IN VITRO CHEMOSENSITIVITY PATTERNS OF CARCINOMA OF
THE LUNG IN HUMAN TUMOR CLONOGENIC ASSAY

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(Received January 6, 1986)

One hundred and sixty-eight different specimens of human carcinoma of the lung were tested for in vitro drug sensitivity using the human tumor clonogenic assay (HTCA) originally described by Hamburger and Salmon. One hundred and twenty-two (73%) specimens grew adequately for chemosensitivity testing. Most tumors were resistant to chemotherapeutic drugs, but in vitro sensitivity, regardless of the type of drugs, varied markedly from specimen to specimen. Although response rates to individual drugs ranged between 9% and 23%, half the specimens tested were sensitive in vitro to at least one drug. A higher in vitro sensitivity rate was observed in small cell lung carcinoma (31%) than in non-small cell lung carcinoma (17%). The frequency of in vitro sensitivity was greater for patients who had received no prior chemotherapy than those who were in relapse. These in vitro results are similar to current clinical experience. There was a significant association between in vitro sensitivity of cells from a primary tumor as compared to its metastases. Overall HTCA appears to be useful in selecting appropriate chemotherapy for individual patients with carcinoma of the lung.

Keywords—human tumor clonogenic assay; in vitro antitumor activity; anticancer drug; lung cancer

INTRODUCTION

The human tumor clonogenic assay (HTCA) is a bi-layer soft agar system for growing fresh human tumor specimens in vitro to determine drug sensitivity and improve our understanding of tumor biology.1-30 Recent clinical correlations of 60% accuracy for predicting a positive clinical response and 90% accuracy for predicting a lack of response to therapeutic agents suggest promising clinical usefulness.4-12 However, a subset of patients, whose tumor colony forming units (TCFUs) exhibit in vitro sensitivity, fail to show objective clinical response (false positives).4,11,13 On the other hand, some tumors were resistant to drugs in vitro, but the patient responded to the drugs (false negative).4,14,15 One important reason for these false positive or false negative results could be due to inappropriate criteria for in vitro sensitivity. There was a definite relationship between decreased survival of TCFUs and increased percentage of clinical response. However, there was no clear absolute cut off point to define in vitro drug sensitivity.50 We have previously explored the factors that influence colony formation of carcinoma of the lung in soft agar.16 This report describes the influence of various factors upon in vitro chemosensitivities and their patterns observed in this series of patients.

MATERIALS AND METHODS

Collection of Tumor Specimens — Tumor specimens were obtained from lung cancer patients seen at National Cancer Center Hospital from 1982 to 1985. Malignant effusions were collected in 500 ml blood bags or 50 ml syringes containing 100 units/ml heparin. Biopsy specimens were collected in McCoy's 5 A medium containing 10% heat-inactivated fetal calf serum and penicillin/streptomycin solution (McCoy's wash), all obtained from Grand Island Biological Company (GIBCO), Grand Island, N.Y. and were immediately transported to the laboratory for disaggregation.

Disaggregation Procedures — The tumor specimens were mechanically dissociated and suspended in McCoy's wash as described in detail previously.17) Final tumor suspensions
were prepared by passing the cells through a sterile stainless steel screen (mesh #120 to 150) (Sanki Kogei Kagaku Co., Ltd., Tokyo, Japan). Viable nucleated cell counts were determined by trypan blue dye-exclusion and were plated at a desired concentration in the HTCA (usually 50,000 cells/plate).

**Culture System for Human Tumor Clonogenic Assay** — The HTCA has been previously described. Briefly, tumor cells were suspended in 0.3% agar with CMRL 1066 medium (GIBCO) and 15% horse serum (GIBCO) and a variety of nutrients. One milliliter of this suspension, as the top layer, was plated on the bottom layer (feeder layer), which consisted of 1 ml of McCoy’s 5A medium in 0.5% agar plated in 35 mm plastic dishes. After preparation of both the bottom and top layers, the plates were examined under an inverted phase microscope to confirm the presence of a good single cell suspension. The plates were then incubated at 37 °C in a 7.5% CO₂ high-humidity atmosphere for two weeks.

