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Comparison of Drug Sensitivity among Tumor Cells within a Tumor, between Primary Tumor and Metastases, and between Different Metastases in the Human Tumor Colony-forming Assay[1]

Nobuhiko Tanigawa,[2] Yoshifumi Mizuno, Takafumi Hashimura, Kazuo Honda, Kisaku Satomura, Yorinori Hikasa, Ohtsura Niwa, Tsutomu Sugahara, Osamu Yoshida, David H. Kern, and Donald L. Morton

Second Department of Surgery, Fukui Medical College, Fukui, Japan [N. T.]; Second Department of Surgery [Y. M., K. H., K. S., Y. H.], Department of Urology [T. H., O. Y.], and Experimental Radiology [O. N.], School of Medicine, Kyoto University, Kyoto, Japan; Kyoto National Hospital, Kyoto, Japan [T. S.]; and Division of Surgical Oncology, UCLA School of Medicine, Los Angeles, California 90024 [D. H. K., D. L. M.]

ABSTRACT

The human tumor colony-forming assay was used to compare chemosensitivity among tumor cells within a primary tumor, between primary tumor and metastases, and between different metastases. No significant differences in cloning efficiency were found in any of the three comparison studies. However, considerable differences in chemosensitivities were observed between different parts of the same tumor and between the primary tumor and metastases. Two different parts of the same tumor were comparably assayed for nine primary tumors. In nine paired samples which allowed *in vitro* drug sensitivity testing, there was no satisfactory correlation of sensitivity to cytostatic drugs. Cell suspensions were prepared from 28 primary tumors and from metastases taken from the same patient. In 14 paired samples which formed sufficient colonies for determination of drug effect, the data showed no satisfactory correlation of chemosensitivity between a primary tumor and its metastases. Both tumor samples from different metastatic sites of the same patient formed sufficient colonies in seven of eight instances. In the seven paired samples, there was strong association of chemosensitivity ($p < 0.005$). The results indicate that the reported discrepancies of *in vitro* and *in vivo* results in clinical trials using the tumor colony-forming assay for predicting resistance or sensitivity to cytostatic drugs may be due to therapeutic heterogeneity among tumor colony-forming units within a primary tumor and between a primary tumor and its metastases.

INTRODUCTION

The predictive value of the human TCFA[3] for predicting clinical response has proved unsatisfactory due to discrepancies between the *in vitro* and *in vivo* responses to specific drugs (2, 19, 20, 26, 28–30, 41–43). Numerous studies have demonstrated that some animal tumors and human tumors are composed of more than one cell clone with different susceptibilities to a variety of cytostatic drugs (3–5, 7, 15, 17, 18, 21, 22, 24). If human TCFUs are heterogenous as regards their sensitivity to cytotoxic drugs, the sensitivity pattern obtained using small biopsies in TCFA is not likely to be representative for the majority of the tumor. Observed discrepancies between *in vitro* and *in vivo* results in the TCFA may be due to such a therapeutic heterogeneity of the human tumors. The aim of this investigation, therefore, was to study whether there was heterogeneity in drug sensitivity among TCFUs within a tumor, between a primary tumor and its metastases, and between different metastases.

MATERIALS AND METHODS

Tumor samples, obtained at operation, were immediately placed in sterile plastic containers in CEM (Microbiological Associates, Inc., Bethesda, MD). When the primary tumors were large, 2 pieces of tumor (one from the central part and the other from the peripheral part in the same tumor) were taken.

The tumor material was cut free from nontumorous tissues and minced into pieces less than 2 mm in diameter. Cell suspensions were prepared enzymatically as in our previous reports (26, 36). In brief, the tumor fragments were placed into a 75-ml trypsinizing flask into which 40 ml of prewarmed Hanks' balanced salt solution (Flow Laboratories, Inc., McLean, VA) containing 0.03% DNase (500,000 units/ml) and 0.14% collagenase type I (all from Calbiochem/Behring Corp., La Jolla, CA) were added. After enzymatic digestion for 30 to 60 min at 37° and the washing procedure, large nucleated cells were counted to assess yield and examined by trypan blue dye exclusion to assess viability.

