



Growth of Human Lung Tumor Cells in Culture

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I. INTRODUCTORY REVIEW

I.1. Cell Culture and Lung Cancer Development

Lung cancer is the most frequent cause of cancer deaths in both men and women [Landis et al., 1998]. Environmental air pollutants, such as tobacco smoke and asbestos, are accepted as the major causes of the disease [Parkin et al., 1994]. Most lung cancers are of epithelial origin, with a few exceptions, such as mesothelioma, which is derived from a non-epithelial cell type (mesothelial cells) attributable to asbestos exposure [Mossman et al., 1990; Rom et al., 1991; Jaurand, 1991; Pass and Mew, 1996]. Epithelial lung cancers are generally classified into two major types: small cell lung cancer (SCLC, about 25% of lung cancers) and non-small cell lung cancer (NSCLC, about 75% of lung cancers). Tumors of the former type disseminate widely and early and are seldom cured by surgical resection, whereas the latter may be cured by surgery if diagnosed early. In addition, SCLC tumors initially have much better response to cytotoxic therapies than do NSCLC. NSCLC consists of at least three major cell types—squamous carcinoma, adenocarcinoma, and large cell carcinoma—in addition to several subtypes. The cell type origin of these cancer cells is still unknown.

Like many other tumors, the development of lung cancer is a multi-step process the nature of which has not been fully elucidated. Although the total annual number of cases has declined recently, probably due to a decreased trend in cigarette consumption, the incidence and mortality rates of lung cancer have increased alarmingly, especially among the female population and populations in developing countries. The nature of this increase is poorly understood. In addition, the overall cure rate for lung cancer remains low, at only about 13%. Reasons for such a low cure rate are many. One of the major reasons is related to the difficulty in the early detection of the disease with current techno-

logies, which are not sufficiently sensitive. It is assumed that the earlier the detection of these tumors, the better the chances for successful treatment. The development of routine techniques for the isolation and in vitro maintenance of these transformed cells, especially at their early stages of transformation, will provide a significant advance in this research area.

1.2. Requirements for Growth Factors and Hormones for Lung Cancer Cells to Grow

Despite deficiency in the development of an in vitro system for studying the multiple stages of lung cancer cell development, cultivation of primary cells from normal and lung cancer specimens has brought some success. These advances may be useful in the future for the development of an in vitro cell culture model, allowing lung cancer cells at different stages of transformation to be studied. Such a system would help to generate biomarkers for the early detection of lung cancer. We, as well as other researchers, initially used a serum-supplemented medium to establish primary cultures of normal specimens and lung tumor specimens. These attempts have proven to be unsuccessful [Wu, 1986; Lechner et al., 1986]. Cell proliferation is either inhibited or unresponsive to growth factors. Furthermore, fibroblast overgrowth is a constant concern whenever a mixed population of cells is grown in serum-containing medium. Deficiency in growth factors or presence of cell growth inhibitors in serum-supplemented medium could explain the poor in vitro growth of these lung cancer and normal epithelial cells. To eliminate the problems created by adding serum to medium, serum-free and hormone- and growth factor-supplemented defined media were developed. Many years ago, Sato and colleagues proposed that most cells in vivo are regulated by hormones and growth factors in interstitial fluid, and not by serum [Barnes and Sato, 1980]. In pursuance of this concept, their laboratories developed various defined media for culturing many different types of cells [Hayashi and Sato, 1976; Bottenstein et al., 1979]. Based on their findings and by subsequent use of trial and error, our laboratory [Wu, 1986] and Lechner's [Lechner et al., 1986] were the first to develop a serum-free, hormone-supplemented medium for primary human airway epithelial cell growth and long-term maintenance in culture. The principal supplements in this medium are insulin, transferrin, hydrocortisone, epidermal growth factor (EGF), retinoid, and a supplement essential for cyclic adenosine monophosphate (cAMP) generation. Later, this medium was modified further, and, with the incorporation of collagen gel substratum and culture at the air-liquid interface [Wu, 1997], the primary cells can now differentiate

properly, similar to the epithelium seen in vivo. In this regard, retinoid is a very important supplement for cell differentiation as well as for cells to grow on a collagen gel.

Using a similar trial-and-error approach, Simms et al. [1980] were the first to develop a serum-free, hormone-supplemented defined medium that supported the continuous replication of established SCLC cell lines. This defined medium initially contained five supplemental growth factors: hydrocortisone, insulin, transferrin, estradiol, and selenium (HITES). Carney et al. [1981] used HITES-supplemented medium to establish cell lines directly from SCLC tumor samples. While HITES-supplemented medium was superior to the routine 10% serum-supplemented medium for the establishment of SCLC cultures, addition of small amounts of serum to HITES-supplemented medium resulted in an increased growth advantage [Gazdar et al., 1990]. With these media, HITES and HITES plus 2% serum, more than 70% of tumor cells derived from SCLC cancers could be cultivated in vitro [Oie et al., 1996].

The culturing of NSCLC presented a different set of problems from the culturing of SCLC. Most surgically removed NSCLC specimens usually contained large amounts of tumor cells with a heterogeneous phenotypes. Before the incorporation of hormones and growth factors into the medium, very few NSCLC cell lines were established in medium supplemented with serum. Most of these cell lines were relatively or completely undifferentiated and were not representative of the tumors from which they were derived. In addition, phenotypic differences among different tumor cells required different culture media and growth supplements. It was realized relatively early that it was easier to establish cultures from adenocarcinomas and large cell undifferentiated carcinomas than from squamous cell carcinomas. By trial and error, Brower et al. [1986] were the first to develop ACL-3 medium for the growth of lung adenocarcinoma cells. This medium was modified further to produce ACL-4, which, with or without serum, is now quite suitable for culturing many types of adenocarcinomas with a success rate of more than 55% [Gazdar and Minna, 1996]. We have initially used the serum-free defined medium [Lechner et al., 1986; Wu, 1986] (without retinoid), originally designed for culturing primary human airway epithelial cells, to grow cells derived from various lung adenocarcinoma specimens with limited fibroblast overgrowth. After this initial manipulation, serum was added to selectively promote the growth of the cancer cells because normal epithelial cells are more sensitive to the inhibition by serum. With this approach, several adenocarcinoma cell lines were established from these limited specimens [Yang et al., 1992; Chu et al., 1997].

