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Heterogeneity in Growth Pattern and Drug Sensitivity of Primary Tumor and Metastases in the Human Tumor Colony-forming Assay[1]

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ABSTRACT

The human tumor colony-forming assay was used to compare primary tumors with their metastases. Cell suspensions were prepared from 38 primary tumors and from metastases taken simultaneously from the same patient. Considerable differences were observed. Cloning efficiency was significantly higher in the cell suspensions taken from metastases than from material from the primary tumor. In 10 paired samples which allowed *in vitro* drug sensitivity testing, the data showed no satisfactory correlation in resistance or sensitivity to cytostatic drugs between primary tumor and metastases. The regression coefficient calculated for the different cytostatic agents (Adriamycin, bis(chloro)ethylnitrosourea, 5-fluorouracil, 4-hydroxyperoxycyclophosphamide) varied between 0.02 and 0.1. The results indicate that experiments designed to analyze chemosensitivity of tumor cells in the tumor colony-forming assay should be interpreted with caution. In particular, *in vitro* sensitivity data obtained from the primary tumor may have severe limitations in planning treatment of metastatic disease.

INTRODUCTION

Several attempts have been made to develop a test that would predict the response of an individual patient's tumor to chemotherapeutic agents. The TCFA[3] is one of the most recent and promising *in vitro* tests (5, 11). Retrospective and prospective clinical trials with the TCFA indicate a 60 to 70% true-positive and a 84 to 98% true-negative *in vitro-in vivo* correlation for a limited number of different tumors and drugs (12, 13). Observed discrepancies of *in vitro* and *in vivo* results may be due to pharmacokinetic *in vitro* differences as well as to differences in the behavior and extent of metastases *in vivo*. The aim of our investigation, therefore, was to elucidate differences in drug sensitivity in the primary tumor and its metastases in the TCFA.

MATERIALS AND METHODS

Solid tumor samples, obtained at the time of surgery, were immediately transported in sterile plastic containers in Hanks' balanced salt solution to the laboratory. Cell suspensions were prepared mechanically by teasing and mincing the tissue with sterile scalpels and needles and forcing it through a wire mesh gauze. After the washing procedure, aliquots of the single-cell suspension were prepared for determination of viability by trypan blue dye exclusion. Papanicolaou smears were used to analyze the proportion of tumor cells. The same number of nucleated viable cells (5×10^5 cells) was plated in each assay, and the cloning efficiency (number of colonies per plate/number of plated tumor cells) was calculated. The cells were cultured according to the technique described in the papers of Hamburger and Salmon (5) and Salmon *et al.* (11). Briefly, the washed cells were suspended in 0.3% Bacto-agar (Difco Laboratories, Detroit, Mich.) containing CMRL-1066 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) with 15% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, Md.) to yield a final cell concentration of 5×10^5/ml. The cells were plated over a 1.0-ml underlayer of tissue culture medium in 0.5% agar. No conditioned medium was used.

By a modification of the original method, the agents to be evaluated by drug sensitivity studies were incorporated into the upper layer of the culture system. Continuous drug exposure was used because an increased inhibition of colony tumor cell growth has been demonstrated recently in some drugs and tumor specimens in comparison to a 1-hr exposure (1). The final concentrations of the various agents used were: Adriamycin, 0.2 μg/ml; bis(chloroethyl)nitrosourea, 0.1 μg/ml; 5-fluorouracil, 6.0 μg/ml. Since cyclophosphamide is inactive *in vitro*, the activated metabolite 4-hydroxyperoxycyclophosphamide was used (0.2 μg/ml). All assays were set up in triplicate. Plates were incubated at 37° in 5% carbon dioxide in humidified air and were not refed in accordance with the original method (11).

The number of colonies (aggregates of more than 30 cells) on control and drug-exposed plates was determined by inverted-phase microscopy at weekly intervals after initiation of the cultures. By methods previously described, granulocyte-macrophage colonies were excluded (9). At least, 100 tumor colonies per control plate were required for an adequate base line for drug sensitivity testing. Statistical differences in cloning efficiency of primary tumors and metastases were tested by the paired-sample *t* test. Correlation analysis was performed to calculate the regression coefficient to compare chemosensitivity in both groups.