Preparation of Soft Agar Plates. The cells were cultured as described previously (26, 36). Briefly, the washed cells were suspended in 0.3% Bacto-agar (Difco Laboratories, Detroit, MI) containing CEM, supplemented with 15% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, MD), penicillin (100 μg/ml), and streptomycin (100 units/ml; Grand Island Biological Co., Grand Island, NY), to yield a final concentration of 5×10^5 large nucleated viable cells/ml. The cells were plated over a 1.0-ml underlayer of CEM in 0.5% agar.

***In Vitro* Drug Exposure.** The tested drugs were incorporated into the upper layer of the culture system, because an increased inhibition of colony growth has been demonstrated by continuous drug exposure as compared to a 1-hr exposure (1). The concentrations of the various agents used were: doxorubicin hydrochloride (Adriamycin), 0.2 μg/ml; bleomycin sulfate, 2.0 μg/ml; bis(chloroethyl)nitrosourea, 2.0 μg/ml; 5-fluorouracil, 10.0 μg/ml; mitomycin C, 3 μg/ml; melphalan, 1.0 μg/ml; and *cis*-platinum, 2.0 μg/ml. Each drug was tested at a dose comparable to the highest concentration pharmacologically achievable in patient serum (26). All platings were done in triplicate. The plates were placed in a humidified incubator at 37° in the presence of 5% carbon dioxide.

Colony Count. Colony growth was checked during the course of the experiment using an inverted microscope at × 100 and × 200. Colonies were defined as aggregates of 50 or more cells. Maximum colony formation was reached between 14 and 21 days of culture, at which time their numbers were recorded, and the mean ± S.D. for each of the triplicate counts was calculated. All counts were corrected for background colony counts by subtracting the colony counts on Day 0. Assays were not considered to be evaluable for determination of drug effect

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: TCFA, tumor colony-forming assay; TCFU, tumor colony-forming unit; CEM, Chee's modification of Eagle's medium.

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unless at least 30 colonies per control plate were seen. Statistical differences in cloning efficiency of paired samples were tested by the paired-sample *t* test. Regression analysis was performed to compare chemosensitivity in the paired tumor samples.

RESULTS

Comparison of 2 Different Parts of the Same Tumor. The central and peripheral parts of the same tumor were comparably assayed for 9 primary tumors: 5 renal cell carcinomas; one nonseminomatous testicular tumor; one liposarcoma; one yolk sac tumor; and one neuroblastoma. Both parts formed sufficient colonies (at least 30/control plate) in all 9 experiments (Table 1). In these 9 paired samples, there were no significant differences in the cloning efficiencies and in viabilities of prepared large nucleated cells. The effect of cytostatic drugs on TCFUs of different biopsies from the same tumor is shown in Chart 1. The regression coefficient was −0.15, and there was no significant association between the biopsies, indicating that the *in vitro* sensitivity results obtained from one part of biopsy were not representative for those of the other part of the tumor in the TCFA.

Comparison of Primary Tumor and Metastases. Twenty-eight primary tumors and metastases obtained simultaneously during the operative procedure were assayed. Both tumor samples formed sufficient colonies in only 14 of the 28 experiments (2 breast, 14 stomach, 8 colon, 3 rectal, and one lymphoma). The source of these 14 tumors is given in Table 2. Their cloning efficiencies did not differ significantly from each other. The results of *in vitro* sensitivity to cytostatic drugs were similar between the paired samples in 6 of the 14 experiments, but not in the other 8. Overall, there was no significant association ($r = 0.26$) between drug inhibition of tumor colony growth in the TCFA, using cells from a primary tumor compared to that using its metastases. Also, there were no significant differences for the different cytostatic agents tested (Chart 2).