**Drug Sensitivity Study** — Stock solutions of standard anticancer drugs were prepared in sterile buffered saline and stored at -20 °C in aliquots sufficient for one assay. Antitumor drugs tested were mitomycin C (0.1 μg/ml), cisplatin (0.2 μg/ml), 5-fluorouracil (1.0 μg/ml), adriamycin (0.04 μg/ml), vindesine (0.005 μg/ml), peplomycin (0.4 μg/ml), etoposide (0.1 μg/ml), 7-N-(p-hydroxyphenyl)-mitomycin C [M-83] (0.1 μg/ml) and L-phenylalanine mustard [L-PAM] (0.4 μg/ml), which have been extensively utilized in lung cancer or are currently being investigated. Drug concentrations were based on pharmacologically achievable concentrations, usually one-tenth of the peak plasma concentration after administration of the standard dose to humans. Tumor cell suspensions were transferred to tubes and adjusted to a final concentration of 5 × 10⁵ cells/ml with the appropriate drug dilution or control medium. Cells were incubated with and without drugs for 1 h at 37 °C in McCoy’s wash. The cells were then centrifuged at 400 g for 5 min, washed twice with McCoy’s wash, and prepared for culture as described above. After plating for 14 d, the number of colonies on the triplicate control plates and drug-treated plates were counted with an inverted phase microscope at a magnification of 40 ×. Aggregates of 50 or more cells were considered to be colonies and aggregates of less than 50 cells were considered to be clusters.

**RESULTS**

**In Vitro Colony Formation**

A total of 168 specimens from 131 patients with primary carcinoma (59) or with advanced metastatic disease (109) were placed in culture. Adequate growth for sensitivity testing (more than 30 colonies grown on control plates) was obtained in 122 (73%), inadequate growth for drug evaluation (5–29 colonies/plate) in 29 (17%), and no colony formation (<5 colonies/plate) in 17 (10%) of 168 viable samples. The

**TABLE I. Number of Specimens of Human Lung Cancer Tested and Their Success Rate**

<table>
<thead>
<tr>
<th>Histology</th>
<th>Adequate growth for chemosensitivity testing &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adequate growth for chemosensitivity testing &lt;sup&gt;a&lt;/sup&gt;</td>
<td>Source</td>
<td>Total (%)</td>
</tr>
<tr>
<td></td>
<td>Primary Site</td>
<td>Lymph node</td>
<td>Pleural effusion</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>22/30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31/41</td>
<td>20/33</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>20/22</td>
<td>5/7</td>
<td>1/2</td>
</tr>
<tr>
<td>Large cell</td>
<td>4/6</td>
<td>2/4</td>
<td>0/1</td>
</tr>
<tr>
<td>Small cell</td>
<td>1/1</td>
<td>2/2</td>
<td>4/6</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>--</td>
<td>1/1</td>
<td>--</td>
</tr>
<tr>
<td>Overall</td>
<td>47/59</td>
<td>41/55</td>
<td>25/42</td>
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</tbody>
</table>

<sup>a</sup> Defined as 30 or more colonies on the control plate and a sufficient quantity of cells harvested to allow drug testing. <sup>b</sup> Success rate; Number with adequate growth/number of specimens tested.
In Vitro Chemosensitivity in HTCA

success rates varied with histology of the specimen; 79/112 (71\%) in adenocarcinoma; 27/33 (82\%) in squamous cell; 6/12 (50\%) in large cell; 8/10 (80\%) in small cell; and 1/1 (100\%) in adenosquamous cell (Table I). The success rates were also found to vary (60\% to 80\%) depending on the site of the tumor. However, there was no significant difference in the frequency of positive cultures.

