Calcitonin as an Indicator of the Response of Human Small Cell Carcinoma of the Lung Cells to Drugs and Radiation

Charles C. Cate, Evan B. Douple, Kim M. Andrews, Olive S. Pettengill, Thomas J. Curchey, George D. Sorenson, and L. Herbert Maurer
Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, New Hampshire 03756

ABSTRACT
A calcitonin (CT)-producing cell line (DMS53) established from human small cell carcinoma of the lung was grown as three-dimensional multicellular spheroids in spinner culture or on agar in multwells, and as tumors in nude (athymic) mice. CT release into the media was directly proportional to spheroid volume. The response of these cells to x-irradiation and Adriamycin, or diazoacetylcholine iodide was assessed by monitoring levels of CT released into the media by individual spheroids. Levels of CT in the blood of nude mice bearing DMS53 xenografts were directly proportional to tumor volume and decreased proportionally with tumor response to x-irradiation and cisplatin treatment. These results suggest that the DMS53 spheroid and xenograft models may be useful systems to monitor responses to therapy utilizing CT as an indicator of tumor burden.

INTRODUCTION
The dismal long-term survival rate for SCCL patients suggests the need for improved methods of therapy, including modulation of intensive conventional regimes which have demonstrated clinical responses and small improvements in survival (1, 22). It would be helpful to be able to precisely monitor the tumor burden in SCCL patients, especially near the end of periods of remission when the tumor cell population may be small and when relapse will be characterized by a critical period of tumor regrowth requiring optimized therapy. Furthermore, the concept that SCCL tumors consist of a heterogeneous mixture of cell clones with chemo- and radiosensitivities unique for each patient is particularly challenging from a clinical point of view, suggesting that if curative therapy is to be designed, it may have to be individualized for each patient (35).

Methods such as the clonogenic stem cell assay are currently being developed for monitoring the response of tumor cells including SCCL to anticancer agents (6, 7, 17). The requirement for single cell preparation in the stem cell assay, together with the low clonogenic cell fraction, raises the question of cell selection. Use of the assay techniques described in this paper in which dissociation of single cells is not a requirement as it is in the clonogenic assay may provide additional means for determining tumor cell response to treatment. New methods are needed to assess tumor cell response including noninvasive techniques for estimating clinical SCCL burden in situ. For this purpose, the monitoring of biochemical markers is attractive, since SCCL tumors are known to secrete several ectopic peptides (4, 16, 30–32).

Human CT is one of the most commonly elevated tumor-produced hormones in SCCL, occurring in about 85% of patients with extensive disease (36). Approximately 50% of cultures established from SCCL tumors secrete CT (32). Since CT may represent a potential biomarker for extent of disease in selected SCCL patients, this study was designed to investigate whether the monitoring of CT levels could be correlated with tumor cell burden in an in vitro and in vivo model for SCCL, using an established SCCL cell line, DMS53, known to be a high producer of human CT in vitro (31).

MATERIALS AND METHODS

Tumor Cells. DMS53, a cell line which had been previously established from a pretreatment lung biopsy, was removed from storage in liquid nitrogen and maintained by growing in plastic 25-sq cm flasks (Costar, Cambridge, MA) at 37°C in 100% humidity and 5% CO2/95% air, with serial passage every 10 days. The medium used was Waymouth's MB 752/1 (Grand Island Biological Co. Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT). Details of the origin, establishment, and characteristics of this cell line have been reported previously (27, 28, 31).

Spheroid Culture. To initiate spheroid formation, DMS53 cells were removed from flasks by trypsinization with 0.25% Pancreatin (Grand Island Biological Co.), and clumps of cells were seeded densely (approximately 100,000/dish) into 60-mm non-tissue culture (bacteriological) dishes (Lab-Tek, Naperville, IL). After 14 days, with one replenishment of medium on Day 7, clumps of cells were transferred into 100-ml spinner flasks (Belco Glass, Vineland, NJ). Spheroids were grown at 37°C in an Incu-cover (Associated Biomedic Systems, Buffalo, NY) spinning at approximately 70 rpm. The spheroids were fed every 4 days by replacing one-half of the medium (50 ml) with fresh medium. For growth curves, spheroids were sized periodically in 2 perpendicular diameters (d) using a calibrated reticule in the eyepiece of an inverted microscope. Spheroid volumes (V) were estimated using the relationship

\[ V = \frac{4}{3} \pi \left( \frac{d_1 + d_2}{2} \right)^3 \]

When spheroids attained appropriate diameters for treatment (typically 400 µm), they were sized, filtered through a nylon mesh, and returned to fresh medium in a clean spinner culture flask. Following treatment with drugs or radiation, the spheroids were washed and placed individually into 6-mm-diameter multwells (Costar), each well containing 2 ml of complete medium. The cells had been coated previously with 0.25 ml of 0.75% agar (Difco Laboratories, Detroit, MI), dissolved in 2x Waymouth's medium with 20% fetal bovine serum. The spheroids were then grown in 100% humidity in a 5% CO2/95% air incubator at 37.5°.