The data suggest that primary-derived tumor colonies may not associate with tumor colonies derived from the corresponding metastases as regards sensitivity to cytostatic drugs.

Comparison of Different Metastases. Both tumor samples from different metastatic sites formed sufficient colonies in 7 of the 8 instances (one stomach, one uroepithelial, 2 colon, one ovarian, one osteosarcoma, and 2 melanoma). These 7 paired samples were detailed in Table 3. Tumor cells from ascites (Table 3, Patient C) were obtained from an advanced ovarian cancer patient at the different times during a period of 6 weeks. The other 6 paired samples were obtained simultaneously during the operation. There were no significant differences in cloning efficiencies between the paired samples in the TCFA. However, when *in vitro* drug sensitivities of cells obtained from different metastatic sites were compared, they were significantly similar to each other ($r = 0.74$; $p < 0.005$) (Chart 3). The data suggest that the association between tumor colonies derived from differ-

Table 1

Comparison of colony growth of different parts (center, periphery) of 9 primary tumors

Patient	Type of tumors	TCFUs/5 × 10^5 cells	
		Center	Periphery
1	Renal cell carcinoma	147 ± 9[a]	171 ± 14
2	Renal cell carcinoma	119 ± 3	99 ± 9
3	Renal cell carcinoma	136 ± 2	41 ± 5
4	Renal cell carcinoma	192 ± 20	294 ± 15
5	Renal cell carcinoma	168 ± 34	49 ± 6
6	Liposarcoma	1323 ± 139	1362 ± 213
7	Neuroblastoma	114 ± 18	71 ± 8
8	Yolk sac tumor	225 ± 36	229 ± 5
9	Nonseminomatous testicular tumor	141 ± 8	38 ± 4

[a] Mean ± S.E.

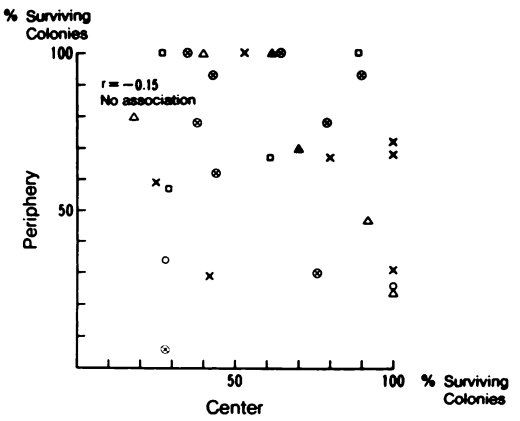

Chart 1. Associations of *in vitro* drug sensitivity of different parts (center, periphery) of 9 primary tumors in the TCFA. No significant correlation occurred. $r = -0.15$; $p > 0.5$. Individual drugs were illustrated by different symbols, respectively: ○, Adriamycin; □, bleomycin; △, 5-fluorouracil; ×, mitomycin C; ▲, melphalan; ⊗, *cis*-platinum.

Table 2

Comparison of colony growth of 14 primary tumors and metastases

Patient	Type of tumors	TCFUs/5 × 10^5 cells	
		Primary	Metastasis
1	Breast carcinoma	79 ± 3[a]	149 ± 17
2	Breast carcinoma	69 ± 6	295 ± 32
3	Gastric carcinoma	65 ± 0	201 ± 63
4	Gastric carcinoma	77 ± 3	43 ± 3
5	Gastric carcinoma	30 ± 3	35 ± 2
6	Gastric carcinoma	355 ± 15	38 ± 3
7	Gastric carcinoma	156 ± 16	135 ± 3
8	Gastric carcinoma	91 ± 16	407 ± 53
9	Colon carcinoma	152 ± 15	30 ± 1
10	Colon carcinoma	109 ± 4	30 ± 2
11	Colon carcinoma	261 ± 11	41 ± 2
12	Colon carcinoma	200 ± 29	202 ± 10
13	Rectal carcinoma	57 ± 5	173 ± 4
14	Malignant melanoma	57 ± 17	35 ± 4

[a] Mean ± S.E.

