In Vivo and in Vitro Biological Activities of Two Human Cell Lines Derived from Anaplastic Lung Cancers

Richard S. Bockman, Anne Bellin, Mary A. Repo, Noreen J. Hickok, and Toru Kameya

ABSTRACT

Cell lines Lu-65 and SK-Luci-6 were established from two patients with anaplastic (non-oat cell) lung cancers. These cell lines showed in vivo and in vitro functional activities that could explain the paraneoplastic syndromes which were clinically manifested. In both patients, elevated white blood cell counts occurred in the absence of any evidence of sepsis. Tumor fragments taken directly from one patient and transplanted to nude mice produced a progressive leukocytosis in the mice. Tissue culture-derived cells from both cell lines enhanced white blood cell numbers following heterotransplantation to nude mice. Cell-free extracts from both cell lines were found to enhance granulocyte-macrophage colony formation in soft agar. Greater colony formation was consistently found with the cell line (SK-Luci-6) that was derived from the patient manifesting the more marked leukocytosis. These data suggest that the tumor cells release colony-stimulating activities. Coincidently, one cell line (Lu-65) synthesized and released large amounts of prostaglandin E2 with little or no other prostaglandin product; the other cell line produced no prostaglandins. When the tumor cell lines were cocultured with explanted fetal rat bones, enhanced bone resorption with excessive calcium release occurred. Bone-resorbing activity correlated with tumor prostaglandin synthesis for the cell line releasing prostaglandin E2. An osteolytic factor that was neither prostaglandin nor parathyroid hormone was released by the SK-Luci-6 cell line. Hypercalcemia was a persistent feature only in the patient from whom the latter tumor line was derived.

INTRODUCTION

Paraneoplastic syndromes associated with ectopic hormone secretion are well-described occurrences with lung cancer (2, 7, 14). Difficulty in establishing permanent cell lines from undifferentiated lung carcinomas has impeded the elucidation and identification of tumor-secreted factors which could account for the observed clinical syndromes. Recently, 2 cell lines have been established into permanent cell culture; these cell lines were derived from patients with anaplastic giant-cell lung cancers (1). As part of an effort to understand the mechanisms for the presenting clinical syndromes, in vitro functional activities of the cell lines have been examined. Each cell line demonstrated the ability to produce factors with biological activities sufficient to explain the clinical syndromes seen in the respective patient. Clinically, both patients had marked leukocytosis in the absence of any evidence of sepsis. A similar leukocytosis could be induced in the nude mouse model to which the patients' tumors were transplanted. Both cell lines could be shown to augment granulocyte-macrophage colony formation in soft-agar cultures of bone marrow cells. In one patient in whom hypercalcemia occurred, an osteolytic activity was released by the cultured cells. Tumor synthesis of PGs3 could not account for the osteolytic activity released by these cells, nor could significant levels of immunoreactive PTH be detected.

MATERIALS AND METHODS

Cell Lines

Lu-65. The Lu-65 culture was derived from a 64-year-old Japanese male (Patient 1) with an anaplastic lung carcinoma. During the patient's clinical course, elevated leucocyte counts consisting primarily of mature neutrophils were persistent and occurred in the absence of any evidence of sepsis. In an effort to obtain a permanent cell culture, a finely minced portion of the initial biopsy specimen was established in nude mice6; subsequently, the tumor was adapted to tissue culture (1). In culture, the cells appeared pleomorphic with 10% giant cells. Cytogenetic studies demonstrated at least 2 cell populations, one diploid and the other polypluid. Only human chromosomes were noted, and polymorphic enzyme analyses showed human enzymic activities that were unique to this cell line (1).

SK-Luci-6. The SK-Luci-6 cell line was derived from a 41-year-old white female (Patient 2) with an anaplastic lung carcinoma. During her clinical course, the patient was noted to have persistent leukocytosis as well as hypercalcemia (range, 11.0 to 17.1 mg/dl). To establish a tumor line, minced tumor fragments from a biopsy specimen of the lung lesion were overlaid onto a feeder layer of human fibroblasts (1). The cells established in culture showed a relatively uniform cell size and were noted by cytogenetic analyses to be hyperdiploid; only human chromosomes were seen. The morphological, histochemical, cytokinetic, and genetic properties of the cell lines have been described previously (1). For both cell lines, the morphology of the tumors grown in the nude mouse model and the cell lines in tissue culture were found to be essentially identical to that of the original biopsy specimens. Both cell lines were shown to be free of Mycoplasma contamination by culture as well as by electron microscopic examination. Both cell lines showed anchorage-independent growth by growing continuously while suspended in 0.3% agarose gel.