The major problem with culturing squamous carcinoma cells of

NSCLC is that fully differentiated epidermal cells do not replicate. The difficulty is in finding the right medium to balance cell differentiation with cell growth. Retinoid is an important regulator that can inhibit terminal differentiation of squamous cells but promote mucous cell differentiation. However, most squamous cancer cells are inhibited by treatment with retinoids. Another approach is to reduce the calcium level in the medium because high calcium is required for the expression of terminal differentiation [Levitt et al., 1990].

3T3 fibroblasts, whose proliferation has been arrested by irradiation or mitomycin C, have been used as a feeder layer to support the continuous cultures of various epidermal keratinocytes [Rheinwald and Beckett, 1981; Allen-Hoffmann and Rheinwald, 1984]. A similar supportive role was seen with feeder layers in culturing squamous cancer cells from lung. By trial and error, it was determined that NSCLC cells derived from squamous cell carcinoma required serum, cholera toxin, and EGF, in addition to the common requirements for insulin, transferrin, and hydrocortisone [Gazdar and Oie, 1986]. Despite this progress, the successful rate in culturing lung squamous carcinoma cells in vitro remains low, at the 25% level [Gazdar and Minna, 1996].

Table 1.1 summarizes a comparison of the growth requirements for normal lung and various lung cancer cells. Several of the supplements are commonly required for these cells, regardless of their origin. These supplements are insulin, transferrin, hydrocortisone, selenium, and various nutrients, as well as ethanolamine and phosphoethanolamine, which are involved in lipid metabolism. However, some supplements are required specifically for individual cell types. For instance, the critical difference between cancer and normal cells is the requirement for retinoid. Retinoids are cytotoxic for most cancer cells, whereas normal cells are dependent on them for cell differentiation and growth on a collagen substratum. Between SCLC and NSCLC cells, the most critical distinction is with EGF and bombesin supplementation. EGF is required for all NSCLC cultures, but is not required by SCLC cells. In contrast, bombesin is needed for SCLC cells, but not required for culturing NSCLC cells. SCLC cells produce bombesin; however, these cells still require the bombesin supplement when they are plated at low cell density. Thus, there is an autocrine/paracrine mechanism involved in the growth of SCLC cells. A major difference between squamous and other NSCLC cells is in the requirement for low calcium to sustain cell proliferation. A high calcium level enhances terminal cell differentiation of squamous cell type.

From this comparison, we can draw a simple conclusion that there are two basic approaches for culturing various lung cancer cells: one for SCLC and the other for NSCLC. In the former approach, the HITES-based supplemented medium is sufficient to initiate primary

Table 1.1. A comparison of growth responses of human normal airway epithelial cells and SCLC and NSCLC cells to nutrients and growth factor/hormone supplements.

Medium components	Normal airway epithelial cells ^{1,3}	SCLC ³	NSCLC ³ Adeno/Large	Squamous
<i>Nutrients</i>				
Calcium: low or high ⁴	low	low/high	low/high	low
Ethanolamine	+	+	+	+
Phosphorylethanolamine	+	+	+	+
Selenium	+	+	+	+
<i>Hormones/Growth factors</i>				
Insulin	+	+	+	+
Transferrin	+	+	+	+
EGF	+	-	+	+
Bombesin	-	+	-	-
Cholera toxin	+	+	+	+
Fetal bovine serum (5%)	-	-	+	+
Hydrocortisone	+	+	+	+
Triiodothyronine	+	-	+	-
Vitamin A	+	-	-	-
17- β -Estradiol	-	+	-	-

^{1,2}Based on Wu, 1986 and Lechner et al., 1986.

³Based on Oie et al., (1996)

⁴Low calcium: <0.1 mM.

⁵Cholera toxin equivalent chemicals, like arginine vasopressin and epinephrine, were used for SCLC and primary human airway epithelial cultures, respectively.

cultures from SCLC tumors. Additional factors, such as bombesin and cholera toxin, or serum, can be added to stimulate cell growth. This approach has been used before with great success [Oie et al., 1996]. The second approach is the use of the serum-free defined medium that was developed for culturing of primary normal airway epithelial cells. This type of medium contains combined supplements ("C" supplements), which are similar to the supplements in the ACL-4 medium developed by Oie et al. [1996], except for the addition of bovine hypothalamus extract (BHE). Using this serum-free and vitamin A-depleted medium, both normal and cancer epithelial cells from NSCLC tissue specimens will survive and proliferate, but not stromal cells. After an initial period of serum-free incubation, serum can be added to induce normal epithelial cells to express terminal differentiation, because the differentiation of most of NSCLC cells is less responsive to serum. The addition of serum may also have growth stimulatory effects on NSCLC cells. This approach has been successful for culturing cells derived from adenocarcinoma [Yang et al., 1992, Chu et al., 1997]. For squamous carcinoma cells, low calcium medium, such as the bronchial epithelial growth medium (BEGM)

Table 1.2. Two types of culture medium for culturing human lung tumor cells.

