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Abstract

The anticancer drug sensitivity of human cancers was tested by the human tumor clonogenic assay (HTCA). Of 152 human cancer specimens tested, 63 (41%) formed more than 30 tumor cell colonies in control plates and could be used to evaluate the drug sensitivity of tumor cells. In 42 (93%) of 45 clinical trials in 24 patients, a parallel correlation was observed between the in vitro anticancer drug sensitivity measured by the HTCA and the clinical response of tumors to anticancer drugs. These results suggest that the HTCA is a good technique for the in vitro test of the anticancer drug sensitivity of human cancers.

KEYWORDS: human tumor clonogenic assay, anticancer drug sensitivity, human cancers

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The anticancer drug sensitivity of human cancers was tested by the human tumor clonogenic assay (HTCA). Of 152 human cancer specimens tested, 63 (41%) formed more than 30 tumor cell colonies in control plates and could be used to evaluate the drug sensitivity of tumor cells. In 42 (93%) of 45 clinical trials in 24 patients, a parallel correlation was observed between the *in vitro* anticancer drug sensitivity measured by the HTCA and the clinical response of tumors to anticancer drugs. These results suggest that the HTCA is a good technique for the *in vitro* test of the anticanccer drug sensitivity of human cancers.

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For patients with advanced cancer, chemotherapy is the first choice among the treatments. However, the response to chemotherapy varies even among patients with the same histologic type of tumor. It is, therefore, important to have an accurate knowledge of the anticancer drug sensitivity of an individual patient's tumor to obtain a successful chemotherapeutic effect. In 1977, Hamburger and Salmon (1, 2) applied the human tumor clonogenic assay (HTCA), using an enriched double-layered soft agar system, to the prediction of the clinical response to chemotherapy in individual patient. They demonstrated a high degree of correlaion between *in vitro* results and clinical responses in cases of ovarian cancer and multiple myeloma. We have extensively tried the application of the HTCA to the management of patients with lung cancer and various other solid tumors since 1980 (3, 4).

The purpose of the present study was to evaluate the usefulness of the HTCA for testing *in vitro* anticancer drug sensitivity of tumor cells taken directly from patients. We describe in this paper the colony growth of a variety of solid tumors in soft agar and the correlation between *in vitro* sensitivities measured by the HTCA and clinical responses to anticancer drugs in the patients from whom the specimens were obtained.

Materials and Methods

Patients and specimens. Patients with well-documented cancer were selected for this study. Specimens were obtained by aspiration of malignant effusions, ascites and metastatic bone marrow and by biopsy of primary and metastatic solid tumors. Malignant effusions, ascites and metastatic bone marrow were collected in a syringe containing preservative-free heparin (100 units/ml), and red blood cells were eliminated by centrifugation on a Ficoll-Conray gradient (specific gravity: 1.077) at 400 g for 20 min. The biopsied specimens were minced into small fragments with scissors under aseptic conditions and single-

cell suspensions were prepared by treatment with 0.8% collagenase II (Sigma Chemical Co.) and 0.002% DNase I (Sigma Chemical Co.) in RPMI 1640 medium (Grand Island Biological Co.) with 1% penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air for 2 h. Cell counts and viability were determined by the trypan blue dye exclusion procedure with a hemacytometer.

In vitro exposure of tumor cells to anticancer drugs. Stock solutions of intravenous formulations of anticancer drugs were prepared in sterile buffered saline and stored at −20°C in aliquots sufficient for individual assays. Drugs tested in the present study were adriamycin (ADM), aclarubicin (ACR), THP-adriamycin (THP), mitoxantrone (MIT), 40497 S, mitomycin C (MMC), cisplatinum (CDDP) and methotrexate (MTX). Cyclophosphamide is inactive *in vitro*, and therefore 40497 S, which is an active compound derived from ifosfamide, was used. THP and MIT are new anthracycline-anthraquinone analogs.

Tumor cell suspensions were transferred to tubes and adjusted to a final concentration of $1\text{-}5\times10^5$ cells/ml. The final concentrations (in μM) of each drug were 0.01, 0.1 and 1.0 for ADM, ACR, THP and MIT, 10, 100 and 1000 for 40497 S, 0.05, 0.5 and 5.0 for MMC and 0.1, 1.0 and 10.0 for CDDP and MTX. These concentrations were pharmacologically achievable in a human. Cells were incubated with and without drugs for 1 h at 37°C. The cells were then washed twice with RPMI 1640 medium and prepared for culture.