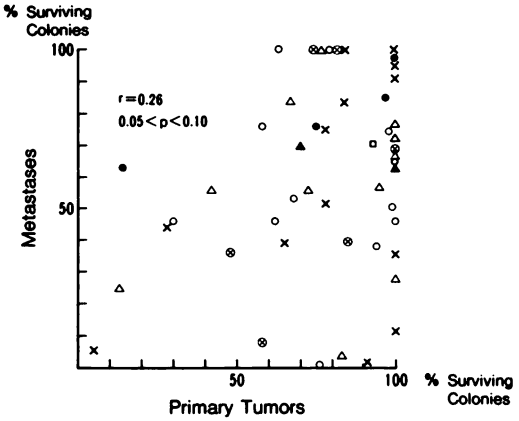

Chart 2. Associations of *in vitro* drug sensitivity of 14 primary tumors and metastatic lesions simultaneously tested in the TCFA. No significant correlation occurred. $r = 0.26$; $p > 0.05$. ●, bis(chloroethyl)nitrosourea; for explanations of other symbols, see the legend to Chart 1.

Table 3
Detail of different metastatic lesions and their colony growth

Patient	Type of tumors	Sites of metastases (TCFUs/5 × 10^5 cells)
A	Gastric carcinoma	Lymph nodes (140 ± 5,[a] 148 ± 8)
B	Colon carcinoma	Ovary (33 ± 11), omentum (35 ± 3)
C	Ovarian carcinoma	Abdominal effusion (151 ± 3, 640 ± 64)
D	Uroepithelial carcinoma	Liver (184 ± 14), abdominal wall (33 ± 3)
E	Osteosarcoma	Lung (120 ± 12), chest wall (50 ± 6)
F	Malignant melanoma	Arm (74 ± 5), breast (352 ± 70)
G	Malignant melanoma	Thigh (78 ± 3), hip (32 ± 3)

[a] Mean ± S.E.

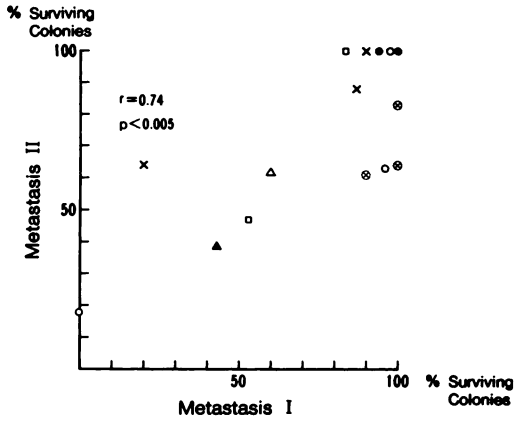

Chart 3. Associations of *in vitro* drug sensitivity of different metastatic lesions in the TCFA. Significant correlation occurred. $r = 0.74$; $p < 0.005$. ●, bis(chloroethyl)nitrosourea; for explanations of other symbols, see the legend to Chart 1.

ent metastatic sites may be closer than that of tumor colonies from a primary tumor and its metastases as regards drug sensitivity under the *in vitro* conditions of TCFA.

DISCUSSION

Tumor stem cells are responsible for tumor repopulation after treatment and also for metastatic growth, and they are therefore the primary target of any cancer chemotherapy (10, 19, 34, 35). The concept of human tumors as stem cell systems suggests that the TCFA may be useful in testing a patient's tumor for chemo- and/or radiosensitivity. The current TCFAs that have been used in many laboratories in efforts to predict the clinical response of a tumor to drug treatment can predict clinical resistance with 84 to 98% accuracy and can predict clinical response with 40 to 72% accuracy (26, 28, 41–43). The predictive value for clinical response of the TCFA is not satisfactory.

