cells of bronchial and bronchiolar epithelium. Am J Med 63:765-771, 1977
5. Reznik-Schuller H: Ultrastructural alterations of APUD cells during nitrosamine-induced lung carcinogenesis. J Path 121:79-82, 1976
6. Blair WH, Heinen J, Tremback K: An in vitro cell line established from "oat-cell like" lung carcinoma experimentally induced in rodents. Proc Am Assoc Cancer Res 21:82, 1980
7. Shimosato Y, Kameya T, Nagai K, et al: Transplantation of human tumors into nude mice. J Natl Cancer Inst 56:1251-1260, 1976
8. Reid L, Shin S: In The nude mouse in experimental and clinical research. Edited by J Fogh, B Giovanella. New York, Academic Press, 1978, pp 313-351
9. Gershwin ME, Ikeda RM, Kawakami TG, et al: Immunobiology of heterotransplanted human tumors in nude mice. J Natl Cancer Inst 58:1455-1461, 1977
10. Reid LM, Minato N, Gresser I, et al: Influence of anti-mouse interferon on the growth and metastasis of virus persistently-infected tumor cells and human prostatic tumors in athymic nude mice. Proc Natl Acad Sci USA 78:1171-1175, 1981
11. Gazdar AF, Carney DN, Sims HL, et al: Heterotransplantation of small cell carcinoma of the lung into nude mice. I. Comparison of intracranial and subcutaneous routes. Submitted for publication
12. Gazdar AF, Carney DN, Russel EK, et al: Establishment of continuous, clonable cultures of small cell carcinoma of the lung which have amine precursor uptake and decarboxylation cell properties. Cancer Res 40:3502-3507, 1980
13. Carney DN, Bunn PA, Gazdar AF, et al: Selective growth of small cell carcinoma of the lung obtained
from patient biopsies in serum-free hormone supplemented medium. Proc Natl Acad Sci USA 78:3185-3189, 1981
14. Hamburger AW, Salmon SE: Primary bioassay of human tumor stem cells. Science 197:461–463, 1977
15. Pavelic ZP, Slocum HK, Rustum YM, et al: Growth of cell colonies in soft agar from biopsies of different human solid tumors. Cancer Res 40:4151–4158, 1980
16. Carney DN, Gazdar AF, Minna JD: 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. Cancer Res 40:1820–1823, 1980
17. Simms E, Gazdar AF, Abrams P, et al: Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor-supplemented medium. Cancer Res 40:4356–4363, 1980
18. Parsee AG, Takor-Takor T: Embryology of the diffuse neuroendocrine system and its relationship to the common peptides. Fed Proc 32:2288–2294, 1979
19. Gazdar AF, Carney DN, Guccion JG, et al: Small cell carcinoma of the lung: Cellular origin and relationship to other pulmonary tumors. In Small cell lung cancer. Edited by A Greco, PA Bunn, R Oldham. New York, Grune and Stratton, 1981, pp 145–175
20. Baylin SB, Weisburger WR, Eggleston JC, et al: Variable content of histaminase, L-dopa decarboxylase and calcitonin in small-cell carcinoma of the lung: Biologic and clinical implications. N Engl J Med 299:105–110, 1978
21. Baylin SB, Abeloff MD, Goodwin G, et al: Activities of L-dopa decarboxylase and diamine oxidase (histaminase) in lung cancers and decarboxylase as a marker for small (oat) cell cancer in culture. Cancer Res 40:1990–1994, 1980
22. Pettengill OS, Sorenson GD, Wurster-Hill DH, et al: Isolation and growth characteristics of continuous cell lines from small-cell carcinoma of the lung. Cancer 45:906–918, 1980
23. Sorenson GD, Brinck-Johnsen T: Hormone production by cultures of small cell carcinoma from lung. Proc Am Assoc Cancer Res 18:248, 1977
24. Radice PA, Dermody WC: Clonal heterogeneity of hormone production by continuous cultures of small cell carcinoma of the lung. Proc Am Assoc Cancer Res 21:41, 1980
25. Gazdar AF, Zweig MH, Carney DN, et al: Levels of creatine kinase in lung cancer tumors and cultures. Cancer Res 41:2773–2777, 1981
26. Abeloff MD, Eggleston JC, Mendelsohn G, et al: Changes in morphologic and biochemical characteristics of small cell carcinoma of the lung. Am J Med 66:757–764, 1979
27. Matthews MJ, Gazdar AF: Pathology of small cell carcinoma of the lung and its subtypes: A clinicopathologic correlation. In Lung Cancer: Advances in research and treatment. Edited by RB Livingston. The Hague, Martinus Nijoff, 1981, pp 283–306
28. Gazdar AF, Carney DN, Baylin SB, et al: Altered morphological, biological and biochemical characteristics in long term cultures and heterotransplanted tumors. Proc Am Assoc Cancer Res 21:51, 1980
29. Wharton J, Polak JM, Bloom SR, et al: Bombesin-like immunoreactivity in the lung. Nature 273:769–770, 1978
30. Becker KL, Monghan KG, Silva OL: Immunocytochemical localization of calcitonin in Kultschitzky cells of human lung. Arch Path Lab Med 104:196–198, 1980