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Correlation of histology and drug response of human tumors grown in native-state three-dimensional histoculture and in nude mice

(intact tissue architecture/[^{3}H]thymidine uptake/histological autoradiography)

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ABSTRACT **An *in vitro* histoculture system in which a native-state collagen-sponge gel supports the three-dimensional growth of tumor tissue has been recently developed that allows the culture and drug response assay for most every tumor type. Important features of the histoculture system include the maintenance of three-dimensional tissue architecture and the use of histological autoradiography to measure nuclear incorporation of [^{3}H]thymidine as an endpoint. We describe in this report *in vitro–in vivo* correlations for drug response and tumor histology by using human tumor xenografts grown in the native-state three-dimensional histoculture system and as xenografts in nude mice. This comparison eliminates many of the confounding variables seen in most correlative clinical trials. Results demonstrate (*i*) a very high preservation of *in vivo* tissue architecture *in vitro*, (*ii*) an 86% accuracy *in vitro* of predicting drug resistance *in vivo* using suprapharmacologic doses of drugs *in vitro*, and (*iii*) an overall predictive frequency of drug resistance and sensitivity ranging from 53% for 5-fluorouracil to 78% for doxorubicin.**

An important need in cancer treatment is an *in vitro* means by which to accurately assess chemosensitivity of all types of human tumors and relevant normal tissue for comparison. There have been many attempts at *in vitro* drug response testing (1–3). As has been pointed out (3), *in vitro* assays thus far performed often do not predict *in vivo* drug response accurately. Many studies have shown that monolayer cultures of cells are often much more sensitive to drugs than the same cells in a three-dimensional configuration (4). We have, therefore, developed a primary culture system in which a native-state collagen-sponge-gel support allows most types of human cancer to grow *in vitro* at <90% frequency with maintenance of tissue architecture, tumor–stromal interaction, and differentiated functions (5–9). The native-state culture system allows multiple endpoint analysis including proliferation indices of cells determined by histological autoradiography after [^{3}H]thymidine incorporation (5–10).

The native-state histoculture system theoretically should have a high potential to be utilized for a predictive assay of tumor chemosensitivity since tumor and normal tissue remain *in vitro* highly similar to the *in vivo* state. In this report we describe experiments to determine the degree to which the native-state system, using [^{3}H]thymidine incorporation as an endpoint, can predict *in vivo* drug response.

We have correlated drug response of human tumor xenografts in histoculture and the same tumors implanted in nude mice, both systems providing highly controlled experimental conditions (11). Tumor histology is also correlated in histoculture and in nude mice.

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MATERIALS AND METHODS

***In Vitro* Drug Sensitivity.** *Histoculture.* Tissues were explanted as has been described (5–10). Briefly, after tissues were surgically removed, they were divided into 1- to 2-mm diameter pieces. Six pieces of tissue were excised from different areas of the original specimen and were then placed (six pieces per gel) on top of previously hydrated flexible sponge gels derived from the extracellular matrix of pigskin. Eagle's minimum essential medium (MEM) containing Earle's salts, L-glutamine (0.3 mg/ml), 10% (vol/vol) fetal calf serum, nonessential amino acids (1:100 dilution of a stock solution from Irvine Scientific), and the antibiotic gentamicin (0.2 mg/ml) was added to culture dishes such that the upper part of the gel was not covered.

In vitro *drug concentrations.* Concentrations of drugs used include doxorubicin at 29 ng/ml, cisplatin at 1.5 μg/ml, melphalan at 1.0 μg/ml, mitomycin C at 100 ng/ml, and 5-fluorouracil (5-FU) at 4 μg/ml. These concentrations are referred to in the manuscript as the 1× concentrations that correspond to clinically achievable doses *in vivo* (6). Also used in this study were 10× concentrations. The exposure time for all drugs was 24 hr *in vitro*.

Autoradiography. Cells within the three-dimensional cultures capable of proliferation were labeled with [^{3}H]thymidine (4 μCi/ml; 1 Ci = 37 GBq) (5–9) for 3 days after the first 2 days in culture; during the second day cells were incubated with chemotherapeutic drugs. Cellular DNA is labeled in any cell undergoing replication within the tissues. After 3 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% (vol/vol) formalin. The cultures were then dehydrated, embedded in paraffin, sectioned, and prepared for autoradiography using Kodak NTB-2 emulsion and counterstained with hematoxylin and eosin. Replicating cells were identified by the presence of silver grains, visualized as bright green with an epipolarization lighting system, over their nuclei due to exposure of the NTB-2 emulsion to radioactive DNA (7–9). Labeled cells were counted either manually or semiautomatically with the "Fas-Com" system as described (7–9), which quantifies the bright pixels of the light reflected from the silver grains.