Human tumor clonogenic assay. Cells were cultured by a modification of the method described by Hamburger and Salmon (1, 2). In brief, single cells were suspended in 0.3% agar in RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS, Flow Laboratories). One ml of the mixture was pipetted onto triplicate 1 ml feeder layers that had hardened in 35-mm plastic Petri dishes (Lux Co., # 5217). The feeder layers consisted of 0.5% agar and RPMI 1640 medium supplemented with 15% FBS. The final concentration of cells in each plate was $1\text{-}5\times10^5$ viable cells. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The number of colonies were counted 14 days after plating under an inverted microscope. Aggregates of 30 or more cells were considered to be colonies. For measurement of *in vitro* drug sensitivity, at least 30 tumor colonies per control plate were required.

Data analysis. The results of the HTCA were expressed as the percentage of survival of tumor colony-forming units (TCFUs) in a particular drug: the ratio between the mean number of colonies surviving on triplicate plates treated with a drug and the mean number of colonies growing on triplicate control plates. Our criteria for *in vitro* sensitivity and resistance were as follows: *in vitro* sensitivity was defined as 70% or more reduction in the survival of TCFUs and *in vitro* resistance as less than 70% reduction in the survival of TCFUs at one-tenth of the peak plasma concentration of each drug.

Clinical responses to anticancer drugs were evaluated as "complete response", "partial response", "stable disease" and "progressive disease". Complete response was defined as the disappearance of all clinically apparent disease for more than one month. Partial response was defined as at least a 50% reduction in the size of all measurable tumors for at least one month. Stable disease was defined as a steady state or the response weaker than partial response. Progressive disease was defined as an increase greater than 50% in the size of any measurable tumors. Only complete or partial responses were considered as sensitive *in vivo*, while stable or progressive diseases were considered as resistant *in vivo*.

Results

Colony growth in soft agar. One hundred fifty-two specimens of lung cancers and various other solid tumors were tested for their *in vitro* drug sensitivity using the HTCA. The types of tumors placed in culture are listed in Table 1. Tumor types of the 152 specimens included carcinomas of the lung (100), the stomach (14), the ovary (10), the breast (6), the pancreas (5), the colon (4), the thyroid (3), the uterus (3) and others (7). Of these 152 specimens, 94 (62%) formed at least 5 colonies per control plate. Carcinomas of the lung, the ovary, the breast and the thyroid had a good growth potential,

Table 1 Correlation between cancer type and colony growth

Cancer type	No. of specimens tested	No. of specimens which gave ≧5 colonies	≧30 colonies
Lung	100	67 (67)[a]	45 (45)
Squamous	20	16 (80)	11 (55)
Small cell	22	16 (73)	12 (55)
Adenocarcinoma	53	33 (62)	21 (40)
Large cell	5	2 (40)	1 (25)
Gastric	14	6 (43)	4 (29)
Ovarian	10	8 (80)	5 (50)
Breast	6	4 (67)	3 (50)
Pancreatic	5	0 (0)	0 (0)
Colon	4	2 (50)	1 (25)
Thyroid	3	3 (100)	3 (100)
Uterine	3	1 (33)	1 (33)
Others[b]	7	3 (43)	1 (14)
Total	152	94 (62)	63 (41)

a: Numbers in parentheses indicate the percentage to the number of specimens tested.

b: Others included the cancers of the kidney, the gallbladder and the urinary bladder and hepatoma, meningioma, thymoma and histiocytoma.

Table 2 Correlation between specimen source and colony growth

Specimen source	No. of specimens tested	No. of specimens which gave ≧5 colonies	≧30 colonies
Primary tumor	51	37 (73)[a]	26 (51)
Metastatic solid tumor	27	21 (78)	14 (52)
Malignant effusion	65	29 (45)	20 (31)
Metastatic bone marrow	9	7 (78)	3 (33)
Total	152	94 (62)	63 (41)

a: Numbers in parentheses indicate the percentage to the number of specimens tested.

but carcinomas of the stomach, the pancreas and the colon showed low growth rates. In regard to the histopathological type of lung cancers, the colony growth was observed in 80% of squamous cell carcinomas, 73% of small cell carcinomas and 62% of adenocarcinomas. Table 2 shows the relation between the colony growth and the specimen source. The colony growth rates of specimens from the primary and the metastatic solid tumors and the bone marrow containing tumor cells were more than 70%, but those of malignant ascites and pleural effusions were only 45%.

Of the 94 specimens which formed at least 5 colonies in soft agar, 63 formed more than 30 colonies per control plate and could be used to evaluate drug sensitivity. Thus, overall, 63 (41%) of the 152 specimens were considered to have adequate colony growth for the drug sensitivity test.

