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In vitro evaluation of anticancer drugs in relation to development of drug resistance in the human tumor clonogenic assay

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Summary. The efficacy of anticancer drugs against ovarian cancer, breast cancer, and colorectal cancer has been evaluated in vitro by the human tumor clonogenic assay developed by Hamburger and Salmon. The in vitro colony assay method used in this study is a minor modification of their method and was used in 83 patients with ovarian cancer, 47 patients with breast cancer, and 13 patients with colorectal cancer. The total numbers of assays performed in vitro were 258 for ovarian cancer, 87 for breast cancer, and 38 for colorectal cancer. The average chemosensitivity rates to single agents tested were 35% and 32% in the untreated patients with ovarian and breast cancer, respectively. In contrast to this result, the chemosensitivity rate of the untreated patients with colorectal cancer was only 16%. Consisting the clinical efficacy of anticancer drugs against these tumors, these results suggest that there is a correlation between chemosensitivity in the human tumor clonogenic assay and clinical responsiveness. In this assay the chemosensitivity in specimens from ovarian cancer patients who had had prior chemotherapy was significantly lower than in those from nonpretreated patients ($P < 0.05$). This seems to indicate the development of drug resistance after treatment with anticancer drugs. These results suggest that the human tumor clonogenic assay is a useful tool for the evaluation of antitumor effects of drugs in vitro.

Introduction

In 1979, the human tumor clonogenic assay was established by Hamburger and Salmon [11, 12], and it has been popularized and extensively studied as a test of chemosensitivity [5, 6, 27]. It is expected that the use of this human tumor clonogenic assay will make it possible to evaluate the antitumor effects of various drugs in vitro, to screen for new anticancer agents, and perform in vitro phase II studies. We have altready reported some results of colony growth in this assay system [14–16, 19]. Our results showed higher success rates for ovarian cancer and colorectal cancer in colony formation and plating efficiency than for sarcomas and gastric cancer, and we concluded that the capacity for colony growth is dependent on tumor type and not dependent on tumor sources. We now report the in vitro antitumor effect of drugs against ovarian cancer, breast cancer, and colorectal cancer and the influence of prior chemotherapy on in vitro chemosensitivity in the human tumor clonogenic assay.

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Materials and methods

Patient specimens. Tumor specimens obtained by biopsy and from malignant effusions were collected from 143 patients: 83 patients with ovarian cancer, 47 patients with breast cancer, and 13 patients with colorectal cancer. The eligibility conditions for tumor samples are as follows: (1) Proven malignancy confirmed by histological or cytological diagnosis; (2) fresh, not frozen condition; (3) possibility of triplicate assays with 500 000 nucleated cells plated per dish. Prior chemotherapy received by patients with ovarian cancer was the CAPF combination (cyclophosphamide, adriamycin, cisplatin, and 5-FU)[13]. Most of the patients with breast cancer had been treated with ACF [17] (adriamycin, cyclophosphamide plus ftorafur) and/or MMF (mitomycin C, methotrexate plus ftorafur).

Collection of cells. Solid tumors were immediately placed in McCoy's 5A medium plus 10% heat-inactivated newborn calf serum, plus 1% penicillin and streptomycin (all from Grand Island Biological Co., Grand Island, NY) and were then mechanically dissociated with scissors and incubated in collagenase (Type I) 0.2 mg/ml, DNase I 0.2 mg/ml, and pronase 0.5 mg/ml, all dissolved in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, at 37 °C for 30 min. Following two washes in phosphate-buffered saline (PBS), tumor cells were passed through a 50-mesh stainless steel cell sieve (Cellector) and 25-gauge needles to produce a single-cell suspension. Ascites and pleural fluid were collected in preservative-free heparinized bottles and centrifuged at 150 *g* for 5 min; tumor cells were then obtained by separation with Ficoll-Conray.