Heterotransplantation

Heterotransplantation of tumor cells was carried out by injecting 1 to 3 x 106 viable tumor cells or tumor fragments s.c. into 6-week-old athymic nude (BALB/c) mice. Tumor engraftment and growth in the nude mice occurred routinely for both cell lines. The passaged tumors could not be induced in the nude mouse model to which the patients' tumors were transplanted. Both cell lines could be shown to augment granulocyte-macrophage colony formation in soft-agar cultures of bone marrow cells. In one patient in whom hypercalcemia occurred, an osteolytic activity was released by the cultured cells. Tumor analysis of the PGs3 could not account for the osteolytic activity released by these cells, nor could significant levels of immunoreactive PTH be detected.

This work was supported in part by grants from the American Cancer Society (PDT-60) and the USPHS through National Cancer Institute Grants CA 3072, CA 06882, and CA 08748. To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, N. Y. 10021.

Received July 21, 1982; accepted June 8, 1983.
be shown to have the same morphological pattern as did the original cultures and biopsy specimens (1). At various times during tumor growth, blood samples were obtained. Serum samples were assayed for CSA as described below, and differential cell counts were determined by cytological smears of peripheral blood.

Granulocyte-Macrophage Colony Formation

The ability of tumor cell-derived factors to initiate the proliferation and differentiation of committed granulocyte-macrophage precursor cells (CFU-c) was tested in a soft-agar assay system which has been described previously (10, 11). Briefly, $10^6$ normal human or mouse (C57BL/6; The Jackson Laboratory, Bar Harbor, Maine) BMC were suspended in McCoy's Medium 5A supplemented with 10% fetal calf serum, 5% pooled human serum, essential and nonessential amino acids, and vitamins (Grand Island Biological Co., Grand Island, N. Y.) to which 0.3% agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.) was added. This cell suspension was overlaid onto a 1- to 2-mi feeder layer containing 0.5% agar. Aliquots of serum, particle-free cell sonicates, and dialyzed or nondialyzed cell-free conditioned media were added to the feeder layer prior to addition of the BMC suspension. The plates were placed in a 37° humidified atmosphere of 10% CO$_2$. After 14 days of culture, the plates were scored for the number of CFU-c colonies containing more than 50 cells/colony. Clusters are defined as grouped cells containing less than 50 cells/group.

PG Synthesis

PG synthesis by the tumor cell lines was tested by measuring the ability of the cells to convert labeled precursor into PG and by radioimmunoassay.

Radioisotope Conversion. Briefly, $10^6$ tumor cells were incubated with [3H]arachidonic acid (5,6,8,9,11,12,14,15-$^3$H)arachidonic acid; New England Nuclear, Boston, Mass.). After 1 to 2 hr the amount of [3H]-arachidonic acid incorporated was determined, and then the cells were washed free of nonincorporated label. The cells were placed in fresh media, and the release of radiolabeled products was followed as described previously (3, 5). Lipids in cell-conditioned media and cells were extracted by the method of Folch (5). The products of PG synthetase were identified by 2-dimensional TLC on silica gel plates (Analtech, Inc., Newark, Del.) and by high-performance liquid chromatography. The distribution of arachidonic acid in cellular lipids was determined by one-dimensional TLC.

Radioimmunoassay. PGE levels of cell-conditioned media were determined as described previously (3, 9). The assay does not distinguish between PGE$_1$ and PGE$_2$ and shows less than a 5% cross-reactivity with the prime metabolites of PGE or with PGF$_2$. 

Bone Resorption

The bone-resorbing activities of the tumor lines were tested in an explanted fetal rat bone system (6). Fetal rat bones were labeled in utero by treating the mother at 18 days of gestation with an injection of 0.2 to 0.5 mCi of $^{45}$CaCl$_2$. The fetal forelimb bones were removed at 20 days and placed in culture on stainless steel rafts bathed in Bigger's medium (Fritton-Jackson modification) containing 5% fetal calf serum (Grand Island Biological Co.). After an 18-hr equilibration period, the supernatants were removed, and fresh media with or without test substances were added to paired bones. After an additional 48 hr of culture at 37° in a 5% humidified CO$_2$ atmosphere, the supernatants were removed to measure $^{45}$Ca release. The data are expressed as the ratio of cpm released from experimental bones (E) divided by the cpm released from paired control bones (C); each reported value represents the mean E/C of at least 4 pairs of explanted bones ± S.E.