	HITES-Based Medium	C-Based Medium
<i>Basal medium</i> ¹	RPMI 1640	DMEM/F 12 or BEGM
Sodium selenite	30 nM	30 nM
Ethanolamine	10 μ M	10 μ M
Phosphorylethanolamine	10 μ M	10 μ M
Sodium pyruvate	—	0.5 μ M
Glutamine	2 mM	2 mM
Adenine	—	0.18 mM
HEPES ²	15 mM	15 mM
<i>Supplement</i>		
Insulin	5 μ g/ml	5 μ g/ml
Transferrin	5 μ g/ml	5 μ g/ml
EGF	—	10 ng/ml
Bombesin	0.1 μ M	—
Cholera toxin	10 ng/ml	10 ng/ml
Hydrocortisone	10 nM	100 nM
Triiodothyronine	—	0.1 nM
Bovine hypothalamus extract	—	15–30 μ g/ml
Bovine serum albumin (Fetal bovine serum) ³	0.2% —	0.5% (5%)

¹Antibiotics added in these media are: penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamicin (5 μ g/ml), and fungizone (5 μ g/ml).

²HEPES stock buffer is at 1.5 M, pH 7.2–7.3.

³Fetal bovine serum can be used after primary culture to enhance NSCLC cancer cell proliferation in culture.

developed by the commercial company Clonetics, can be used as the basal nutrient medium.

2. PREPARATION OF MEDIA AND REAGENTS

Table 1.2 summarizes the compositions of HITES- and C-based media. Most of these chemicals, except bovine hypothalamus extract (BHE), are available commercially (see Section 6). The commercially equivalent products to BHE are endothelial cell growth supplement (ECGS) and pituitary extract (PE).

2.1. Preparation of HITES-Based Medium

2.1.1. Nutrient Medium Preparation

(i) Supplement RPMI 1640 medium with penicillin G (100 U/ml), streptomycin (100 μ g/ml), gentamicin (5 μ g/ml), fungizone (5 μ g/ml), [*N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid] buffer (HEPES, 15 mM, pH. 7.2), ethanolamine (10 μ M), phosphoryl-ethanolamine (10 μ M), and bovine serum albumin (BSA, 2 mg/ml).

(ii) Sterilize through a 0.2 μ m membrane filter and store at 4°C.

(iii) Before use, add glutamine at 2 mM final concentration.

2.1.2. Preparation of Hormone and Growth Factor Supplements

See Sources of Materials table at end of chapter for suppliers of hormones and growth factors.

- (i) Dissolve hydrocortisone at 0.05 mM in 95% alcohol or in phosphate-buffered saline (PBSA) if a water-soluble analogue is used.
- (ii) Dissolve insulin in 1 mM acetic acid at 2.5 mg/ml.
- (iii) Dissolve transferrin in PBSA at 2.5 mg/ml.
- (iv) Dissolve 17- β -Estradiol in 95% alcohol at 0.1 mM.
- (v) Prepare sodium selenite at 10 mM in PBSA.
- (vi) Dissolve cholera toxin in PBSA at 10 μ g/ml.

2.1.3. Additional Growth Supplements for HITES-Based Medium

Prepare bombesin at 1 mM in PBSA containing BSA, 1 mg/ml.

2.1.4. Complete HITES-Based Medium

- (i) Before use, add aseptically complete RPMI-1640 nutrient medium from above preparation (2.1.1) with various hormones and growth factors for the final concentrations as shown in Table 1.2.
- (ii) Add bombesin (diluted to 0.1 mM in PBSA with BSA, 1 mg/ml) to a final concentration of 0.1 μ M, if needed.
- (iii) Store this complete HITES-based medium at 4°C for a maximum of one week.

2.2. Preparation of C and C-Modified Media

The major difference between C and C-modified nutrient media is the calcium level. C medium is a 1:1 mixture of Dulbecco's Modified Eagle's medium (DMEM) and Ham's F12, whereas C-modified medium is a low-calcium-based bronchial epithelium growth medium (BEGM) medium developed by Clonetics. These media are prepared and supplemented with antibiotics, HEPES, ethanolamine, phosphoethanolamine, glutamine, and BSA as described (see section 2.1.1).

2.2.1. Preparations of Hormone and Growth Factor Supplements

Prepare insulin, transferrin, hydrocortisone/dexamethasone, and sodium selenite (see section 2.1.2.). In addition, dissolve 100 μ g EGF in 10 ml PBSA to give 10 μ g/ml, and triiodothyronine (T3) in PBSA at 10 μ M.

2.2.2. Preparation of Bovine Hypothalamus Extract (BHE)

Prepare BHE as described previously [Robinson and Wu, 1991], summarized as follows:

- (i) Briefly homogenize 100 g of bovine hypothalamus in 200 ml ice-cold PBSA at 4°C in a blender for approximately 3–6 min.
- (ii) Pass the homogenates through 2 to 3 layers of cheesecloth, and stir for 1–2 h at 4°C.
- (iii) Centrifuge the homogenates at 13,800 g for 40 min at 4°C, and collect the supernatants and add streptomycin sulfate at 0.5 g/ml.
- (iv) Stir the mixture at 4°C for an additional 1 to 2 h.
- (v) Centrifuge the mixture again at 13,800 g for 40 min. This process can remove the lipid fraction in the extract.
- (vi) Filter-sterilize the supernatant through a 0.2 µm filter membrane.
- (vii) Store aliquots of 5 ml per vial at –20°C for 2 to 3 months.

2.2.3. Complete C and C-Modified Media

Before use for cell culture, C or C-modified nutrient media containing various antibiotics, HEPES, ethanolamine, phosphorylethanolamine, glutamine, and BSA as described above in section 2.1.1(i), is supplemented with various hormones, growth factors, and BHE as described in Table 1.2. The complete C and C-modified media can be stored at 4°C for no more than one week.