In vitro drug exposure of tumor cells. The tumor cells were exposed to standard anticancer drugs for 1 h at the following final concentrations (μg/ml): adriamycin 0.04; bleomycin 0.1; cisplatin 0.2; 5-fluorouracil (5-FU) 1.0; L-phenylalanine mustard (L-PAM) 0.4; and mitomycin C 0.1. The final concentrations of investigational drugs (μg/ml) are as follows: hexamethylmelamine (HMM) 1.0 and 4'-0-tetrahydropyranyladriamycin (THP-adriamycin) 0.5. These concentrations correspond to approximately one-

tenth of the peak plasma concentration for each drug dose in clinical use (Table 1). Stock solutions of both standard and investigational drugs were prepared in sterile physiological saline, water (mitomycin C and THP-ADM), or ethanol (L-PAM) and stored in a freezer (−20 °C) for up to 2 months. Tumor cell suspensions were adjusted to a final concentration of 5×10^5 cells/tube with medium and incubated with the drugs or control solution (saline) for 1 h in a water bath at 37 °C with shaking in McCoy's 5A medium plus 10% heat-inactivated newborn calf serum. They were then centrifuged at 150 *g* for 5 min, washed twice with phosphate-buffered saline, and cultured as described below.

Assay for colony formation. The culture system used in this study is a minor modification of the method of Hamburger and Salmon [11, 12]. Conditioned medium was not used. In brief, the cells to be tested were incubated with drugs at 37 °C for 1 h. Following two washes in McCoy's 5A medium plus 10% newborn calf serum, 5×10^5 cells were suspended in 0.3% agar in enriched Connaught Medical Research Laboratories Medium 1066 (CMRL medium; Grand Island Biological Co.) supplemented with 15% horse serum and nutrient. Prior to plating, asparagine (6.6 mg/ml), DEAE-dextran (50 mg/ml), and 2-mercaptoethanol (0.5×10^{-3} *M*) were added to enriched CMRL medium. Then 1 ml of this mixture was plated over 1 ml enriched McCoy's 5A medium with 10% heat-inactivated fetal calf serum plus 5% heat-inactivated horse serum plus nutrients in 0.5% agar in 35-mm plastic petri dishes. Before plating, tryptic soy broth (3%) plus asparagine (6.6 mg/ml) plus DEAE-dextran (50 mg/ml) were added to enriched McCoy's 5A medium. Plates were routinely monitored after plating for single-cell suspensions, and cell control and drug plates were prepared in triplicate. The plates were then incubated at 37 °C in a 7.5% CO_2 humidified atmosphere. The number of colonies was determined by counting the colonies with the automatic colony analyzer (CA-7A type) manufactured by Oriental Instruments, Ltd, Tokyo, Japan at 10–14 days after plating; at least 30 tumor colonies per plate were required in the control plates. Colonies were defined as aggregates of more than 50 cells. Positive identification of tumor cells was based on characteristics observed on Papanicolaou-stained slides.

Data analysis. Colony counts of the three plates counted for each tumor at the single drug concentration were averaged to obtain one data point. The results of the human tumor clonogenic assay were expressed as the percentage decrease in tumor colony-forming units (TCFUs) for a particular drug compared with control. The percentage decrease in TCFUs was calculated as the ratio between the mean number of colonies on treated plates and that on control plates. The significance of differences in chemosensitivitiy (ratio of decrease in TCFUs) was analyzed by the Chi-square test, and sensitivity rates were shown with the confidence limits (P) at the 95% confidence coefficient. The statistical significance of any difference between drug sensitivity curves was tested by the Kolmogorov-Smirnov (KS) test (two samples), and the P values were calculated by two-sided analysis.

Results

A total of 143 tumors (ovarian cancer, breast cancer, and colorectal cancer) were cultured and eight anticancer drugs were tested against three tumors. A total of 84 of the 143 specimens (59%) cultured formed ≧30 colonies per control plate and were evaluable for drug sensitivity analysis. The following are the details of the evaluable cases for ovarian cancer 49/83 (59%), breast cancer 26/47 (55%), and colorectal cancer 9/13 (69%). The cases evaluable for drug sensitivity included 31 with ovarian cancer, 16 with breast cancer, and 7 with colorectal cancer none of whom had received prior chemotherapy. The median numbers of colonies for the three tumor types evaluated in this study are; ovarian cancer, 200; breast cancer, 105; colorectal cencer, 198 colonies per dish (Table 2).