RESULTS

Thirty-eight primary tumors and metastases obtained simultaneously during the operative procedure were assayed. Both tumor samples formed colonies (at least 15 per control plate) in only 21 of the 38 experiments. The source of these 21 tumors is given in Table 1.

The mean percentage of tumor cells in the cell suspension of metastases was lower than that observed for the primary tumors (Table 2). Despite the lower number of tumor cells in the samples of metastases, the cloning efficiency was significantly increased ($p < 0.1\%$). Morphological differences were recognized. Frequently, the colonies derived from metastatic lesions were larger and contained more cells than colonies from the primary tumors. Also, clusters (cell aggregation of 8 to 20 cells) were observed more frequently in primary than in metastatic tumor samples. Maximum colony formation was reached between 14 and 21 days of culture independent of

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[3] The abbreviation used is: TCFA, tumor colony-forming assay.

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Table 1

Type of primary tumors and metastases and percentage of tumor cells in the prepared cell suspension of each sample used for colony-forming assay

	Primary tumor		Metastatic lesion	
Patient	Type of primary tumor	% of tumor cells in prepared cell suspension	Type of metastases	% of tumor cells in prepared cell suspension
1	Breast carcinoma	70	Lymph node	55
2	Breast carcinoma	80	Lymph node	60
3	Breast carcinoma	70	Lymph node	55
4	Breast carcinoma	75	Lymph node	60
5	Breast carcinoma	70	Lymph node	50
6	Gastric carcinoma	85	Peritoneum	40
7	Gastric carcinoma	30	Lymph node	15
8	Gastric carcinoma	50	Peritoneum	5
9	Gastric carcinoma	55	Peritoneum	60
10	Gastric carcinoma	80	Peritoneum	60
11	Colon carcinoma	65	Liver	45
12	Colon carcinoma	90	Lymph node	30
13	Colon carcinoma	85	Lymph node	40
14	Colon carcinoma	60	Lymph node	55
15	Rectal carcinoma	70	Lymph node	30
16	Seminoma	65	Lymph node	35
17	Pulmonary carcinoma	75	Lymph node	55
18	Renal carcinoma	85	Soft tissue	20
19	Ovarian carcinoma	90	Peritoneum	65
20	Ovarian carcinoma	65	Peritoneum	40
21	Malignant melanoma	40	Lymph node	35

tumor cell source. Sufficient numbers of tumor colonies (>100 tumor colonies per control plate) to allow statistical evaluation of the effect of cytostatic agents were obtained in 10 instances with 21 paired samples (Table 2). In these 10 experiments, the reduction of tumor colony growth by cytostatic drugs differed between primary tumor and metastatic lesions under the *in vitro* conditions of the TCFA (Chart 1). The regression coefficient for the different cytostatic agents varied between $r = 0.02$ and $r = 0.1$ and was not significant for any cytostatic agents tested (Adriamycin, bis(chloroethyl)nitrosourea, 5-fluorouracil, 4-hydroxyperoxycyclophosphamide). The data suggest that metastasis-derived tumor colonies are probably more sensitive *in vitro* to cytostatic agents than are tumor cell colonies derived from the corresponding primary tumor. Similar results were obtained when the assays were examined at weekly intervals.

DISCUSSION

The present study was designed to compare results obtained with the TCFA in primary tumor and metastases. The cloning efficiency of cell suspensions derived from primary tumors was significantly lower compared to those derived from metastases. No satisfactory correlation regarding resistance or sensitivity to cytostatic agents was observed between primary tumor and metastases in our experiments using the TCFA.

These findings may be discussed with respect to technical problems of the TCFA, to biological properties of clonable cells, and to their chemosensitivity in the assay. A major technical problem in the clinical application of TCFA is caused by the fact that sufficient colonies to allow drug sensitivity studies can be obtained from only a minority of available tumor material (10, 13). The design of our study required adequate colony growth from 2 tumor samples from each patient. This condition could be met in only 10 of 38 cases. Therefore, technical restriction becomes more pronounced if more extensive analysis of the *in vitro* drug sensitivity of a tumor is attempted, using material from several sites. Although the number of experiments is relatively small, the observed differences between primary tumor and metastases are relevant to the interpretation of the TCFA.