In vitro *drug response.* The number of [^{3}H]thymidine-labeled cells was counted per field using ×200 magnification. For each drug concentration the one to three fields containing the highest number of labeled cells were counted to identify the areas in the heterogeneous tumor cultures having the least drug response. The control cultures were evaluated in the same manner. Six replicate cultures were evaluated for each drug concentration to determine *in vitro* response. The treatment value of [^{3}H]thymidine-labeled cells was divided by the control value. A reduction in the treatment value of 50%

Abbreviation: 5-FU, 5-fluorouracil.
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indicated *in vitro* sensitivity to a drug. *In vitro* drug response experiments were repeated for most of the tumor types and results were chosen from the experiments demonstrating the least response to a particular drug.

***In Vivo* Chemotherapy.** In vivo *drug doses and schedules.* Human tumors evaluated include the gastric cancers St-4, St-15, St-40, H-111, SC-2-JCK, and Exp-4; the colon cancers Co-3, Co-4, Co-6, and Co-8; the breast cancers MCF-7 and MX-1; the lung cancers Lu-130, Lu-24, and H-69; and the hepatoma Li-7, all of which are established xenograft lines (12, 13). *In vivo* all the drugs were dissolved in 0.2 ml of physiological saline and administered at a schedule of every 4 days for 12 days i.p. except doxorubicin, which was given i.v. The doses administered were 3 mg/kg for mitomycin C, 4 mg/kg for doxorubicin, 50 mg/kg for 5-FU, 80 mg/kg for cyclophosphamide, which were determined as the maximum tolerable doses for nude mice when they were injected on a schedule of every 4 days for 12 days (3, 12). Melphalan was used as the *in vitro* surrogate for cyclophosphamide, which requires *in vivo* metabolic activation and cannot be tested readily *in vitro*.

In vivo *drug response*. Two tumor-size fragments, approximately 3 × 3 × 3 mm in size, were inoculated into the subcutaneous tissue of the back of nude mice under ether anesthesia by means of a trocar needle. Tumors were measured (length and width) with sliding calipers three times a week by the same person. The tumor weight (W in mg) was calculated from the linear measurements using the formula: W = length (mm) × [width (mm)]^2/2. When tumors reached 100–300 mg, usually 2–3 weeks after the tumor inoculations, tumor-bearing mice were randomized into test groups consisting of six mice per group. The relative mean tumor weight (RW) was calculated as W_i/W_o, where W_i is the mean tumor weight of a group at any given time and W_o is the mean tumor weight at the initial treatment. The antitumor effects of the drugs were evaluated in terms of the lowest T_{rw}/C_{rw} during the experiment, where T_{rw} is the relative mean tumor weight of the treated group and C_{rw} is the relative mean tumor weight of the control group at the same time. The antitumor activity was evaluated as positive when the lowest T_{rw}/C_{rw} during the experiment was less than 42% of control reflecting a 25% reduction of the diameter of the tumor (3, 12).

RESULTS AND DISCUSSION

Comparison of *in Vitro* and *in Vivo* Histology. When hematoxylin-and-eosin-stained histological preparations were made from the xenografts before and after histoculture and compared, a striking similarity was revealed indicating the degree to which native-state histoculture can conserve tissue architecture (see Fig. 1).

Stomach cancer. Fig. 1 *A* and *B* shows stomach tumor H-111 growing *in vivo* and *in vitro*, respectively, demonstrating relatively well-differentiated structures under both conditions. Note green grains over cells in Fig. 1*B*, indicating