In vitro chemosensitivities of ovarian cancer to six commercially available drugs and two investigational drugs were analyzed (Table 3). A 50% level of inhibition of colony formation was used as the cutoff value to discriminate between those of these populations that were sensitive and those that were resistant in vitro. Of the cases without prior chemotherapy 35% ($34.6 < P < 35.9$), or 60/170 drug assays revealed sensitivity on average, compared with 22% ($21.1 < P < 21.9$), or 19/88 drug assays in cases with prior chemotherapy. The difference in the chemosensitivity rate is thus significant ($P < 0.05$). In cases with no prior chemotherapy the sensitivity rates to each drug were higher than those in cases with prior chemotherapy, but this difference was not statistically significant. Table 4 details the in vitro

Table 1. Anticancer drugs investigated in vitro

Drug tested	Preparation	Concentration used in study (μg/ml)	Dosage in clinical use	References
Adriamycin	PS [a]	0.04	45 mg/m²	[4]
Bleomycin	PS	0.1	15 U/m²	[2, 10]
Cisplatin	PS	0.2	100 mg/m²	[22]
5-fluorouracil	PS	1.0	15 mg/kg	[9]
L-phenylalanine mustard	Ethanol	0.4	30 mg/body	[3]
Mitomycin C	Water	0.1	20 mg/body	[21]
Hexamethylmelamine	PS	1.0	150 mg/m²	[7, 8]
THP-adriamycin [b]	Water	0.5	40 mg/m²	[20]

[a] Physiological saline
[b] 4'-0-Tetrahydropyranyladriamycin

Table 2. Growth of tumor colonies from ovarian, breast, and colorectal cancers

	Ovarian Ca	Breast Ca	Colorectal Ca
No. of samples	83	47	13
No. of evaluable samples	49 (59)[a]	26 (55)	9 (69)
No. with no prior chemotherapy	31	16	7
No. with prior chemotherapy	18	10	2
No. of colonies per dish			
Median	200	105	198
Mean	557	634	435
Range	33–4000	37–4000	66–2331
SD	954	1190	680

[a] ( ): %

Table 3. Effect of prior chemotherapy on in vitro chemosensitivities of anticancer drugs in ovarian cancer

Drug tested	Cases without prior chemotherapy		Cases with prior chemotherapy	
	Total	Sensitive[a] (%)	Total	Sensitive (%)
Adriamycin	24	9 (38)	14	2 (14)
Bleomycin	21	6 (29)	8	1 (13)
Cisplatin	26	9 (35)	16	4 (25)
5-fluorouracil	21	6 (29)	11	1 (9)
L-phenylalanine mustard	20	10 (50)	14	5 (36)
Mitomycin C	18	7 (39)	11	4 (36)
Hexamethylmelamine	19	5 (26)	7	0 (0)
THP-adriamycin	21	8 (38)	7	2 (29)
Total	170	60 (35)[b]	88	19 (22)[b]

[a] $\geqq$ 50% inhibition of colony formation
[b] $P < 0.05$

Table 4. Effect of prior chemotherapy on in vitro chemosensitivities of anticancer drugs in breast cancer

Drug tested	Cases without prior chemotherapy		Cases with prior chemotherapy	
	Total	Sensitive[a] (%)	Total	Sensitive (%)
Adriamycin	12	5 (42)	8	1 (13)
Bleomycin	1	1 (100)	4	0 (0)
Cisplatin	7	2 (29)	4	1 (25)
5-fluorouracil	9	2 (22)	4	2 (50)
L-phenylalanine mustard	9	2 (22)	5	1 (20)
Mitomycin C	9	3 (33)	8	2 (25)
THP-adriamycin	3	1 (33)	4	0 (0)
Total	50	16 (32)	37	7 (19)

[a] $\geqq$ 50% inhibition of colony formation

Table 5. Effect of prior chemotherapy on in vitro chemosensitivities of anticancer drugs in colorectal cancer

Drug tested	Cases without prior chemotherapy		Cases with prior chemotherapy	
	Total	Sensitive[a] (%)	Total	Sensitive (%)
Adriamycin	7	1 (14)	2	0 (0)
Bleomycin	2	0 (0)	1	0 (0)
Cisplatin	4	1 (25)	1	0 (0)
5-fluorouracil	6	1 (17)	1	0 (0)
Mitomycin C	7	1 (14)	1	0 (0)
THP-adriamycin	5	1 (20)	1	0 (0)
Total	31	5 (16)	7	0 (0)

[a] ≧ 50% inhibition of colony formation

chemosensitivities to seven anticancer drugs of the 16 breast cancer cases with adequate growth. The in vitro sensitivity rate for the cases without prior chemotherapy was 16 out of 50 drug assays (32%, $19 < P < 47$) and that for the cases with prior chemotherapy was 7 out of 37 drug assays (19%, $9 < P < 35$) (not statistically significant). In contrast to the cases with ovarian cancer and breast cancer, the in vitro chemosensitivity rate among 9 patients with colorectal cancer was only 16% ($8 < P < 34$), or 5/31 drug assays, which is a significantly low rate compared with the previous two tumor types ($0.05 < P < 0.1$) (Table 5). There is no significant difference in sensitivity between any two drugs in breast cancer or colorectal cancer.