In Vitro Drug Sensitivity

Based on previously reported prospective and retrospective clinical correlation studies,\textsuperscript{5,12} we arbitrarily defined drug sensitivity of a given tumor as 50\% inhibition of colony formation (50\% survival) in the presence of the drug at the standard dose compared to colony formation in the absence of the drug.\textsuperscript{16,17}

The responses of each specimen of carcinoma of the lung against each drug are shown in Fig. 1 in the order of decreasing effectiveness. For adenocarcinoma cells, 87 instances of in vitro sensitivities were seen in 583 tests (15\%) with a variety of active agents, including cisplatin (8/76), vindesine (13/76), L-PAM (12/60), peplomycin (10/60), adriamycin (10/66), 5-fluorouracil (10/65), mitomycin C (8/76), M-83 (8/41), and etoposide (8/63). No drug was significantly more active than any other. Forty-one of 79 specimens (52\%) were not sensitive in vitro to any of the drugs tested. Of the remaining 38 tumors (48\%) showing sensitivity to any drug, 14 tumors were sensitive to only one drug, 10 tumors were sensitive to 2 drugs each and 14 tumors were sensitive to three or more drugs.

Twenty-seven specimens of squamous cell had sufficient growth for drug testing. Among them, 15 specimens (56\%) were sensitive in vitro to any of the drugs tested as shown also in Fig. 1. A smaller number of specimens of large cell carcinoma was tested for chemosensitivity. Only 5 instances of 45 tests (11\%) demonstrated in vitro drug sensitivity. Eight specimens of small cell carcinoma had sufficient growth. Seven of eight specimens tested (82\%) were sensitive to one or more drugs studied in vitro. This response rate was higher than that in non-small cell lung carcinoma (50\%). On the basis of site of the tumor, there was no obvious difference overall in chemosensitivity between the primary site and metastatic deposit (Table II).

The mitomycin C derivative, M-83,\textsuperscript{18} was the most active agent in vitro with cytotoxic activity seen in 23\% of tests. Melphalan, adriamycin, vindesine, 5-fluorouracil, etoposide, peplomycin and mitomycin C were active in vitro in 14 to 20\% of test. Cisplatin, which is a component of clinically effective regimens, had a disappointingly low response rate of 9\%.\textsuperscript{19} The in vivo clinical response rate to cisplatin has been reported to be close to 14\%.\textsuperscript{20–22} Sensitivity to

<table>
<thead>
<tr>
<th>VDS</th>
<th>ADM</th>
<th>FU</th>
<th>L-PAM</th>
<th>MMC</th>
<th>PLM</th>
<th>VP16</th>
<th>M-83</th>
<th>CDDP</th>
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FIG. 1. Response in 122 Specimens of Carcinoma of the Lung to Various Antitumor Drugs

Each column circle represents the response of an individual patient’s cells including 79 adenocarcinomas, 27 squamous cells, 6 large cells and 8 small cells, which were treated with various drugs (VDS; vindesine (0.005 μg/ml), ADM; adriamycin (0.04), FU; 5-fluorouracil (1.0), L-PAM; L-phenylalanine mustard (0.4), MMC; mitomycin C (0.1), PLM; peplomycin (0.4), VP-16; etoposide (0.1), M-83; 7-N (p-hydroxyphenyl)-mitomycin C (0.1) and CDDP; cisplatin (0.2)) at the concentration indicated in parentheses for 1 h, then cultured under optimized conditions for 2 weeks. Closed and open circles indicate response and non-response, respectively. Response was defined as more than 50\% decrease in tumor colony forming units. Figures in the bottom row show the number of drugs to which each specimen responded.
cisplatin may be underestimated under the condition of one-hour drug exposure in the HTCA, as the activity of cisplatin is dependent on exposure time.23) **Drug Sensitivity of Primary Tumor Versus Metastases**

Twenty-two primary tumors and metastases obtained simultaneously were assayed. Both tumor specimens formed sufficient colonies in 13 of the 22 experiments (8 adenocarcinoma, 3 squamous cell, one large cell and one small cell). Cloning efficiencies of primary and metastatic site did not differ significantly from each other.16) The results of in vitro sensitivity to anti-

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FIG. 2. **Correlation of Drug Sensitivity of Carcinoma of the Lung of Primary Sites and Metastatic Lesions simultaneously Tested in the Human Tumor Clonogenic Assay**