Statistical Analysis

Student's t test to compare means was carried out using the significance limits of a 2-tailed test.

RESULTS

Clinical Course. There was rapid progression of disease in both patients, this being marked in Patient 2. Neither patient showed significant response to therapy. Both patients manifested a persistent leukocytosis in the absence of any evidence of intercurrent infection (Chart 1); persistently higher WBC were seen in Patient 2. The serum calcium level in Patient 1 remained normal during the course of his disease. Further, he was without any evidence of flushing, asthma, or diarrhea. Patient 2 was persistently hypercalcemic, and the serum calcium level was controlled by repeated administration of mithramycin.

Heterotransplantation. Engraftment of Lu-65 and SK-Luci-6...
in nude mice was followed weekly. Beginning at the fourth week posttransplant, 3 of 3 animals bearing Lu-65 tumors showed a progressive increase in measurable tumor size. The increase in tumor size was associated with a progressive increase in WBC that represented an absolute increase in polymorphonuclear cells

(Chart 2A). Tumor excision in 2 of 2 animals with tumor volumes of 10,000 cu mm was associated with a rapid fall in WBC from 270,000 to 10,000 cells/cu mm. Tumor growth was rapid in the animals successfully engrafted with the SK-Luci-6 cell line. Within 3 weeks, large tumors were noted in 3 of 3 animals. Persistent leukocytosis was noted during tumor engraftment characterized by a progressive absolute monocytosis (Chart 2B). Abscess formation was not evidenced on the tumors examined at excision or postmortem. Thus, tumor engraftment in the nude mice recapitulated the paraneoplastic syndrome seen in the patients; in addition, SK-Luci-6 appeared to be the faster-growing tumor, as evidenced by the more malignant course of Patient 2.

CFU-c. Serum samples from tumor-bearing animals 5 to 7 weeks posttransplantation were tested for CSA in the soft-agar culture system. Serum samples from tumor-bearing nude mice were found to increase the number of cell clusters and colonies arising from committed granulocyte precursors in normal mouse (C57BL/6) BMC when compared to control sera (Table 1). The differences were significant at all doses tested for the SK-Luci-6 samples but only for cluster formation and at the higher serum (50-µl) dose for Lu-65. It should be noted that greater CSA was demonstrated in the serum of mice bearing SK-Luci-6 tumors and correlates with the higher WBC noted in the patient (Patient 2) from whom the latter cell line was derived.

To determine if the tumor cells released CSA, lysed tumor cells and media conditioned by the tumor cell lines were tested in the soft-agar culture system. Approximately 2 to 4 x 10⁵ tumor cells were lysed by ultrasonic disruption. Cell debris was sedimented by centrifugation, and the supernatant media were tested for CSA. Tumor cell lysates showed significant colony-forming activity (Table 2). The SK-Luci-6 cell line proved to have more CSA when compared to Lu-65.

CSA could be shown to be released into the media by testing cell-conditioned media in the soft-agar culture system (Table 3). SK-Luci-6 cells appeared to release more CSA than did Lu-65; however, it was noted that the levels of PGE, a known inhibitor of CFU-c, were significantly greater in the Lu-65 cell cultures (Table 3). Dialysis of the cell-conditioned media reduced measurable PGE levels. Unexpectedly, cluster- and colony-forming activity in Lu-65-conditioned media was reduced following dialysis; little effect was noted for SK-Luci-6. Dialyzed conditioned

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**Table 1**

<table>
<thead>
<tr>
<th>Colony-forming activity* in serum*² of tumor-bearing nude mice</th>
<th>Alquot (µl)</th>
<th>Clusters</th>
<th>Colonies</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu-65</td>
<td>20</td>
<td>31 ± 3⁶</td>
<td>3 ± 1</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>63 ± 10⁶</td>
<td>10 ± 1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>SK-Luci-6</td>
<td>20</td>
<td>137 ± 8⁷</td>
<td>16 ± 3⁶</td>
<td>9</td>
</tr>
<tr>
<td>50</td>
<td>228 ± 14⁷</td>
<td>48 ± 5⁵</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Control plates (no serum)</td>
<td></td>
<td>25 ± 7</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Control serum</td>
<td>20</td>
<td>36 ± 10</td>
<td>0.7 ± 1</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>39 ± 4</td>
<td>7 ± 2</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers of granulocyte-macrophage colonies containing 50 cells measured at 14 days in soft-agar suspension of 2 x 10⁵ BMC from C57BL/6 mice in 1.0 ml 0.3% agar containing McCoy's Medium 5A supplemented with 10% fetal calf serum and 5% human serum.