Drug Treatment. Stock solutions of chemotherapeutic drugs were added to spinner cultures so that the desired final concentrations were
attained, and the cells were exposed to the drugs for 2 hr. Stock solutions of Adriamycin (doxorubicin hydrochloride; Adria Laboratories, Columbus, OH) containing 2.5 mg/ml in Waymouth's medium were added to spinner cultures to give a final concentration of 0.25 to 2.5 μg/ml. DACI was synthesized using the general method of House and Blankley (19). The corresponding bromide is a known compound (14). The spheroids were exposed to DACI concentrations of 0.01 to 1 μM for 2 hr. After exposure, the spheroids were removed and washed in fresh medium, distributed singly into multiwells, and placed in the incubator.

The cisplatin was dissolved in 0.9% NaCl solution and injected i.p. into tumor-bearing nude mice in doses of either 5 or 12 mg/kg.

Irradiation. Spheroids of approximately equal size were transferred by pipet into plastic Petri dishes and irradiated after they had settled to the bottom of the dish. The irradiation source was a Maxitron 300 (General Electric Co., Milwaukee, WI) operating at 140 kVp, 20 ma, and 2.7 mm Al (half-value layer). The dose rate was 301 rads/min, as measured with thermoluminescent dosimetry. After irradiation, spheroids were returned to multiwells, one spheroid per well.

Xenografts. DMS53 (~5 x 10^6) cells were collected from monolayers or by teasing serially passaged tumors and injected s.c. into the flanks of BALB/c-derived nude (athymic) mice (Harían Industries, Madison, WI). This implantation of SCCL cells resulted in tumor incidences of greater than 90%, with tumors appearing in 2 to 4 weeks. Mice were maintained in an isolated facility with controlled airflow, humidity (35 to 50%), and temperature (24–25°), and housed in autoclavable plastic cages fitted with sealed filter tops. Prior to irradiation, mice were moved into a portable laminar-flow animal hood (Anigard; Baker Co., Sanford, ME). Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Elkins-Sinn, Cherry Hill, NJ), and the 1.5-cm-diameter cone of the X-ray machine was placed over the tumor. The skin containing the tumor was mobile enough to permit exposure of the tumor with little exposure to the normal tissues of the mouse. Tumor diameters were measured twice weekly with sterile calipers. Tumor volumes were estimated using the relation

\[
V = \frac{4}{3} \pi \left( \frac{d_1 \times d_2 \times d_3}{2} \right)^{3/2}
\]

where \(d_1\), \(d_2\), and \(d_3\) are the dimensions of the tumor in the three principal axes. Multiple suborbital bleedings were collected from the tissues of the mouse. Tumor diameters were measured twice weekly to obtain plasma for measuring CT.

This implantation of SCCL cells resulted in tumor incidences of greater than 90%, with tumors appearing in 2 to 4 weeks. Mice were maintained in autoclavable plastic cages fitted with sealed filter tops. Prior to irradiation, mice were moved into a portable laminar-flow animal hood (Anigard; Baker Co., Sanford, ME). Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Elkins-Sinn, Cherry Hill, NJ), and the 1.5-cm-diameter cone of the X-ray machine was placed over the tumor. The skin containing the tumor was mobile enough to permit exposure of the tumor with little exposure to the normal tissues of the mouse. Tumor diameters were measured twice weekly with sterile calipers. Tumor volumes were estimated using the relation

\[
V = \frac{4}{3} \pi \left( \frac{d_1 \times d_2 \times d_3}{2} \right)^{3/2}
\]

where \(d_1\), \(d_2\), and \(d_3\) are the dimensions of the tumor in the three principal axes. Multiple suborbital bleedings were collected from the tissues of the mouse. Tumor diameters were measured twice weekly to obtain plasma for measuring CT.