3. SAFETY PRECAUTIONS

Normally, lung cancer tissues are obtained from patients, with consent, by surgery or by bronchoscopic biopsies for medical diagnosis. The pathological stage of the tissues and status of the patient is not always clear. To avoid the transmission of diseases or infectious agents associated with the tissue, extra care in handling these materials, the wastes, and the culture, is needed. Great care should be taken to handle these biohazardous materials using good laboratory practice. Consult national and local safety guidelines before initiating this work [See also Caputo, 1996].

4. STEP-BY-STEP PROTOCOLS

4.1. Tissue Handling and Shipping

Immediately after surgical removal of tissues from patients, tissues should be immersed in a nutrient medium containing antibiotics and be kept cold while being transported to the lab. Normally, we use minimal essential medium (MEM). Others used RPMI-1640 or L-15 [Leibovitz, 1986]. We found no difference in the choice of medium for

this step. Immersion in cold medium seems to be the most critical requirement for keeping cells viable.

Protocol 1.1. Processing of Lung Tumor Samples

Reagents and Materials

Sterile

- MEM: Eagle's MEM without bicarbonate
- Petri dishes, 10 cm
- Forceps, fine
- Scissors, fine

Protocol

- (a) Clean tumor tissue further, once it has arrived at the lab, by gently swirling around with MEM.
- (b) Place the tumor specimen in a sterile 10 cm Petri dish, and add a small amount of medium to keep the tissue wet.
- (c) Remove necrotic areas, fatty tissue, blood clots, and connective tissue with forceps and scissors or crossed scalpels.
- (d) Rinse the remaining tumor tissue further with fresh MEM.

4.2. Cell Dissociation

For cell isolation, MEM is supplemented with BSA, 10 mg/ml, to prevent cell damage caused by mechanical and enzymatic treatments. The presence of BSA can also serve as a detoxification reagent to neutralize toxins potentially released by dead or dying cells. Two methods for cell dissociation will be described and, if the tissue size is large enough, both these cell-disaggregating methods should be tried. The first method is the simplest, fastest, and least traumatic method of obtaining cells from tumor tissue for culture by the mechanical spillout method [Leibovitz, 1986]. The second method is an enzymatic method.

Protocol 1.2. Harvesting Cells from Lung Tumor by Mechanical Spillout

Reagents and Materials

Sterile

- Fine scissors or scalpels, #11
- MEM-BSA medium: Eagle's MEM without bicarbonate, supplemented with BSA, 10 mg/ml
- Ficoll/metrizoate solution, e.g., Lymphosep, density, 1.077 g/cc
- Universal containers or 20 ml centrifuge tube

Protocol

- (e) Mince the tumor into very small pieces with sterile scissors or by cross-cutting with two scalpels. This mechanical teasing approach will release tumor cell aggregates, which usually adhere loosely to stromal tissue.
- (f) After repeated resuspension with a wide-bore pipette and further mincing, tumor cells are released into the medium.
- (g) Collect tumor cells by centrifugation at 200 g for 5 min.
- (h) Resuspend pellet in 5 ml medium.
- (i) Perform Ficoll/metrizoate centrifugation to eliminate erythrocytes, dead cells, and other tissue debris.
- (j) Add 5 ml Ficoll/metrizoate to universal container or centrifuge tube
- (k) Layer cell suspension carefully, without mixing, over 5 ml Ficoll/metrizoate.
- (l) Centrifuge for 10 min at 100 g.
- (m) Collect cells from interface.
- (n) Dilute cells to 10 ml in medium and centrifuge at 100 g for 5 min.
- (o) Suspend the final cell pellet at $2 \times 10^5 - 1 \times 10^6$ cells/ml in the complete HITES-based, C-based, or C-modified medium.
- (p) Seed into 25-cm² flask(s) according to the pathological status of the tumor specimen (see section 4.4).

The Enzymatic Method

Because tumor cells are loosely adherent to the stromal layer, these cells can be dislodged by incubating tissue fragments with 0.1% collagenase (in MEM).

Protocol 1.3. Disaggregation of Lung Tumor Tissue in Collagenase

Reagents and Materials

Sterile

- Collagenase (type IV, Sigma cat #C5138 or equivalent) solution, 0.1% in MEM
- MEM-BSA medium: MEM without bicarbonate, supplemented with BSA, 10 mg/ml
- Growth medium, e.g., complete HITES-based, C-based, or C-modified medium
- Ficoll/metrizoate
- Scalpels
- Universal containers or 20 ml centrifuge tubes

Protocol

- (a) Cut tissues into small pieces (about 2–5 mm diameter), and immerse in the collagenase solution.
- (b) After 30 min incubation at 37°C, mince these tissues as described in the mechanical spillout method and rinse with MEM-BSA medium.
- (c) Collect these media and centrifuge (200 g, 5 min) and resuspend in the medium.
- (d) Carry out a Ficoll/metrizoate gradient centrifugation (see Step (e) in Protocol 1.2) to remove cell debris, erythrocytes, and tissue fragments, etc.
- (e) Collect the cell pellet by centrifugation and suspend into various culture media.

There are other enzymes, such as neutral protease, elastase, trypsin, and glycoconjugate-degrading enzymes, and chelating chemicals, such as ethylene diamine tetraacetate (EDTA) or ethyleneglycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which are used in cell dissociation from tissue. Some of these enzymes and chemicals cause cytotoxic effects, but some of them do not. Thus, the selection of these dissociating enzymes and chemicals is based on trial-and-error.

4.3. Cell Culture Conditions

To initiate primary lung cancer cell culture, it is advisable to culture serum-free to avoid the overgrowth of stromal cell types. Normally, if the pathological status of the tumor tissue is known, one can use HITES-based medium for SCLC tumor cells, C medium for adenocarcinoma, and large cell carcinoma cells or C-modified medium for squamous cell carcinoma. However, if the pathological status is unclear, tumor cell suspensions should be divided and suspended into these three types of medium for culture to maximize the chance of growing tumor cells.