To allow analysis of the development of resistance after chemotherapy for cancer patients, the relationship of in vitro chemosensitivity and prior treatment for ovarian cancer patients has been plotted in Figs. 1–4. The ordinate is the percentage decrease in TCFUs and the abscissa the

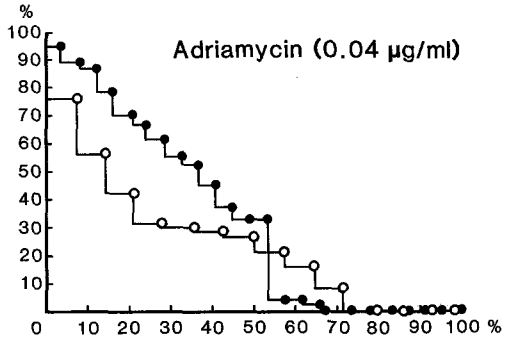

Fig. 1. Relationship between percentage of decrease in tumor colony-forming units (TCFUs) and cumulative percentage of cases treated with adriamycin (0.04 μg/ml) in vitro. *Closed circles*, cases with no prior chemotherapy; *open circles*, cases with prior chemotherapy

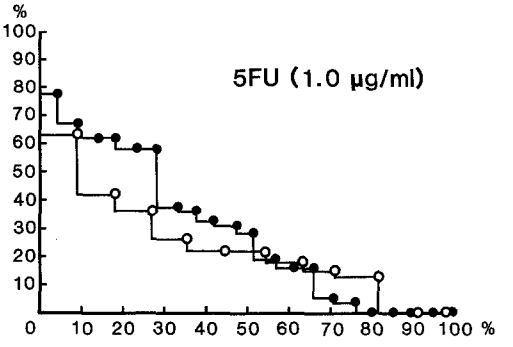

Fig. 2. Relationship between percentage of decrease in TCFUs and cumulative percentage of cases treated with 5-fluorouracil (1.0 μg/ml) in vitro *Closed circles*, cases with no prior chemotherapy; *open circles*, cases with prior chemotherapy

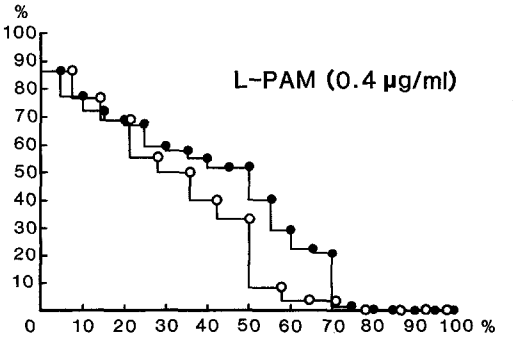

Fig. 3. Relationship between percentage of decrease in TCFUs and cumulative percentage of cases treated with L-phenylanine mustard (L-PAM) (0.4 μg/ml) in vitro. *Closed circles*, cases with no prior chemotherapy; *open circles*, cases with prior chemotherapy

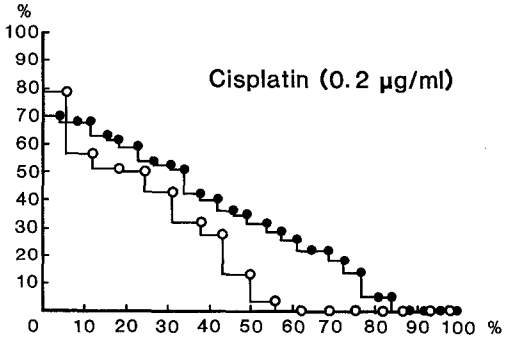

Fig. 4. Relationship between percentage of decrease in TCFUs and cumulative percentage of cases treated with cisplatin (0.2 μg/ml) in vitro. *Closed circles*, cases with no prior chemotherapy; *open cirlces*, cases with prior chemotherapy

cumulative percentage of ovarian cancer patients. Following treatment with adriamycin (Fig. 1), 5-FU (Fig. 2), and L-PAM (Fig. 3), no statistical significance (tested by the KS test) was seen in the drug sensitivity curves for adriamycin ($P=0.256$), 5-FU ($P=0.633$), or L-PAM ($P=0.720$). When the in vitro chemosensitivity of cisplatin is considered (Fig. 4), the difference between the group with no prior treatment and that with prior treatment is seen to be greater than that for the three other drugs, but not statistically significant ($P=0.175$).