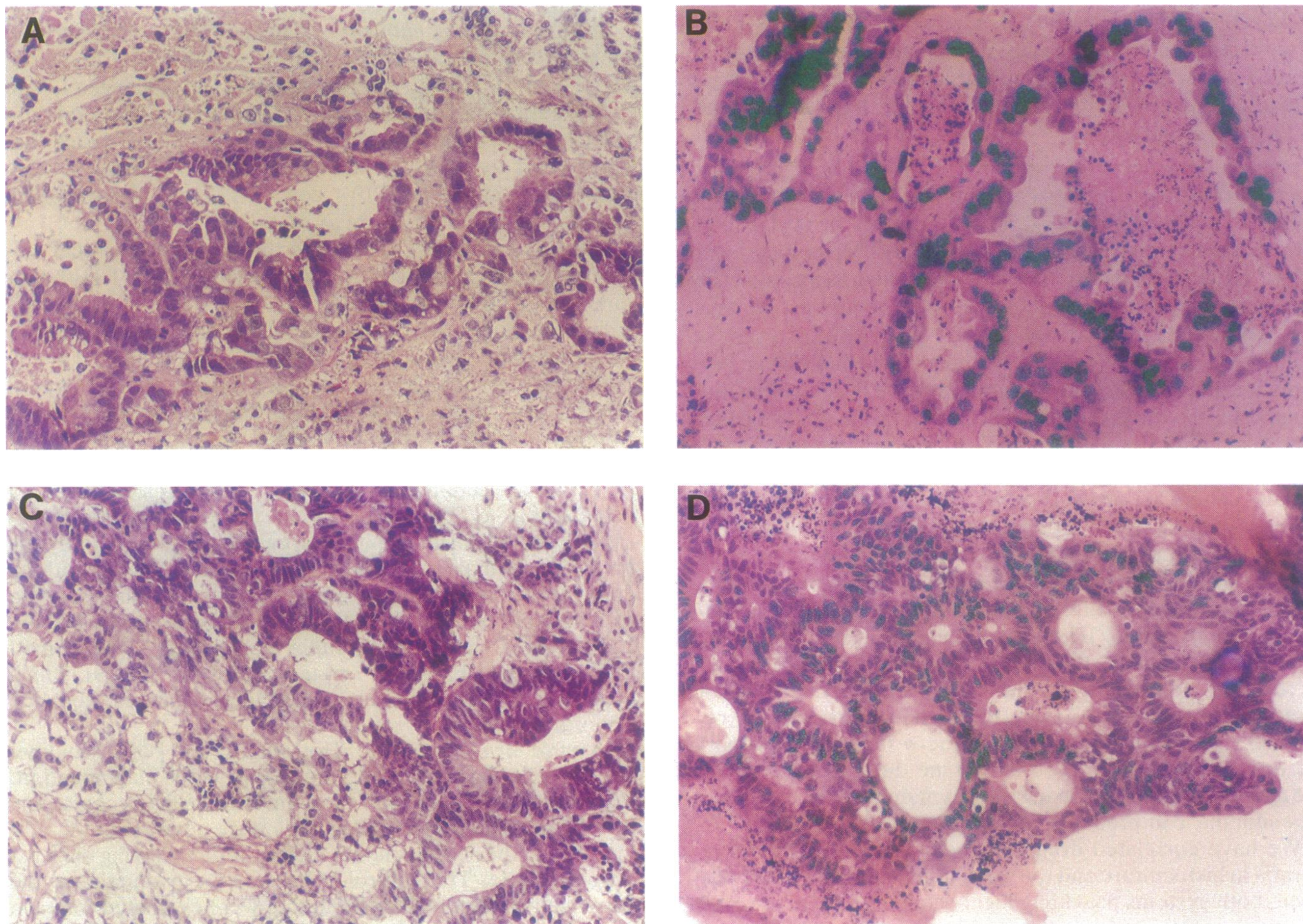

FIG. 1. Comparison of histology of human tumor xenografts grown in a three-dimensional culture in a native-state sponge-gel matrix with the same human tumors grown in nude mice. See text for details. (*A*) Human stomach tumor H-111 grown in nude mouse. (Hematoxylin and eosin, ×700.) (*B*) H-111 grown in histoculture in a native-state sponge-gel matrix. Autoradiogram. (Hematoxylin and eosin, ×700.) (*C*) Human colon cancer Co-3 grown in nude mouse. (Hematoxylin and eosin, ×700.) (*D*) Co-3 grown in histoculture in a native-state sponge-gel matrix. See *B* for details.

DNA synthesis seen by polarization microscopy using autoradiography after uptake of $[^3H]$thymidine.

Colon cancer. Fig. 1 *C* and *D* shows colon tumor Co-3 growing *in vivo* and *in vitro*, respectively, demonstrating relatively well-differentiated structures under both conditions. Note green grains over cells in Fig. 1*D*, indicating DNA synthesis seen by polarization microscopy using autoradiography after uptake of $[^3H]$thymidine.

Comparison of *in Vitro* and *in Vivo* Drug Response. Table 1 shows the *in vitro–in vivo* correlations of all *in vitro* drug-sensitivity determinations made at the 1× concentration of drugs. Drugs used were mitomycin C, 5-FU, doxorubicin, mitomycin, and cisplatin. As can be seen from Tables 1 and 2, the *in vitro–in vivo* correlations were 53% for 5-FU, 60% for melphalan, 67% for cisplatin, 70% for melphalan/cyclophosphamide, and 78% for doxorubicin (Adriamycin) for 15 xenografts of human tumors tested as tissue in the native-state assay and in nude mice. With regard to false positives, the frequency was highest for 5-FU and low for all other drugs. With regard to false negatives, the rate was highest for mitomycin C and low for all the other drugs.