RESULTS

CT levels in the medium from multiwells containing individual SCCL spheroids were measured using RIA, and the levels obtained during the first 108 hr are shown in Chart 1A. When the levels of CT were normalized to a spheroid volume (pg CT/cu μm per ml), the concentrations of CT measured in the medium became constant after approximately 24 hr in the multiwell, as illustrated in Chart 1B. These results suggest that the rate of secretion of CT was directly proportional to spheroid volume between Days 1 and 5. When a heterogeneous population of spheroids was used, the concentration of CT in the medium was proportional to tumor volume, as indicated in Chart 2 for spheroids between 3 x 10^7 and 1.5 x 10^8 cu μm. With much larger spheroids, the proportionality was not maintained due to loss of cell viability in developing necrotic centers.

Spheroids received graded radiation doses (0 to 30 Gy), and the concentrations of CT in the medium are measured every 2 days. The levels of CT attained are illustrated in Chart 3A. Significant differences in CT levels were obtained between control spheroids and spheroids which received 5 and 10 Gy. However, at radiation doses between 10 to 30 Gy, differences...
were virtually identical to that of the untreated controls over 9 days. DACI concentrations of 0.03 and 0.3 mg/ml produced significantly depressed levels of CT secretion at 2 days following treatment. The response was only transient at 0.03 mg/ml, however, since it disappeared by Day 5. The spheroids which received the higher DACI dose (0.3 mg/ml) showed signs of progressive disaggregation and gross cytotoxicity by Day 5.

To determine if the levels of CT secreted could be correlated with tumor burden in vivo, levels of CT were measured by RIA in the plasma of nude (athymic) mice bearing DMS53 xenografts. In an early experiment, CT levels in 8 tumor-bearing mice were demonstrated to be approximately 30-fold greater (5613 ± 346 (S.E.) pg/ml) than that of 7 age-matched untreated controls (182 ± 50 pg/ml). The results of a second experiment (Chart 4), in which multiple samples of plasma CT from 8 additional tumor-bearing mice were assayed, showed that CT levels correlated highly with tumor volumes (correlation coefficient, 0.9753). Serial comparisons of mean plasma CT levels for control and tumor-bearing mice are summarized in Table 1. In a third experiment,

between the levels of CT for the different radiation doses were not significant.

A response similar to the effect of radiation was observed for spheroids exposed to various doses of Adriamycin for 2 hr. Depressed secretion of CT into the medium was observed for Adriamycin (0.25 and 1.0 μg/ml), but raising the concentration to 2.5 μg/ml did not produce a significantly greater depression when compared to 1.0 μg/ml (Chart 3B). A mixed response was seen in a third experiment following exposure of spheroids to DACI concentrations of 0.003, 0.03, and 0.3 mg/ml. At the lowest dose (0.003 mg/ml), CT concentrations per unit spheroid volume

MARCH 1984

Table 1

<table>
<thead>
<tr>
<th>Time postinjection (days)</th>
<th>Control mice (6)</th>
<th>Xenograft bearers (8)</th>
<th>Mean tumor volume (8) (sq cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>121</td>
<td>199</td>
<td>0.045</td>
</tr>
<tr>
<td>15</td>
<td>173</td>
<td>360</td>
<td>0.019</td>
</tr>
<tr>
<td>29</td>
<td>125</td>
<td>1575</td>
<td>0.160</td>
</tr>
<tr>
<td>42</td>
<td>131</td>
<td>5545</td>
<td>0.755</td>
</tr>
<tr>
<td>48</td>
<td>136</td>
<td>9188</td>
<td>1.156</td>
</tr>
<tr>
<td>54</td>
<td>NE²</td>
<td>NE</td>
<td>1.446</td>
</tr>
</tbody>
</table>

² Numbers in parentheses, number of mice.
³ NE, not evaluated.
established tumors were exposed to either 10 or 50 Gy X-irradiation. The resulting decrease in tumor volume with time after treatment is shown in Chart 5A, and the corresponding decrease in CT levels in the blood of these animals is shown in Chart 5B. Note that the animals with larger tumors at the time of treatment had the larger CT levels. On Day 15, the mice received i.p. injections of cisplatin, at doses of either 5 or 12 mg/kg. As the tumors decreased in size following treatment with either of these doses, the CT levels continued to fall. When the tumor in mouse No. 2 was no longer palpable, levels of CT continued to decrease and reached control levels (180 pg/ml). Furthermore, when the tumors started to regrow, the levels of circulating CT also showed a corresponding increase effectively reflecting relapse in 2 mice (Chart 5). In an additional study following intracranial inoculations of DMS53 cells into 2 nude mice, elevated CT levels (1590 and 2880 pg/ml, respectively) indicated the presence of undetectable tumors that were subsequently confirmed histologically.

