GIANT CELL CARCINOMAS OF THE LUNG PRODUCING COLONY-STIMULATING FACTOR IN VITRO AND IN VIVO

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Two culture cell lines (C-Lu65, C-Lu99) were established from human giant cell carcinomas of the lung transplanted in athymic nude mice (BALB/c, nu/nu). During early passage in tissue culture, C-Lu65 grew as a loosely adherent monolayer with some piling-up and with floating cells. After 30 successive subcultures, C-Lu65 began to grow in suspended cell clusters, showing a faster growth rate. C-Lu65 was characterized by multinucleated giant cells with large abnormal nuclei and prominent nucleoli. C-Lu99 grew as adherent cells, and fewer multinucleated giant cells were observed. C-Lu65 and C-Lu99 showed some ultrastructural differences in cell surface and cytoplasmic features. Chromosomal analysis revealed numerical and structural abnormalities in both cell lines. Cell-free supernatants from both cell lines stimulated the colony formation of mouse bone marrow cells in vitro. In addition, mice bearing tumors induced by transplanting C-Lu65 and C-Lu99 showed remarkable leukocytosis without evidence of infection. These results suggest that these two cell lines release colony-stimulating factor both in vitro and in vivo.

Key words: Tissue culture — Nude mouse — Colony-stimulating factor — Lung cancer

The incidence of lung cancer in Japan is gradually increasing. Lung cancer can be divided into two major groups, small cell carcinoma (SCC) and non-small cell carcinoma (NSCC), which are distinct from each other in clinical manifestations, response to chemotherapy and biological behavior.1 Because of their higher malignancy with rapid growth and extensive metastases, however, small cell carcinoma and giant cell carcinoma (one histological type of NSCC) have many clinical problems in common, particularly incurability.1-5

Since the study of Nash and Stout in 1958,6 giant cell carcinoma of the lung has been considered to be a distinct entity. It is a rare anaplastic carcinoma characterized by large, bizarre, multinucleated cells7 and is included in group IV, large cell carcinoma, in the WHO classification (1981).8 Clinically, this tumor often shows aggressive growth, resistance to therapy and poor prognosis.3-5 In 1961 Ozzello and Stout9 reported the epithelial origin of giant cell lung cancer, based on a short-term tissue culture study. However, the histogenesis of this tumor is still obscure.

It is well known that a number of lung cancer patients often have associated paraneoplastic syndromes. Patients with giant cell carcinoma often show leukocytosis without any evidence of infection. The explanation for this seems to be that tumor cells produce a factor stimulating in vivo myelopoiesis, called colony-stimulating factor (CSF).10-12 For further analysis of this factor, cultured cell lines of giant cell carcinomas would be useful.

There are several reports on establishment of cell lines derived from small cell carcinoma of the lung.13 However, few cell lines have been derived from giant cell carcinoma. Recently Anger et al.14 reported two cell lines from large cell anaplastic carcinoma; one of
them (Lu65) was derived from a serial xenotransplant established in our laboratory. Since 1975, we have studied heterotransplantation of human lung cancers into nude mice extensively and have established 19 strains, including three giant cell carcinomas (Lu65, Lu99 and Lu116). Following the success of Anger et al., we cultivated the xenografted tumors in vitro and have established two cell lines of giant cell carcinoma. The morphology, cell kinetics, cell genetics, and CSF production of the cell lines, C-Lu65 and C-Lu99, are reported here. These cell lines are presently being used for various research projects.

MATERIALS AND METHODS

Heterotransplantation Heterotransplantation of lung cancer and serial maintenance of human tumors in nude mice were previously described. Briefly, some small fragments of tumor tissues were inoculated subcutaneously into 6- to 8-week-old athymic mice (BALB/c, nu/nu) and the tumors formed were serially transplanted in the same manner.

Tumorigenesis of cultured cells was also examined in nude mice by subcutaneously inoculating 1 to 2 x 10^6 cells suspended in 0.2 ml of RPMI-1640. Tissue Culture Tissues of tumor xenografts were minced and passed through a steel mesh. Tumor cells were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and 10mM Hepes buffer and seeded into 60 mm plastic culture dishes. Dishes were kept in a humidified incubator at 37° in 5% CO2. Cells were subcultured almost bi-weekly with pipetting and resuspension in new growth media without using any enzyme treatment.