When drugs were tested *in vitro* at the suprapharmacological 10× levels, the tumor xenografts that were resistant *in vitro* were also resistant *in vivo* in 86% of the determinations (Table 3). Our data, as well as those of Kern and Weisenthal (3), indicate that *in vitro* drug response testing is of great value in pointing out highly drug-resistant tumors for the avoidance of the use of inactive agents. The prediction of resistance, therefore, allows the elimination of chemotherapy-related morbidity in patients with tumors that are resistant to drugs tested. The prediction of resistance can also allow investigational treatment of patients whose tumors are resistant to standard therapies.

Our summary results indicate varying accuracy in predicting *in vivo* drug sensitivity depending on the drug used. The high false-positive frequency shown by 5-FU may be explained in part by the differences in its metabolic degradation and excretion *in vivo* and *in vitro*. *In vivo*, 5-FU is primarily cleared by hepatic degradation. In the *in vitro* system this is not possible. Additionally, *in vivo*, 10–15% of 5-FU is excreted intact in the urine, usually within the first hour of exposure (14). This was not taken into account in the *in vitro* system at the time of the experiment. These differences would suggest that there may be a more prolonged exposure to 5-FU in the *in vitro* system than would normally occur *in vivo*, therefore, allowing for the possibility of different responses. Another possible explanation for the high false-positive frequency shown by 5-FU may be due to the endpoint used. Although we see an inhibition of $[^3H]$thymidine incorporation, which suggests the lack of DNA synthesis, we cannot confirm that the cells have reached end-stage using only this one endpoint. It is possible that, given time after 5-FU exposure, the cells could have recovered in its absence.

The high false-negative frequency shown by mitomycin C may be explained by the different oxygen concentrations

Table 2. Summary of drug response comparisons of xenografts *in vitro* and *in vivo* for 1× drug concentrations *in vitro*

	Mitomycin	Doxorubicin	5-FU	Cisplatin	Melphalan
True positives,* no.	7	1	1	4	1
True negatives,† no.	2	10	7	4	6
False positives,‡ no.	2	2	6	2	2
False negatives,§ no.	4	1	1	2	1
% accuracy	60 (9/15)	78 (11/14)	53 (8/15)	67 (8/12)	70 (7/10)

Summary of drug response comparison of 15 human xenografts grown *in vivo* in nude mice and *in vitro* in three-dimensional native-state histoculture. Percent accuracy = [(no. true positives + no. true negatives)/no. total] × 100. Numbers in parentheses are no. true positives + no. true negatives/no. total. The overall accuracy was 65.2% (43/66). See Table 1 for other details.

*True positive no. = no. sensitive *in vitro* and *in vivo*.

†True negative no. = no. resistant *in vitro* and *in vivo*.

‡False positive no. = no. sensitive *in vitro* and resistant *in vivo*.

§False negative no. = no. resistant *in vitro* and sensitive *in vivo*.

Table 1. Drug response comparisons of xenografts *in vitro* and *in vivo* for 1× drug concentrations *in vitro*