The discrepancies between *in vitro* and *in vivo* results may be due to differences of distinct tumor stem cells and clonable cells in the TCFA or to differences in drug sensitivity among TCFUs residing within a tumor. The present study was, therefore, designed to compare chemosensitivity results assessed with the TCFA between different parts of the same primary tumor, between a primary tumor and its metastasis, and between tumor samples from different metastatic sites.

No correlation regarding resistance or sensitivity to cytostatic agents was observed between 2 different parts (center and periphery) of the same primary tumor. This appears to be due to heterogeneity which could be triggered by local nutritional differences, variations in tumor necrosis, and contaminations by other nonmalignant cells (9, 33). These factors should cause differences in the cloning efficiency of tumor cells in the TCFA. No significant changes in their cloning efficiencies, however, were found, suggesting that the discrepancies in their drug sensitivities would not be largely affected by those environmental factors of tumor cells. Meanwhile, it is considered that the results of the TCFA may be influenced by the presence of 2 or more clones of tumor cells with different drug sensitivities. The presence within a primary tumor of subpopulations of cells with different drug sensitivities has been documented recently in clinical (3, 5, 7, 24, 37–39) and experimental tumors (4, 15, 17, 18, 21, 22, 25, 27, 31, 32) with usual conventional *in vitro* culture of tumor cells but not with the TCFA.

The results of this study may suggest, like the conclusion of Schabel *et al.* (32), that many methods to evaluate drug sensitivity of tumors may be misleading, since they are based on the assumption that tumors are homogeneous. However, tumor specimens tested in this study were all relatively larger than are the usual human neoplasms, because the capacity of separate biopsies technically depended on the size of the tumor samples. In general, as a primary tumor increases in size, the differences in biological characteristics of various parts of the same tumor also seem to increase. The data suggest that the sensitivity pattern of one biopsy is not likely to be representative for the other part of the same tumor, when assessed by the TCFA in the larger tumors.

The results of drug sensitivity of tumor colonies derived from primary tumors were not significantly associated with those of the corresponding metastases. The results of TCFA for tumor samples from different metastatic sites, however, indicated strong association in sensitivity to some cytostatic agents. There are previous reports of biological differences among tumor cells from human primary tumors, metastatic lesions, and various metastases (14–16, 23, 31, 32, 40). From the results of an investigation of the capacity of i.v.—inoculated B16 melanoma tumor cells to form pulmonary metastases in C57BL/6 mice, Fidler and Kripke (11, 13) and Hager and Heppner (16) postulated that metastases result from the survival and proliferation of specialized subpopulations of cells that preexist within the primary neoplasm and that different metastases may develop from different progenitor cells. Evidence for this cellular diversity has been provided by some other works with human tumors (6, 8). Similar to previous studies using TCFA (33), we found no significant correlations in drug sensitivity between primary tumors and their metastases.

Of interest was the finding in this study that there was a strong association in drug sensitivity between tumor colonies derived from different metastases of the same patient. Other investigators have used *in vitro* culturing of the entire tumor cell population instead of the TCFA and have suggested the existence of heterogeneity in drug sensitivity among various metastatic lesions (12, 14, 40). Thus, it appears that metastatic lesions may be composed of subpopulations of tumor cells with nonidentical susceptibilities to cytostatic drugs. In our studies, the association of drug sensitivities between different metastases in the TCFA suggests that drug sensitivity of clonal tumor cells shows a better correlation compared to assays that utilize the whole-tumor cell population (29, 43). For this reason, we suggest that *in vitro* drug sensitivity results in the TCFA obtained from the primary tumor may have severe limitations in selection of appropriate drugs against metastatic lesions, whereas the results from

a metastatic lesion may have more profound implications for the predicting of potential agents for the treatment of other metastatic lesions of the same patient.

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