Correlation between in vitro sensitivity and clinical response. The correlation between the *in vitro* drug sensitivity measured by the HTCA and the clinical response to anticancer drugs in 24 patients from whom the specimens were obtained are summarized in Table 3. The *in vitro-in vivo* correlations were studied in 45 trials including 16 prospective trials.

Of the 45 trials performed in these patients, 6 showed the clinical responses as the HTCA predicted (true positive). In 3 trials, the HTCA indicated that the tumor would respond to the drug, but it did not clinically (false positive). Thus the true positive ones were 6 out of 9 (67%). There were no cases in which the tumor was resistant to the drug *in vitro*, but responded clinically (false negative). In 36 trials, the

Table 3 Correlation between *in vitro* sensitivity and clinical response

No. of clinical trials[a]	Positive		Negative	
	True[b,c]	False[b]	True[b,c]	False[b]
45	6	3	36	0

a: Clinical trials were done in 24 patients.

b: True positive, sensitive both *in vitro* and *in vivo*; False positive, sensitive *in vitro* and resistant *in vivo*; True negative, resistant both *in vitro* and *in vivo*; False negative, resistant *in vitro* and sensitive *in vivo*.

c: True positive rate, $6/9 \times 100 = 67\%$; True negative rate, $36/36 \times 100 = 100\%$.

tumor was resistant to the drug *in vitro* and also *in vivo* (true negative). Thus the true negative rate was 100%. Overall, 42 (93%) of the 45 trials showed a parallel correlation between the *in vitro* sensitivity and the clinical response.

Discussion

For the past many years, a number of clinical investigators have pursued the development of an *in vitro* test that could accurately predict the response of an individual patient's tumor to anticancer drugs. Methods of *in vitro* sensitivity testing reported up to the present include the measurements of labeling index, the doubling time, the chromium release, the dye exclusion and the colony formation (1,2, 5-7). Roper and Drewinko (8) evaluated these methods using a human immunoglobulin-producing cell line and concluded that the colony-forming assay provides the most reliable dose-dependent index of drug-induced cell lethality.

Since the HTCA has been reported to be useful for predicting the clinical response to anticancer drugs in patients with ovarian cancer and multiple myeloma (1, 2), the HTCA has been applied to various types of tumors. Salmon (9) and Von Hoff *et al.* (10) have extensively used the HTCA as a predictor of clinical response to chemotherapy and demonstrated a good correlation between the *in vitro* sensitivity and the clinical response. A comprehensive review of the results of *in vitro-in vivo* correlations reported by Bradley *et al.* (11) showed that the true positive rate was 71% and the true negative rate was 94% in 1,667 trials.

In the present study, the *in vitro-in vivo* correlation was studied in 45 trials including 16 prospective ones. In 42 (93%) of the 45 trials, a parallel correlation was observed between *in vitro* results and clinical responses. The true positive and negative rates for predicting a respose or lack of response of an individual patient's tumor to anticancer drugs were 67% and 100%, respectively. These results were very similar to those reported by others (9-11). While it is disappointing that only 6 *in vitro*-positive cases showed a positive response to the drugs clinically, the extremely high accuracy (100% true negative rate) in predicting the clinical drug resistance indicates that the HTCA can be used to exclude anticancer drugs which will not be clinically useful. More recently, Von Hoff *et al.* (12) and Alonso (13) performed prospective clinical trials to evaluate strictly the usefulness of the HTCA for selecting anticancer drugs for patients with advanced cancer. They reported true positive rates of 60-65% and true negative rates of 85-90%. Thus, the HTCA showed a good *in vitro-in vivo* correlation even in the prospective clinical trials.

While the results obtained by clinical studies are encouraging, there are several problems in the feasibility of applying the HTCA on a routine basis. These problems include the low colony-forming efficiency, the possible inappropriate *in vitro* drug incubation conditions and the arbitrary criteria for *in vitro* sensitivity or resistance. The most important of these is the low colony-forming efficiency of tumor cells taken from patients. In the present study, 94 (62%) of the 152 specimens formed at least 5 colonies per control plate, and only 63 (41%) formed more than 30 colonies per control plate to afford interpretable *in vitro* drug sensitivity data. Our results were closely comparable to those reported by others (9,10,12). Several investigators have studied the growth conditions of the assay system in order to improve colony-forming efficiency (14-16), but the optimum conditions have not been determined yet.

In summary, the HTCA is a useful technique for testing *in vitro* sensitivity of individual patients' tumors to anticancer drugs and is accurate in predicting the drug sensitivity and the drug resistance. However, further technical developments yielding higher colony-forming efficiency are necessary to make it more practical for routine clinical use.

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