*² Serum from tumor-bearing nude mice sacrificed 5 to 7 weeks following tumor transplantation.

* Mean ± S.E.

* Six-two Student's t test of means compared to control serum, p < 0.001.

*⁷ Two-tailed Student's t test of means compared to control serum, p < 0.05.

*⁸ Two-tailed Student's t test of means compared to control serum, p < 0.01.

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**Chart 2.** WBC (A, B), tumor volume (A, B), and differentials (A', B') for nude mice bearing tumors derived from Lu-65 (A) and SK-Luci-6 (B) cell lines. Mo, monocytes; Lym, lymphocytes; Seg, segmented neutrophils.
media from Lu-65 were tested in the soft-agar system using normal human BMC. The addition of Lu-65-conditioned media to the human BMC increased CFU-c numbers [5 ± 1 (S.E.) colonies and 126 ± 7 clusters] when compared to control samples which produced no colonies or clusters in the absence of CSA. Conditioned media from a variety of tumor cell lines not producing CSA do not cause colony formation when tested in this assay system (12). Based on these findings, it would appear that there is a circulating CSA in the sera of mice bearing the Lu-65 and SK-Luc-6 tumors. The tumor cells contain a CSA and spontaneously release the activity into the media during cell culture. These data suggest that tumor cell production of CSA was responsible for the leukocytosis manifested by the patients and by the nude mice.

**PG Synthesis.** Both cell lines could be shown to rapidly incorporate [3H]arachidonic acid into membrane phospholipids. However, only the Lu-65 cells were capable of converting the [3H]arachidonic acid into PG, and remarkably there appeared to be a single class of PG produced, PGE2 (Table 4). Essentially, background levels of other tritiated PG except for PGE2 were released into the media after 4 hr of culture of the prelabeled Lu-65 cells. Cell homogenization of Lu-65 increased total cpm release as PG, but 5 to 10 times the amount of tritiated PGE2 was released. SK-Luc-6 released levels of tritiated PG products that could not be distinguished from background. Greater than 97% of the counts recovered from lysed SK-Luc-6 cells were identified as arachidonic acid. The data from 2-dimensional TLC and high-performance liquid chromatography would suggest that a single PG synthetase complex with a unique isomerase was present in the Lu-65 cells. These observations were quantitatively confirmed by radioimmunoassay where a dose-dependent increase of PGE2 was measured with increasing Lu-65 cell number placed into culture; no changes in PGE2 levels above background level contained in the fetal calf serum were noted with the SK-Luc-6 cell line (Table 5).

**Bone Resorption.** Fetal rat bones cultured with tumor cells or tumor-cell-conditioned media from both cell lines caused a marked augmentation in 45Ca release compared to controls. For the Lu-65 cell line, increased 45Ca release was correlated with tumor cell PG synthesis. Coculture of Lu-65 cells (106 cells/culture) with fetal rat bones augmented 45Ca release (E/C, 2.46 ± 0.09), and this effect could be abrogated by the addition of indomethacin (2.5 µM) (17) to the cocultures (E/C, 1.36 ± 0.02), the difference being highly significant (p < 0.001). Consistent with these findings, dialysis of Lu-65 media significantly reduced PGE levels in the media and resulted in a significant loss of bone-resorbing activity (E/C, 1.44 ± 0.09, falling to 1.17 ± 0.02 after dialysis; p < 0.025). Media conditioned by SK-Luc-6 caused augmented 45Ca release from the bone explants (E/C, 1.37 ± 0.09; n = 6; p < 0.001 compared to control). Bone resorption by SK-Luc-6 was not due to tumor cell production of PG, since insufficient PGE levels were measured by radioimmunoassay and little conversion of tritiated arachidonate could be demonstrated.
Giant-cell tumors of the lung has recently been reported (1). We (2, 7, 14). A description of 2 newly established cell lines from induction have been reported with undifferentiated lung cancers the Lu-65 cell line may be specific for granulocyte precursors administration (13). Thus, it is possible that the CSA released by enhancement of colony formation in either cell line. Therefore, it is possible that both tumor lines produced and committed granulocyte precursor cell proliferation and differen these paraneoplastic syndromes. In vitro studies with the Lu-65 (12,15); at present, no explanation exists for the coincidence of proteins and human chorionic gonadotropin.6

In 2 recent reports, patients with squamous cell carcinomas associated with leukocytosis and hypercalcemia were described (12, 15); at present, no explanation exists for the coincidence of these paraneoplastic syndromes. In vitro studies with the Lu-65 and SK-Luci-6 may provide some insight as to whether a unique factor or several factors mediate the pathophysiological changes seen in these patients.