Protocol 1.4. Culture of SCLC Cells**Reagents and Materials***Sterile*

- Serum-free HITES-based medium (see Section 2.1)
- Fetal bovine serum (FBS)
- Dimethyl sulfoxide (DMSO)
- Cryo-tubes

Protocol

- (a) Following disaggregation seed the tumor cell suspension at 1×10^6 cells/ml in serum-free, HITES-based medium.
- (b) After 1–2 weeks incubation, most SCLC cells grow as “floating aggregates,” whereas normal cells will attach to the dish to proliferate. Thus, it is very easy to recognize if the primary culture has SCLC cells. Recover these floating aggregates from the medium by centrifugation at 200 g for 5 min).
- (c) If too much cell debris contaminates the culture, recover the viable cells by Ficoll/metrizoate centrifugation (see Step (e) in Protocol 1.2).
- (d) Occasionally, SCLC cells adhere to the dish surface. Detach these cells by gently rapping the flasks and recovering the floating cells.
- (e) During passage, it is necessary to avoid a low seeding density. Normally, maintain the seeding density at $5\text{--}10 \times 10^5$ cells/ml for each passage.
- (f) To boost cell proliferation in subsequent culture, supplement the HITES-based medium with 2–5% FBS.
- (g) Preserve SCLC cells (at $1\text{--}5 \times 10^6$ cells/ml) cryogenically in the HITES-based medium supplemented with 10% DMSO and 10–50% FBS, and store in a liquid N₂ tank.

Protocol 1.5. Culture of Tumor Cells from Adenocarcinoma and Large Cell Carcinoma**Reagents and Materials***Sterile*

- C-based medium
- Trypsin, 0.1% solution in PBSA with 1 mM EDTA
- Trypsin inhibitor, 1% solution in C medium

Protocol

- (a) Plate primary tumor cells in a 25 cm²-flask in C-based medium. The seeding density should be 5×10^6 cells/ml. After 2–4 days incubation, most of the tumor cells, as well as normal epithelial cells and stromal cells, adhere to the surface of the flask. Normally, there are few stromal cells seen in this initial culture. However, if present, these cells either will not grow or will have a very low proliferative activity. In contrast, normal epithelial cells can grow rapidly.
- (b) After a week or two in culture, trypsinize the adherent cells with 0.1% trypsin, 1 mM EDTA solution in PBSA.
- (c) After the cells have been detached, add an equal volume of 1%

- trypsin inhibitor solution prepared in C medium and recover cells by centrifugation (200 g, 5 min).
- (d) Suspend cell pellet in a fresh C medium at 1×10^6 cells/ml, and seed into tissue culture dishes or flasks.
 - (e) Change medium the next day, and then change medium continuously once every other day until confluence is reached.
 - (f) Passage confluent cultures again as before, and seed in serum-free C medium.
 - (g) To eliminate normal epithelial cells that can contaminate the cultures, treat cultures with C medium supplemented with 10% FBS.
 - (h) After a one-week incubation, passage cultures again and maintain in the serum-free C medium.

Depending on the outcome of the culture, additional serum-treatment may be needed. This approach should preserve most of adenocarcinoma and large cell carcinoma cell types in culture. If there is contamination with stromal cells in culture, the following two procedures are recommended. One is to trypsinize the culture partially, by using a low-concentration trypsin solution ($<0.05\%$), because fibroblasts are easier to detach than epithelial cells. The second approach is to perform differential attachment, because fibroblasts attach to the dish surface faster than do epithelial cells. Plate cells briefly in a tissue culture dish (2–6 h), after which the unattached cells are recovered and plated into a new dish. If the fibroblast contamination persists, these two procedures should be used repeatedly. Otherwise, a cell cloning approach (see Section 4.4.3), such as using limited dilution and clonal plating, should be used. After several passages, these tumor cells can be cryogenically preserved in C medium supplemented with 10% DMSO and 10–50% FBS and stored in a liquid N_2 tank.

4.4.1. Culturing Tumor Cells from Squamous Carcinoma

The procedure to culturing tumor cells from squamous carcinoma tumor is the same as for tumor cells from adenocarcinoma and large cell carcinoma, except that a low-calcium (0.1 mM), serum-free, C-modified medium is used. After several passages, serum (10% FBS) can be added to the squamous cancer cell cultures to boost cell proliferation.

4.4.2. Culture of Tumor Cells from Mixed Types of Lung Tumors

Occasionally, the lung tumor obtained contains mixed types of carcinoma. For growing these cells, these three types of media should be used: HITES-based and C and C-modified media. The culture conditions are the same as described above.

4.4.3. Cloning

After several passages, lung cancer cultures can be purified further by clonal selection. Clonal selection is also used for cultures with mixed morphologies.

Protocol 1.6. Cloning Cultures from Lung Carcinoma

Reagents and Materials

- Trypsin
- Trypsin inhibitor
- Conditioned medium (CM): fresh medium 1:1
- 96-well plate
- 24-well plate
- 6-well plate

Protocol

- (a) To achieve high efficiency in clonal selection, prepare CM from confluent cultures of the same cells. Briefly, media are conditioned in confluent cultures for 1–2 days. Filter these CM to remove cell debris, and then dilute with fresh culture medium at a 1:1 ratio. Use this 50% CM medium for cloning.
- (b) Dilute trypsinized cells serially from 1000 cells/ml to 1 cell/ml and plate into 96-well plates (200 μ l/well).
- (c) After 1–2 weeks' incubation, harvest colonies from those wells in each 96-well plate carrying one colony, and replate into separate wells of a 24-well plate.
- (d) After confluence, transfer the 24-well plate cultures to 6-well plates, and then to 10 cm dishes, and so on. This gradual dilution will enhance the survival of tumor cells during each transfer.