DISCUSSION

Cultured SCCL cells as well as those obtained from solid tumors are frequently difficult to dissociate into a single-cell suspension. The spheroid model, exploited so effectively in radiobiological studies by Sutherland and Durand (33), and which does not require a single cell for initiation of cultures, provides a useful alternative culture technique for in vitro evaluation of treatment protocols. Several human tumors have been reported to grow as 3-dimensional spheroids (5, 9, 20, 29, 37), and spontaneous aggregation and spheroid formation by certain established SCCL cell lines has been observed by others (3, 13, 15, 25, 34), as well as in our own laboratory (27). In many ways, the spheroid model system may better represent some metabolic, morphological, and geometric characteristics of tumors in vivo, factors which might influence the effects of radiation or chemotherapy. For these reasons, development of an appropriate spheroid model for testing treatment effects on SCCL cells is clearly advantageous. However, because of the problems associated with disaggregation and measurements of cell viability or clonogenicity, in order to fully exploit the spheroid model it would be beneficial to derive the latter from a cell line that had a relatively commonly associated, readily measurable biomarker that behaved similar in vitro and in vivo.

CT is a tumor-produced hormone that is frequently seen in SCCL, particularly in patients with extensive disease (36). In our institution, one-half of all SCCL patients studied, including those with local as well as extensive disease, demonstrated circulating CT levels that were higher than the normal reference value. Furthermore, about the same percentage of established SCCL cell lines have also been shown to secrete CT in vitro (32). The levels of CT production are quite variable, however. In patients, CT is usually increased less than 4-fold above the upper limits of the normal range (112 pg/ml), although occasionally values may occur that are more than 10 times normal. In cultures, CT-secreting cell lines may vary more than 400-fold in the amount produced per cell per day. It is not clear that all cells produce hormone at the same rate, and the extent of production probably reflects the output of a heterogeneous population of high, low, and nonproducers. There is also a spectrum of molecular forms of immunoreactive CT produced by patients and by tumor cells in culture (4, 10, 32) which may contribute to the wide range of detected values reported (10). Nevertheless, CT is commonly associated with SCCL and is quite easily measured by radioimmunoassay at the pmol level.

The cell line, DMS53, was selected for the present study because, although normally grown as attached cells, it was readily induced to undergo spheroid formation, formed transplantable tumors in nude mice, and consistently produced remarkably high concentrations of CT in all 3 milieux. In particular, the excellent, almost linear, relationships maintained in mice between circulating CT levels and tumor volumes, and between untreated spheroid volumes and medium CT concentrations provided usable systems for treatment assessment based on CT measurements. This was particularly important in which the
treatment of spheroids was followed by varying degrees of cell dissociation making tumor volume measurements difficult. Similar drug-induced dissociation of SCCL cultured cells (enlargement and deterioration of multicellular aggregates, dissolution of spheroids) accompanied by a dramatic reduction in biomarker activity has been reported by Luk et al. (23). In addition, in their system, decreased viability manifested by cell loss and disruption of the morphology of cell aggregates after treatment with α-difluoromethylornithine was reflected by an 85% loss in L-dopa decarboxylase activity, an enzyme marker for O-H-1 SCC cells.

In those cases of human SCCL in which significantly elevated CT levels have been detected early, serial determinations have been shown to be highly useful for estimating tumor load, monitoring therapy, and general patient management (16, 21, 24, 26, 36). However, variations in peptide production among tumor sites (2), increases in circulating levels after tumor regression in patients with normal pretreatment levels (18), and undetectable or normal levels in about 40% of SCCL patients (32) combine to limit the use of circulating CT as an indicator of tumor burden to selected clinical situations. Nevertheless, because CT production is such a stable characteristic of DMS53 cells and tumors, measurement of CT in the spheroid-xenograft models may provide a rather sensitive method for detecting effects of a variety of therapies designed for SCCL, and comparison studies with other drug screening systems (such as the clonogenic tumor "stem cell" assay) would seem to be warranted.

The results of the present study demonstrate that decreasing levels of CT are released into the medium from 3-dimensional DMS53 spheroid cultures following treatment with radiation or chemotherapeutic drugs and that such reduced CT concentrations reflect proportional losses of cell viability. Similar observations were made in the nude mouse xenograft model.

We have described a preliminary test in the spheroid system of a potentially useful chemotherapeutic agent targeted at SCCL. As suggested by their putative cell of origin (K-type cell, APUD system), decreased viability manifested by cell loss and disruption of the high cholinesterase levels in SCCL would seem to be warranted. Other drug screening systems (such as the clonogenic tumor "stem cell" assay) would seem to be warranted.

REFERENCES


Calcitonin as an Indicator of the Response of Human Small Cell Carcinoma of the Lung Cells to Drugs and Radiation