The observed differences in cloning efficiency between primary tumor and metastases may be due to methodolcgical and biological characteristics. Differences in cloning efficiency have been reported in simultaneous or consecutive assays for primary tumor and tumor cells from ascites (4) for tumor samples from different metastatic sites (9) and for material with different degree of differentiation from the primary tumor (8). Clonogenicity of tumor cells in the TCFA is especially influenced by other, nonmalignant cells in the plating mixture (3). Differences between primary tumor and metastases in the TCFA may be enhanced by using a differing of malignant cell:nonmalignant cell ratio in the cell suspension. In addition to these technical problems, the results of the TCFA are mainly influenced by the biological heterogenicity of tumor cells. Neoplasms are composed of cells with different proliferative and invasive properties (6). It has been postulated that the cells which produce colony formation in the TCFA represent the subpopulation of cells responsible for tumor growth and propagation (5). The differences in cloning efficiency and morphological appearance of the colonies in our experiments indicate heterogenicity within the clonable tumor cell compartments of a primary tumor and its metastases. This heterogenicity will influence the sensitivity studies performed on material of various tumor manifestations. It has already been shown that distinct nonclonogenic tumor cells are differentially susceptible to cytostatic agents (2, 7). Our data show that the clonogenic cells from a primary tumor and its metastases differ also in their drug sensitivity. The primary reason may also lie in the biological heterogenicity of the clonable tumor cells. In addition, a

Table 2

Comparison of colony growth and cloning efficiency of 21 primary tumors and metastases 21 days after plating procedure

	Primary tumor		Metastatic lesion	
Patient	TCFU[a]/5 × 10^5 plated cells	Cloning efficiency	TCFU/5 × 10^5 plated cells	Cloning efficiency
1	76 ± 13[b]	0.022	142 ± 11	0.052
2	139 ± 14	0.035	220 ± 15	0.073
3	280 ± 18	0.080	380 ± 29	0.138
4	185 ± 10	0.049	240 ± 12	0.080
5	250 ± 16	0.071	360 ± 30	0.144
6	54 ± 3	0.013	82 ± 7	0.041
7	18 ± 4	0.012	44 ± 3	0.059
8	42 ± 5	0.017	33 ± 5	0.132
9	91 ± 6	0.033	157 ± 28	0.052
10	58 ± 10	0.015	147 ± 20	0.049
11	97 ± 13	0.030	148 ± 20	0.066
12	35 ± 5	0.008	40 ± 10	0.027
13	110 ± 20	0.026	180 ± 15	0.090
14	140 ± 15	0.047	168 ± 16	0.061
15	48 ± 3	0.014	68 ± 9	0.045
16	55 ± 6	0.017	116 ± 13	0.066
17	53 ± 25	0.014	102 ± 18	0.037
18	102 ± 20	0.024	157 ± 16	0.157
19	138 ± 14	0.031	169 ± 30	0.052
20	153 ± 16	0.047	214 ± 25	0.107
21	143 ± 13	0.072	194 ± 10	0.111

[a] TCFU, tumor colony-forming units.
[b] Mean ± S.E.