In some cases, when the serial dilution approach cannot be used; selection has to be performed on a confluent culture.

- (a) Place a drop of trypsin solution on top of the colony of interest.
- (b) After a brief incubation, pipette a drop of medium on top of the colony from a Pasteur pipette carrying culture medium with trypsin inhibitor.
- (c) Using the same pipette to scrape the colony, recover the detached cells and plate in a 24- or 96-well plate.

4.4. Characterization of Neoplastic and Differentiated Functions

Cultured cells should be characterized for their epithelial nature, neoplastic phenotype, and differentiated functions. The epithelial na-

ture can be characterized by anti-keratin antibody or by transmission electron microscope (TEM). Briefly, for floating cell aggregates, such as SCLC cultures, cells are cytocentrifuged onto glass slides and fixed with ice-cold methanol or other fixatives. For adherent cells, a direct fix with methanol is sufficient. These fixed cells are stained with anti-keratin antibody (from Sigma) according to standard immunohistochemical procedure. Fluorescence staining is preferred because a cytoskeletal filament structure is more apparent. Epithelial cell types are positive for cytokeratin staining. The staining should be negative for all stromal cells. For TEM, cultured cells are fixed in glutaraldehyde and processed for TEM according to the standard protocol. The epithelial cell type should have keratin fibers, tonofilaments, and desmosomes.

Neoplastic properties should be determined in two ways. One is an *in vitro*, soft agar colony-forming assay; the other is an *in vivo* tumorigenicity assay in either severe combined immunodeficiency (SCID) mice or immune-deficient nude mice. For the colony-forming assay on soft agar, exponentially growing cells are suspended in complete growth medium containing 0.3% agar and overlaid on 1% agarose in 10 cm tissue culture dishes ($1 \times 10^3 - 1 \times 10^4$ cells/dish). These dishes are maintained at 37°C in a CO₂ incubator for 2 weeks. The number of visible colonies is counted to determine mean values for colony-forming efficiency. Normal cells cannot grow on soft agarose, and they should be used as a negative control in this assay. Known tumor cells and cell lines that can grow on soft agarose should be included in the assay as positive controls. For *in vivo* tumorigenicity assay, harvest cells and suspend in PBSA at 2.5×10^7 cells/ml. Inject 0.2 ml from each tumor cell line dorsally into each of five 6- to 8-week old SCID or nude mice. These mice are then observed for 8 weeks for the development of tumors. The tumor can be characterized further for morphology and immunohistochemical analyses. Tumors are fixed briefly in formaldehyde and are embedded in paraffin. The histological sections of tumors are examined for cell morphology, nuclear atypia, and the presence of duct-like structures. In addition, various antibodies can be used to characterize the nature of the tumor. These antibodies, such as anti-small proline-rich protein (SPRR1B) antibody [Lau et al., 2000] and anti-involucrin antibody (Pierce), can be used to identify squamous cells. This can be supported further by the finding of the formation of a cornified envelope, which is insoluble in the presence of sodium dodecyl sulfate (SDS) and reducing agent. Anti-mucin antibody [Lin et al., 1989] and Alcian blue-PAS stain will confirm identification as adenocarcinoma.

Antibodies specific to L-dopa decarboxylase production [Baylin et

al., 1980] and to bombesin-like immunoreactivity [McMahon et al., 1984] are used to confirm an SCLC type of cancer. Where these are not expressed, i.e. in variant SCLC cell lines, creatine kinase BB isozymes (CKBB) and neuron specific enolase (NSE) may be used [Carney et al., 1985; Gazdar et al., 1985].

4.5. Variations

Other enzymatic procedures have been used successfully for lung epithelial cell isolation. These are trypsin, pronase (neutral protease), and elastase. Trypsin is used routinely for passaging cell cultures, and it can dissociate cancer cells from stromal tissues. However, reports indicate that the viability of dissociated cells is poor. Pronase and the neutral protease (e.g., Sigma's Type XIV protease) have been used for the isolation of epithelial cells from lung tissues. However, we have found that the treatment is best performed at 4°C overnight, because higher temperature treatment decreases cell viability. Elastase is used routinely for alveolar epithelial cell isolation [Dobbs and Gonzales, 2002], and it can be potentially useful if tumor cells adhere to stroma containing elastin.

The rationale for using serum-free medium instead of serum-supplemented medium is that the growth of stromal cells is limited in the former growth condition. However, this approach may also limit the growth of cancer cells, so, after several passages in serum-free medium, serum can be added to boost the proliferation of the cancer cells. However, this treatment must be performed with some caution, as the treatment may also stimulate terminal differentiation, especially of squamous cells. The other possibility is that cancer cells may produce growth factors, such as platelet-derived growth factor, to stimulate fibroblast growth. In that case, fibroblast contamination is inevitable. It is then necessary to perform a cloning or differential adherence approach to select cancer cells.

It was found from the SCLC cultures that cells can change their differentiated functions. For example, SCLC cells can be changed from a bombesin-secreting type to a non-secreting type [Carney et al., 1985; Gazdar et al., 1985]. This change is associated with the virulence of the cancer cells, which also become refractory to chemotherapy. This change also occurs in vivo. The same is true for NSCLC cells, which can be changed between a mucin-secreting cell type and a squamous cell type or can become undifferentiated. The nature of these changes is not clear and could be spontaneous or due to further mutations. For instance, *c-myc* amplification is associated with the change from classical SCLC cells to variant cells [Johnson et al.,

1986]. Because of this phenomenon, it is necessary to characterize periodically the differentiated functions of the cultured cells.