Storage Cultured cells and minced tissues of tumor xenografts in growth media, to which 10% dimethyl sulfoxide (Sigma, St. Louis, Mo.) had been added, were frozen using a program freezer (CRYO-MED) and stored in liquid nitrogen.

Light Microscopy The histology of the tumor xenografts was examined on hematoxylin and eosin stained sections. Cultured cells were examined by phase-contrast microscopy and, in addition, cells grown on coverslips were stained by Papanicolaou's method.

Scanning Electron Microscopy Tumor cells grown on coverslips were fixed in 2.5% phosphate-buffered glutaraldehyde for 30 min, washed in the same buffer and post-fixed in 1% osmium tetroxide. After dehydration with graded ethanol solutions, the preparations were dried at the critical point, coated with gold and examined with a Hitachi 2R scanning electron microscope.

Transmission Electron Microscopy Pellets of cultured cells were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide in the same way. After dehydration, the specimens were embedded in Epon 812 resin. Thin sections were cut, stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope.

Cell Kinetics Each well of 24-well tissue culture plates (Costar) was seeded with 0.8 x 10^6/ml cells and the numbers of cells were counted in triplicate using a hemocytometer every 24 hr for one week.

Cell Genetics Karyotype analysis was done by the trypsin-Giemsa and ASG methods as described.

Colony Formation Assay Culture supernatants of the tumor cells were centrifuged at 2000 rpm for 10 min and passed through 0.45 μm filters. Femoral bone marrow cells (5 x 10^6/ml) of 7- to 8-week-old C3H/HeN mice were suspended in McCoy medium 5A with 15% FCS, 10% horse serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.3% Bacto-agar, to which a one-tenth volume of cell-free culture supernatant was added. After incubation for 7 days in 35 mm plastic plates at 37° in 5% CO2, colonies (more than 50 cells) and clusters (more than 5 cells and less than 50 cells) were counted in triplicate.

RESULTS

Source of Tumor Tissue Lu65 was derived from a 64-year-old Japanese male (case 1) who was admitted to the National Cancer Center Hospital, Tokyo in 1978. He had a tumor in the right lower lobe of the lung and enlarged supraclavicular lymph nodes. Peripheral blood cell counts of the patient showed persistent leukocytosis in the range of 11,000 to 20,000/mm³ without any evidence of infection during his clinical course. This patient died ten weeks after admission due to pulmonary insufficiency. Lu99 was derived from a 63-year-old Japanese male (case 2) who was admitted to the Matsudo National Sanatorium, Chiba because of right chest pain in 1979. He had a tumor in the right upper lobe of the lung and right pleural effusion. He had also remarkable leukocytosis in the range of 40,000 to 80,000/mm³ without any evidence of infection. He had undergone thoracotomy, but the tumor was unresectable due to invasion to the thoracic

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He died eight weeks later because of pulmonary insufficiency. Specimens were biopsied from the metastatic right supravacuicular lymph nodes of case 1 and from a metastatic pleural tumor of case 2. Both cases were histologically diagnosed as giant cell carcinoma of the lung.

Establishment in Tissue Culture Residual tissues of the biopsied specimens were used for transplantation into nude mice. Both Lu65 and Lu99 became serially transplantable in nude mice. The transplanted tumors showed the histology of anaplastic carcinoma with multinucleated giant cells, which resembled the histology of the original tumors.

Tissue culture of Lu65 and Lu99 cells was relatively easy. Lu65 cells have been maintained for approximately 2 years, and Lu99 cells for 1 year. Lu65 cells have been subcultured biweekly 144 times and Lu99 cells, 82 times. They were designated as C-Lu65 and C-Lu99. Both C-Lu65 and C-Lu99 cells produced solid tumors more than 2 cm in diameter within 6 weeks when 1 to $2 \times 10^7$ cells were inoculated into the subcutaneous tissue of nude mice. These tumors resembled the original tumors histologically (Fig. 1).