Discussion

By means of a human tumor clonogenic assay developed by Hamburger and Salmon in 1977, various malignant tumors, including those of ovarian cancer, breast cancer, gastric cancer, and hematological malignancies, can be cultured and examined in the form of in the bilayer soft-agar assay. In the field of tumor biology, this assay system can be used as chemosensitivity test [5, 6, 27], a screening system for investigational anticancer agent [24], or an in vitro phase II study [1, 15, 23, 26], for the prediction of clinical prognosis [18], or as a tool for early detection of relapse [25]. As we have already reported, a high growing and plating efficiency is seen in ovarian cancer, breast cancer, and colorectal cancer. These tumors are good candidates for the human tumor clonogenic assay [14, 16]. In this paper, we have reported the in vitro antitumor effect of eight drugs and the development of drug resistance in vitro in patients with three cancers. Using a $\geqq 50\%$ decrease in TCFUs as the criterion of 'sensitivity', the in vitro chemosensitivity rate of specimens of ovarian cancer and breast cancer from patients with no prior chemotherapy are 35% (60/170 drug assays) and 32% (16/50 drug assays), respectively. These chemosensitivity rates are compatible with or a little higher than the clinical response rates reported for the same drugs in the literature. For colorectal cancer, the in vitro chemosensitivity rate was only 16% (5/31 drug assays), and it tended to be lower than that for the other two cancers ($0.05 < P < 0.1$). We regard these results as evidence that the in vivo antitumor effect of drugs can be predicted by using the human tumor clonogenic assay in vitro. These data may provide a basis for the application of this method in the in vitro phase II study and screening of new anticancer drugs.

Shoemaker [24] reported that 25 of 103 compounds which were negative in in vivo P388 leukemia screening showed activity, with in vitro response rates ranging from 13% to 100% in stage I of the human tumor clonogenic assay (continuous exposure to compounds at a concentration of 10 μg/ml). In all, 76% (78/103) of compounds were negative. Furthermore, 14 out of these 25 compounds had response rates of 20% or greater in the five dose-response experiments in six target tumors (breast, colorectal, kidney, lung, melanoma, and ovary) (stage II). On the basis of these data Shoemaker suggested that this system could be valuable in the drug screening system.

In the case of ovarian cancer, in vitro chemosensitivity to the drugs tested of the specimens obtained from previously treated patients was significantly lower than that of specimens from patients with no prior chemotherapy ($P<0.05$). In the comparison of cumulative percentage with reference to percentage decrease in the number of TCFUs between the patients with and without prior treatment, however, the group with prior treatment shows the same sensitivity as the group without prior treatment to adriamycin ($P=0.256$), 5-FU ($P=0.633$), and L-PAM ($P=0.720$), but in the case of cisplatin the difference was greater than that to the other three drugs ($P=0.175$) (statistically not significant). Concerning the relationship of development of drug resistance and prior chemotherapy, we have already reported that in vitro sensitivity of adriamycin and bleomycin is markedly reduced by prior chemotherapy with the same drugs [19]. We interpreted these data as an indication that drug resistance appears after exposure to a chemotherapeutic regimen. In cancer chemotherapy the first treatment with which complete response is achieved is most important when the development of drug resistance after chemotherapy is considered. Thus, if medical oncologists selected the drug to be used for the treatment of patients on the basis of the result of a clonogenic assay, the selection could be more informed. Furthermore, the collection of in vitro chemosensitivity data for patients with prior chemotherapy may produce useful information for selection of a second-line chemotherapy. In summary, our results presented here support the concept that the human tumor clonogenic assay can be used to evaluate the antitumor effect of drugs and, potentially to predict the development of drug resistance in patients who have received prior chemotherapy.

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