The other variation applicable to this protocol is in the *in vivo* tumorigenesis assay. It has been shown previously that cancer cells can repopulate a denuded tracheal graft. "Denuded" means that the lining epithelium has been removed [Terzaghi and Klein Szanto, 1980]. Klein Szanto and his colleagues showed that cancer cells will maintain some differentiated features while expressing malignancy in the graft [Momiki et al., 1991]. The information generated from this approach is particularly relevant and useful because airway material was used in.

5. DISCUSSION

The development of an *in vitro* model system for lung cancer cells permits a direct examination of the life cycles, differentiated functions, and genetics of these tumor cells. The life cycle study allows the understanding of growth rate, growth behavior, growth factor requirement(s), and cell cycle control mechanisms of these tumor cells. These analyses, when they are compared with normal epithelial cells, may yield information as to why the tumor cells have an unlimited life span and unregulated growth control.

Study of the differentiated function will allow an understanding of the intrinsic and ectopic phenotypic expression, the regulation of expression, particularly of the biomarkers unique to these tumor cells. The biomarkers are useful not only for early detection of these tumor cells but also as targets for immuno-therapeutic treatments. Genetic studies will permit the understanding of the nature of the mutations associated with the development of these tumors; for example, which chromosomal loci are involved. Genetic analysis will also allow the elucidation of which oncogenes and tumor suppressor genes are up- and down-regulated, and what is the control mechanism involved in the regulation of gene expression in these tumor cells. Thus, much information can be generated from the successful culturing of tumor cells *in vitro*. Such information may be difficult to obtain from *in vivo* procedures because of inaccessibility and limited cell recovery. Furthermore, the heterogeneity of cell types *in vivo* also make interpretation of data difficult.

In addition to obtaining fundamental information on regulatory mechanisms, the *in vitro* system also allows the initiation of various studies on the development of therapeutic treatment for these cancer cells. One can test directly the effects of various drugs on defined target cells and provide a rational basis for selection of drugs for chemo-

therapy. In addition, results based on in vitro chemotherapy studies of SCLC cells have led to the conclusion that there are both variant and classic types in SCLC, a finding which has significance in the treatment of this type of cancer.

Lastly, cell culture information may also help in the development of culture conditions suitable for the growth and maintenance of a pre-neoplastic cell population. Theoretically, these cells should be poised between the normal and the cancer cell types, and their growth behavior and other properties, except malignancy, characteristic of this lineage. Using pre-neoplastic cell cultures may give a better understanding of the process of carcinogenesis, and its regulation. In addition, the identification of pre-neoplastic cell populations could provide the means to identify environmental risk factors associated with particular genetic aberrations.

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SOURCES OF MATERIALS

Item	Supplier
Agar	Difco (Becton Dickinson)
BEGM	Clonetics (BioWhittaker)
BHE	see ECGS and PE
Bovine hypothalamus	Pel Freeze
BSA	Sigma
Cholera toxin	List Biologics
ECGS	Collaborative Research (Becton Dickinson)
EGF	Upstate Biotechnology
Ethanolamine	Sigma
Ficoll/metrizoate	Amersham Pharmacia, ICN, Nygaard
Fungizone	Sigma
Gentamycin	Sigma
Growth factors	Sigma
HEPES	Sigma
Hormones	Sigma
Lymphosep	ICN
MEM	Gibco (Invitrogen)
Penicillin	Gibco (Invitrogen)
Pituitary extract (PE)	Gibco (Invitrogen)
Phosphoethanolamine	Sigma
RPMI 1640	Gibco (Invitrogen)
Streptomycin	Sigma
Triiodothyronine (T3)	Sigma
Trypsin	Gibco (Invitrogen)
Trypsin inhibitor	Sigma T0800

REFERENCES

- Allen-Hoffmann, B.L., and Rheinwald, J.G. (1984) Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. *Proc. Natl. Acad. Sci. USA* 81:7802–7806.
- Barnes, D., and Sato, G. (1980) Serum-free cell culture: a unifying approach. *Cell* 22:649–655.
- Baylin, S.B., Abeloff, M.D., Goodwin, D., Carney, D.N., Gazdar, A.F. (1980) Activities of L-dopa decarboxylase and diamine oxidase (histaminase) in human lung cancers and decarboxylase as a marker for small (oat) cell cancer in cell culture. *Cancer Res.* 40:1990–1994.
- Bottenstein, J., Hayashi, I., Hutchings, S., Masui, H., Mather, J., Mc Clure, D.B., Ohasa, S., Rizzino, A., Sato, G., Serrero, G., Wolfe, R., Wu, R. (1997) The growth of cells in serum-free hormone-supplemented media. In Jakoby, W.B., and Paston, I.H. (eds) *Methods in Enzymology*. New York, NY: Academic Press, Vol. 58, pp. 94–109.
- Brower, M., Carney D.N., Oie H.K., Gazdar A.F., Minna J.D. (1986) Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.* 46:798–806.
- Caputo, J.L. (1996): Safety Procedures. In Freshney, R.I., Freshney, M.G., eds., *Culture of Immortalized Cells*, New York, John Wiley & Sons, pp. 25–51.
- Carney, D.N., Bunn P.A., Gazdar A.F., Pagan J.A., Minna J.D. (1981) Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc. Natl. Acad. Sci. USA* 78:3185–3189.
- Carney, D.N., Gazdar, A.F., Bepler, G., Guccion, J.G., Marangos, P.J., Moodt, T.W., Zweig, M.H., Minna, J.D. (1985): Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res.*, 45:2913–2923.
- Chu, Y.W., Yang, P.C., Yang, S.C., Shyh, Y.C., Hendrix, M.J.C., Wu, R., Wu, C.W. (1997) Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. *Am. J. Respir. Cell Mo. Biol.* 17:353–360.
- Dobbs, L.G., Gonzalez, R.F. (2002): Isolation and culture of pulmonary alveolar epithelial type II cells. In Freshney, R.I., Freshney, M.G., eds., *Culture of Epithelial Cells*, 2nd ed., New York, John Wiley & Sons, pp. 303–335.
- Gazdar, A.F., and Minna, J.D. (1996) NCI series of cell lines: an historical perspective. *J. Cell Biochem. Suppl* 24:1–11.
- Gazdar, A.F., and Oie, H.K. (1986) Cell culture methods for human lung cancer. *Cancer Genet. Cytogenet* 19:5–10.
- Gazdar, A.F., Carney, D.N., Nau, M., Minna, J.D. (1985). Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological, and growth properties. *Cancer Res.*, 45:2924–2930.
- Gazdar, A.F., Oie, H.K., Shackleton, G.H., Chen, T.R., Triche, T.J., Myers, C.E., Chrousos, G.P., Brennan, M.F., Stein, C.A., La Rocca R.V. (1990) Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosyntheses. *Cancer Res.* 50:5488–5496.
- Hayashi, I., and Sato, G.H. (1976): Replacement of serum by hormones permits growth of cells in a defined medium. *Nature*, 259:132–134.
- Jaurand, M.C. (1991) Observations on the carcinogenicity of asbestos fibers. *Ann. NY Acad. Sci.* 643:258–270.
- Johnson, B.E., Battley, J., Linnoila, I., Becker, K.L., Makuch, R.W., Snider, R.H., Carney, D.N., Minna, J.D. (1986) Changes in phenotype of human small cell lung cancer cell lines after transfection and expression of the c-myc proto-oncogene. *J. Clin. Invest.* 78:525–532.
- Landis, S.H., Murray, T., Bolden, S., Wingo, P.A. (1998) Cancer statistics, 1998. *CA Cancer J. Clin.* 48:6–29.
- Lau, D., Xue, L., Hu, R., Liaw, T., Wu, R., Reddys, S. (2000) Expression and regu-