Morphological Studies C-Lu65 cells, at first, grew as a loosely adherent monolayer, with some piling-up and with a few floating cells (Fig. 2A). However, through 30 to 40 repeated subcultures, the cells gradually began to form loose cell clusters floating freely in the culture medium (Fig. 2B). C-Lu65 cells were large and pleomorphic with increased N/C ratio and formed some multinucleated giant cells. Large bizarre nuclei had one or two prominent nucleoli. The cell surface was equipped with numerous microvilli, and many stacked rough endoplasmic reticula filled with amorphous materials, polysomes and mitochondria were observed in the cytoplasm (Fig. 3A and 3B).

C-Lu99 cells grew as a tight adherent monolayer. Variation in cell size and morphology was not marked, but a few giant multinucleated cells were seen. These cells contained large nuclei with prominent nucleoli and an abundant cytoplasm, and also

Fig. 1. Histology of a tumor formed by transplanting C-Lu65 cells into a nude mouse. It was an anaplastic carcinoma with scattered multinucleated giant cells and leukocyte infiltration. Hematoxylin and eosin stain.

wall and mediastinum.
Fig. 2. (A) Phase contrast microscopy of 10th passage C-Lu65 cells. Most C-Lu65 cells grew as adherent cells. (B) In the 93rd passage, most C-Lu65 cells formed loose cell clusters and floated freely in the culture medium.
Fig. 3. (A) Scanning electron microscopy of C-Lu65 cells. Numerous microvilli can be seen on the cell surface. (B) Transmission electron microscopy of C-Lu65 cells. Many stacked rough endoplasmic reticula filled with amorphous materials, polysomes and mitochondria can be seen in the cytoplasm.
Fig. 4. (A) Scanning electron microscopy of C-Lu99 cells shows many blebs on the cell surface. (B) Transmission electron microscopy of C-Lu99 cells. Lamellar rough endoplasmic reticula and polysomes in the cytoplasm are well developed.
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showed many blebs on the cell surface and many polysomes, mitochondria and lamellar rough endoplasmic reticula in the cytoplasm (Fig. 4A and 4B).

Tonofibrils, desmosomes and secretory granules, which would indicate differentiation toward squamous cells or glandular cells, were not detected in either C-Lu65 or C-Lu99 cells.

**Growth Kinetics** As shown in Fig. 5, both C-Lu65 and C-Lu99 grew rapidly in vitro, and the doubling times of these cell lines were 36 to 48 hr. Comparison of the 6th and 93rd passage cells of C-Lu65 recovered from liquid nitrogen showed that the latter had a more rapid growth rate and higher saturation density than the former.

**Chromosome Analysis** The karyotypes of C-Lu65 and C-Lu99 are shown in Fig. 6A and 6B. Both exhibited numerous numerical and structural abnormalities. Some of the abnormalities appeared in more than 50% of cells, and therefore, were referred to as marker chromosomes. C-Lu65 was hyperdiploid (52+2, n=48) with several markers, such as t(?;1) and 17p+. One-third of the cells had double minute chromosomes. C-Lu99 was hypotriploid (60+2, n=44) with marker chromosomes, such as 1p−, 10q+, 11p+ and 12p+ (Fig. 6B).

The #1 chromosome was involved as a marker chromosome in both cell lines. The translocation observed in C-Lu65 took place on the long arm, whereas interstitial deletion of C-Lu99 was observed on the short arm of #1 chromosome. Therefore, no common pattern of abnormality was noted.

**CSF Production** Conditioned media from both C-Lu65 and C-Lu99 cell lines stimulated colony formation of mouse bone marrow cells in soft agar. C-Lu99 cells, however, showed higher colony-stimulating activity than C-Lu65 cells (Table I). In addition, when tumors were produced by injecting C-Lu65 and C-Lu99 cells into nude mice, the tumor-bearing mice showed remarkable leukocytosis compared with normal mice (Table II). Furthermore, in agreement with the in vitro results, mice bearing Lu99 tumors showed more pronounced leukocytosis than mice bearing Lu65 tumors.