In summary, 2 new cell lines derived from human anaplastic large-cell lung cancers have been established. These cell lines demonstrate several functional activities when tested in vitro and in vivo. The latter activities could explain the paraneoplastic clinical syndromes described in the patients from whom the tumors were derived and thus provide unique models for study.

Table 5

<table>
<thead>
<tr>
<th>No. of cells (x 10⁶)*</th>
<th>ng PGE released/4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu-65</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.69</td>
</tr>
<tr>
<td>5</td>
<td>7.16</td>
</tr>
<tr>
<td>10</td>
<td>15.90</td>
</tr>
<tr>
<td>SK-Luci-6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.92</td>
</tr>
<tr>
<td>5</td>
<td>0.91</td>
</tr>
<tr>
<td>10</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* Number of cells used to condition media for 24 hr at 37°.

Since removal of PGE₂ did not augment colony formation. Both cell lines rapidly incorporated [³²P]arachidonic acid into membrane phospholipids, and the label was found to distribute among the major classes of membrane phospholipids. Only the Lu-65 cell line was able to convert arachidonate to PG. Surprisingly, a single class of PG product was synthesized, PGE₂. Such a finding strongly suggests that a single species of PG synthetase exists, one that is associated with a unique PG isomerase activity. Since little is known about PG isomerases and the cellular controls of isomerase activity, the Lu-65 cell line and its single PG synthetase provide a rare opportunity to examine the enzyme system and its regulation. To our knowledge, this represents the first report of a human cell line that produces ng amounts of PGE₂ per 10⁶ cells.

Both cell lines demonstrated the ability to secrete an osteolytic factor when studied in the explanted fetal rat bone culture system. The activity from the Lu-65 appeared to be closely correlated to the ability of the cells to release PGE₂, since inhibition of PG synthetase abrogated the osteolysis induced by the tumor-conditioned media. PGE₂ is known to be a potent osteolytic agent in the in vitro rat system utilized (4, 8, 16). Therefore, it seems probable that PG production is the prime mechanism by which osteolysis was induced by the the Lu-65 cell line. In vivo, PGE₂ is rapidly converted by the lungs, liver, and kidneys to the prime circulating metabolite 13,14-dihydro-15-ketoprostaglandin E₂. We have demonstrated previously that the 15-ketoprostaglandin E₂ derivative has no osteolytic activity (4), and this is one possible explanation for the inability of tumor PGE production to cause cancer hypercalcemia in the patient. Unfortunately, neither 15-ketoprostaglandin E₂ in the serum nor the prime urinary metabolite of PGE₂ was measured in the patient from whom the Lu-65 line was derived. The osteolytic activity released by the SK-Luci-6 cell line clearly was not related to tumor PGE synthesis since the latter cell line could not be shown to convert arachidonate to PG. It is unlikely that ectopic PTH secretion would account for the hypercalcemia seen in the patient. Radioimmunoassay of the patient’s serum and of tumor cell homogenates and conditioned media demonstrated barely detectable levels of immunoreactive material. No PTH mRNA could be detected in lysed tumor cell preparations.8 Further characterization of the osteolytic factor derived from SK-Luci-6 is now in progress. It is of interest to note that Lu-65 releases large amounts of material that cross-react in parallel fashion with specific antibody to the α-subunit of the pituitary glycoproteins and human chorionic gonadotropin.6

In 2 recent reports, patients with squamous cell carcinomas associated with leukocytosis and hypercalcemia were described (12, 15); at present, no explanation exists for the coincidence of these paraneoplastic syndromes. In vitro studies with the Lu-65 and SK-Luci-6 may provide some insight as to whether a unique factor or several factors mediate the pathophysiological changes seen in these patients.

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ACKNOWLEDGMENTS

The technical assistance of R. Ferguson is acknowledged with gratitude.

REFERENCES


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