- lation of a molecular marker SPR1, in multistep bronchial carcinogenesis. *Am. J. Respir. Cell Mol. Biol.* 22:92–96.
- Lechner, J.F., Stoner, G.D., Yoakum, G.H., et al. (1986) In vitro carcinogenesis studies with tracheobronchial tissues and cells. In Schiff L.J. (ed) *In Vitro Models of Respiratory Epithelium*. Boca Raton, FL: CRC Press, pp. 143–159.
- Leibovitz, A. (1986) Development of tumor cell lines. *Cancer Genet. Cytogenet* 19:11–19.
- Levitt, M.L., Gazdar, A.F., Oie, H.K., Schuller, H., Thatcher, S.M. (1990) Cross-linked envelope-related markers for squamous differentiation in human lung cancer cell lines. *Cancer Res.* 50:120–128.
- Lin, H., Carlson, D.M., St. George, J.A., Plopper, C.G., Wu, R. (1989) An ELISA method for the quantitation of tracheal mucins from human and nonhuman primates. *Am. J. Respir. Cell Mol. Biol.* 1:41–48.
- McMahon, J.B., Schuller, H.M., Gazdar, A.F., Becker, K.L. (1984) Influence of priming with 5-hydroxytryptophan on APUD characteristics in human small cell lung cancer cell lines. *Lung* 162:261–264.
- Momiki, S., Baba, M., Caamano, J., Lizasa, T., Nakajima, M., Yamaguchi, Y., Klein Szanto, A.J.P. (1991) In vivo and in vitro invasiveness of human lung carcinoma cell lines. *Invas. Metastasis* 11:66–75.
- Mossman, B.T., Bignon, J., Corn, M., Seaton, A., Gee, J.B.L. (1990) Asbestos: scientific developments and implications for public policy. *Science* 247:294–301.
- Oie, H.K., Russell, E.K., Carney, D.N., Gazdar, A.F. (1996) Cell culture methods for the establishment of the NCI series of lung cancer cell lines. *J. Cell Biochem. Suppl* 24:24–31.
- Parkin, D.M., Pisani, P., Lopez, A.D., Masuyer, E. (1994) At least one in seven cases of cancer is caused by smoking. Global estimates for 1985. *Int. J. Cancer* 5904:494–504.
- Pass, H.I., and Mew, D.J.Y. (1996) In vitro and in vivo studies of mesothelioma. *J. Cell Biochem. Suppl* 24:142–151.
- Rheinwald, J.G., and Beckett, M.A. (1981) Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous carcinomas. *Cancer Res.* 41:1657–1663.
- Robinson, C.B., and Wu, R. (1991) Culture of conducting airway epithelial cells in serum-free medium. *J. Tiss. Cult. Meth.* 13:95–102.
- Rom, W.N., Travis, W.D., Brody, A.R. (1991) Cellular and molecular basis of the asbestos-related diseases. *Am. Rev. Respir. Med.* 143:408–422.
- Simms, E., Gazdar, A.F., Abrams, P.G., Minna, J.D. (1980) Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor supplemented medium. *Cancer Res.* 40:4356–4363.
- Terzaghi, M., and Klein Szanto, A.J.P. (1980) Differentiation of normal and cultured preneoplastic tracheal epithelial cells in rat: importance of epithelial-mesenchymal interactions. *J. Natl. Cancer Inst.* 65:1039–1045.
- Wu, R. (1986) In vitro differentiation of airway epithelial cells. In Schiff, L.J. (ed) *In Vitro Models of Respiratory Epithelium*. Boca Raton, FL: CRC Press, pp. 1–26.
- Wu, R., Zhao, Y.H., Chang, M.M. (1997): Growth and differentiation of conducting airway epithelial cells in culture. *Europ. Respir. J.*, 10:2398–403.
- Yang, P.C., Luh, K.T., Wu, R., Wu, C.W. (1992) Characterization of the mucin differentiation in human adenocarcinoma cell lines. *Am. J. Respir. Cell Mol. Biol.* 7:161–171.