Table I. Colony Formation Assay of Conditioned Media of C-Lu65 and C-Lu99 Cells

<table>
<thead>
<tr>
<th>Conditioned media</th>
<th>Colonies</th>
<th>Clusters</th>
</tr>
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<tbody>
<tr>
<td>C-Lu65</td>
<td>1.3±0.9</td>
<td>37.7±3.0</td>
</tr>
<tr>
<td>C-Lu99</td>
<td>12.7±1.4</td>
<td>50.5±10.2</td>
</tr>
<tr>
<td>Media alone</td>
<td>0.0±0.0</td>
<td>0.7±0.7</td>
</tr>
</tbody>
</table>

Data are expressed as means±SD of assays performed in triplicate.

Table II. Peripheral Blood Leukocytes of Nude Mice Bearing Tumors Induced by Cultured Cell Transplantation

<table>
<thead>
<tr>
<th></th>
<th>C-Lu65</th>
<th>C-Lu99</th>
<th>Control nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of passages of cultured cells</td>
<td>5</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Days after transplantation</td>
<td>36</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>5.2</td>
<td>5.5</td>
<td>6.1</td>
</tr>
<tr>
<td>Leukocytes (× 10⁹/mm³)</td>
<td>24.6</td>
<td>21.2</td>
<td>67.2</td>
</tr>
</tbody>
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a) means±SD in 5 mice.
Fig. 6. Karyotypes of C-Lu65 (A) and C-Lu99 (B) cells.
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DISCUSSION

In this study, two different human cell lines from giant cell carcinoma of the lung were established from xenografts in nude mice. It is generally accepted that human tumor xenografts are good sources for tissue culture. Both C-Lu65 and C-Lu99 cells formed somewhat similar multinucleated giant cells, but they differed in cell surface and subcellular ultrastructures, suggesting the presence of considerable morphological differences among cases of giant cell carcinoma.

After repeated passages C-Lu65 changed from predominantly adherent cells to floating cell aggregates with accelerated growth rate. This change may have occurred by selection of subclones with relatively rapid growth or possibly by a spontaneous mutation in C-Lu65. However, the mechanism by which cells adapt to tissue culture is not known and should be studied further to identify tissue culture-specific changes vis-a-vis the characteristics of the original tumor.

Lu65 and Lu99 induced leukocytosis in nude mice, as had been recognized clinically in the patients, and conditioned media of these cultured cells stimulated the colony formation of mouse bone marrow cells in soft agar. Therefore, these cell lines appeared to be useful for studies on CSF. Further characterization of CSFs produced by Lu65 and Lu99 is necessary.

The fact that serum-free LA medium containing several human hormones and growth factors also allowed optimal cell growth of C-Lu65 and C-Lu99 cells (data not shown) suggests that this medium will be useful for studies of CSF and other tumor products under contamination-free conditions.

The aneuploid nature of the lung carcinoma cell lines presented here is consistent with other reports. The abnormalities on chromosome #3, which were observed by Loh et al. in 4 lung cancer cell lines, however, could not be confirmed in these lines. We found structural abnormalities of #1 chromosome in C-Lu65 and C-Lu99. However, it was suggested by Sandberg that #1 chromosome abnormality is involved frequently in a number of different solid tumors, such as cancer of the breast, melanoma and cancer of the bladder. Therefore, none of the abnormalities observed in this study seems to be specific for giant cell carcinoma. It is still desirable to investigate further the relation between chromosomal rearrangement and the phenotypes observed in the hope that some general patterns will emerge.

Lu65 is reported to have two amplified oncogenes, K-ras and myc. Double minute chromosomes may carry the amplified myc gene because the concomitant appearance of double minute chromosomes and the amplification of myc gene was observed in several other cell lines.

Several lines of investigation, including oncogene analysis and specific monoclonal antibody production are in progress using these cell lines. Using Lu65 and C-Lu65 as immunogens, we have produced mouse monoclonal antibodies reactive against non-small cell lung cancer but not reactive against small cell lung cancer (unpublished data). These monoclonal antibodies failed to react with C-Lu99, suggesting that some cell surface immunological differences exist between the two cell lines.

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