Specimens numbered 1–8, 9–11, 12 and 13 were adenocarcinoma, squamous cell, large cell and small cell, respectively. Both cells of primary tumor and its metastases were treated with various drugs. Each paired TCFUs of primary tumor and metastases were treated with various drugs. Each paired TCFUs of primary tumor and metastases were plotted in the vertical and horizontal axis, respectively. Significant correlation appeared in specimens numbered 2 and 5 ($p < 0.001$), 3, 10 and 11 ($p < 0.05$), and also in totality ($p < 0.001$).
tumor drugs were similar between the paired specimens in 7 (specimen 2, 3, 5, 6, 7, 10, 11) of the 13 instances as can be seen in Fig.2. In five paired samples, there was a statistically significant correlation between the chemosensitivity of a primary tumor and its metastases. Overall, there was a significant association ($r = 0.449$, $p < 0.001$) between drug inhibition of tumor colony growth of cells from a primary tumor compared to that of its metastases.

**Effect of Prior Chemotherapy**

![Graph showing in vitro response ratio (%) for different drugs.](image)

**DISCUSSION**

The bi-layer agar culture system is a relatively new technology. With time, refinements will invariably improve the utility of the assay. We have recently shown that the number of clonal cells liberated from a tumor can be enhanced by altering the method of obtaining cell suspensions. The disaggregation of tumor specimens using mechanical means may prove superior to enzymes for obtaining clonogenic cells from tumor speci-
mens. Finally, it has been established that adequate growth for sensitivity testing (> 30 colonies/plate) was obtained in 122 cases (73%) of the 168 samples of carcinoma of the lung. In our study we demonstrated that the cloning efficiencies of cells derived from primary tumors were higher than those of cells derived from metastatic tumors, and that cloning efficiencies varied with the location of metastatic site but these differences were not statistically significant. However, the cloning efficiencies were not affected by tumor histology, grade of differentiation, patient age, stage of disease, or prior chemotherapy.

The chemosensitivity results of the present study demonstrate in vitro activity of many of the agents currently used clinically. The results provide substantial evidence for patient to patient differences in sensitivity of tumor cells to anticancer drugs, even among tumors of the same histopathology. Most tumor specimens exhibit resistance to standard anticancer drugs up to dose levels which exceed pharmacologically achievable plasma concentrations. Drugs were active in vitro in only 16% of tests. However, more than 50% of patients were sensitive to any one of the drugs tested. This suggests that the clinical response rate may be improved to approximately 50% by individualized choice of drugs for each patient.

The highest in vitro sensitivity rate was seen in small cell carcinoma, a disease in which patients are now effectively treated with multiple drug therapy.\(^{24,25}\) On the other hand, the response rate in non-small cell lung carcinoma (17%) was less than that in small cell lung carcinoma (31%). The pattern of in vitro sensitivity for each tumor type is similar to current clinical experience.\(^{26}\)

There was a satisfactory correlation of chemosensitivity between a primary tumor and its metastases in 54% of cases. However, in 6 paired specimens of 13 patients (46%), there was no correlation between these two specimens. Other investigators\(^{10}\) have also reported that there was no statistically significant correlation of sensitivity to cytostatic drugs among tumor cells within a primary tumor, between primary tumor and metastases, and between different metastases. These results indicate that the reported discrepancies of in vitro and in vivo results in clinical trials using the HTCA for predicting resistance or sensitivity to cytostatic agents may be due to heterogeneity among TCFUs derived from a primary tumor or its metastases.

We are currently pursuing prospective clinical trials designed to assess the relationship between in vitro sensitivity of carcinoma of the lung and the in vivo clinical response of these tumors to drugs tested in the HTCA.

Acknowledgements This work was supported in part by Grant-in-Aid for Cancer Research (56-S-1, 58-12) from the Ministry of Health and Welfare and from the Comprehensive 10-Year Strategy for Cancer Control, Japan. Anne W. Hamburger was a visiting scientist, and Jun-ichi Ishihara was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan.

REFERENCES

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