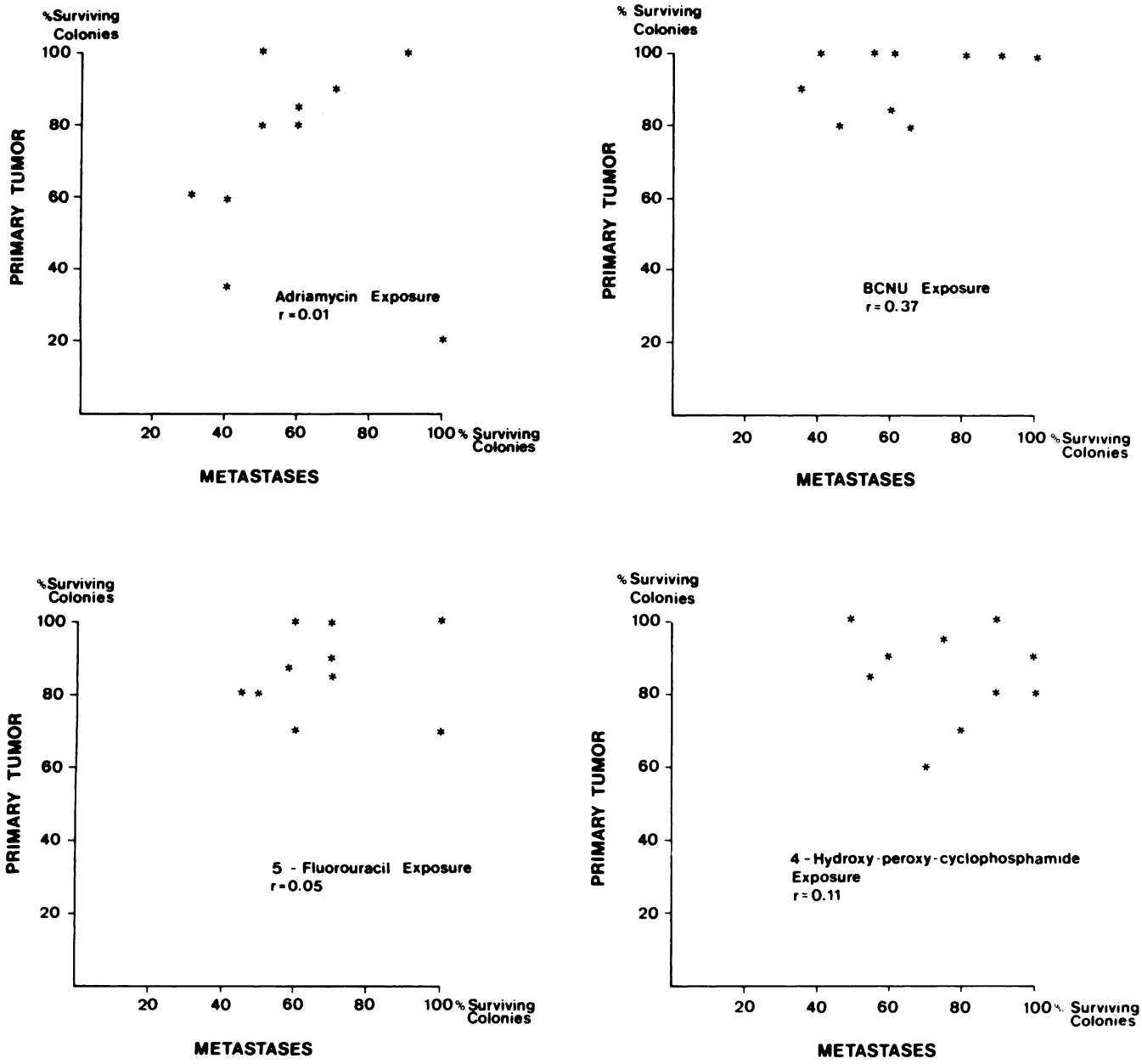

Chart 1. Regression analysis of *in vitro* sensitivity of 10 primary tumors and metastasic lesions simultaneously tested in the colony-forming assay to various cytostatic agents. Results 21 days after initiation of the culture. Adriamycin (0.2 μg/ml), bis(chloroethyl)nitrosourea (*BCNU*) (0.1 μg/ml), 5-fluorouracil (6.0 μg/ml), and 4-hydroxyperoxycyclophosphamide (0.2 μg/ml) were incorporated into the agar at the time of plating procedure. No significant correlation occurred.

cell suspension obtained from the primary tumor and the corresponding metastases will contain a different ratio for clonable and nonclonable tumor cells and for normal cells; different sensitivity in these fractions may modify colony growth under drug exposure (3, 4). Experiments designed to analyze surviving tumor cell colonies after *in vitro* drug sensitivity testing may therefore be affected by changes in clonogenic cells. Therefore, testing the chemosensitivity of a human tumor by the TCFA influenced by a complex array of biological and technical determinance and its interpretation for clinical use should be done with caution.

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