	% resistance relative to control									
	Mitomycin		Doxorubicin		5-FU		Cisplatin		Melphalan	
Tumor	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*
MX-1	13.0 (s)	7.9 (s)	50.0 (s)	39.8 (s)	98.0 (r)	52.4 (r)	21.0 (s)	3.4 (s)	ND	0.8 (s)
SC-2-JCK	73.0 (r)	18.0 (s)	100 (r)	69.4 (r)	27.0 (s)	56.2 (r)	43.0 (s)	29.4 (s)	53.0 (r)	44.8 (r)
St-40	50.0 (s)	7.3 (s)	100 (r)	64.9 (r)	57.0 (r)	98.1 (r)	86.0 (r)	23.6 (s)	86.0 (r)	64.9 (r)
St-4	100 (r)	65.0 (r)	100 (r)	48.3 (r)	88.0 (r)	87.6 (r)	71.0 (r)	91.7 (r)	64.0 (r)	45.6 (r)
H-69	17.0 (s)	28.3 (s)	30.0 (s)	ND	0.4 (s)	56.0 (r)	0.8 (s)	84.0 (r)	20.0 (s)	ND
H-111	100 (r)	45.5 (r)	100 (r)	54.4 (r)	68.0 (r)	31.3 (s)	78.0 (r)	67.8 (r)	100 (r)	51.0 (r)
Co-4	21.0 (s)	13.8 (s)	22.0 (s)	52.1 (r)	6.4 (s)	13.4 (s)	30.0 (s)	15.8 (s)	22.0 (s)	74.9 (r)
Lu-24	48.0 (s)	20.4 (s)	84.0 (r)	33.3 (s)	100 (r)	68.0 (r)	66.0 (r)	65.6 (r)	47.0 (s)	8.3 (s)
Lu-130	48.0 (s)	6.4 (s)	74.0 (r)	66.1 (r)	51.0 (r)	90.6 (r)	41.0 (s)	86.8 (r)	70.0 (r)	76.1 (r)
Co-6	100 (r)	2.6 (s)	100 (r)	69.4 (r)	100 (r)	44.2 (r)	89.0 (r)	52.9 (r)	100 (r)	ND
St-15	82.0 (r)	22.7 (s)	60.0 (r)	66.2 (r)	8.4 (s)	61.4 (r)	42.0 (s)	27.2 (s)	74.0 (r)	51.5 (r)
Exp-4	44.0 (s)	37.0 (s)	0.0 (s)	63.5 (r)	4.2 (s)	50.3 (r)	62.0 (r)	19.1 (s)	8.3 (s)	47.0 (r)
Co-3	30.0 (s)	71.9 (r)	62.0 (r)	55.4 (r)	45.0 (s)	44.8 (r)	24.0 (s)	ND	ND	92.0 (r)
Co-8	49.0 (s)	69.8 (r)	100 (r)	64.6 (r)	100 (r)	58.7 (r)	100 (r)	ND	100 (r)	ND
MCF-7	69.0 (r)	23.4 (s)	82.0 (r)	64.5 (r)	41.0 (s)	63.5 (r)	ND	ND	93.0 (r)	36.3 (s)

Drug response comparison of 15 human xenografts grown *in vivo* in nude mice and *in vitro* in three-dimensional native-state histoculture. Human tumor xenograft material was sent from Tokyo to San Diego. The xenograft material was explanted as 1-mm^3 cubes on collagen-gel sponges. *In vitro* sensitivity was scored when a drug inhibited $[^3H]$thymidine incorporation 50% or more of control as measured by histological autoradiography. *In vivo* drug response testing was carried out at the Keio University School of Medicine by T.K. and his group (12, 13). *In vivo* sensitivity of the human tumor xenografts in nude mice was scored when the tumor growth was 42% or less of control. *In vivo* values indicate the remaining tumor mass compared to control. Sensitivity to a drug is indicated when the treated values are equal to 42% of control or less. *In vitro* values indicate the number of cells within the cultured tumor synthesizing DNA compared to the number in the control. Sensitivity to a drug is indicated when the treated values are equal to 50% of control or less. s, Sensitive response; r, resistance; ND, no data.

Table 3. *In vitro* vs. *in vivo* drug response comparisons of tumor xenografts resistant to 10× drug concentrations *in vitro*

Tumor	Drug	% resistance relative to control	
		In vitro	*In vivo*
MX-1	Doxorubicin	53.0 (r)	39.8 (s)
	5-FU	100 (r)	52.4 (r)
SC-2-JCK	Doxorubicin	80.0 (r)	69.4 (r)
	Melphalan	83.0 (r)	44.8 (r)
St-4	Mitomycin	72.0 (r)	65.0 (r)
	Doxorubicin	100 (r)	48.3 (r)
	5-FU	100 (r)	87.6 (r)
H-111	Mitomycin	80.0 (r)	45.5 (r)
	Doxorubicin	70.0 (r)	54.4 (r)
	5-FU	100 (r)	31.3 (s)
	Melphalan	73.0 (r)	51.0 (r)
Lu-24	5-FU	100 (r)	68.0 (r)
Lu-130	Doxorubicin	66.0 (r)	66.1 (r)
	Cisplatin	60.0 (r)	86.8 (r)
St-15	Melphalan	78.0 (r)	51.5 (r)
Co-8	Doxorubicin	100 (r)	64.6 (r)
	5-FU	98.0 (r)	58.7 (r)
Co-6	Mitomycin	81.0 (r)	2.6 (s)
	Doxorubicin	100 (r)	69.4 (r)
	5-FU	100 (r)	44.2 (r)
	Cisplatin	100 (r)	52.9 (r)

Eighteen true negatives and three false negatives were identified for an overall accuracy of 86% (18/21). *In vivo* values indicate the remaining tumor mass compared to control. Sensitivity to a drug is indicated when the treated values are equal to 42% of control or less. *In vitro* values indicate the number of cells within the cultured tumor synthesizing DNA compared to the number in the control. Sensitivity to a drug is indicated when the treated values are equal to 50% of control or less. s, Sensitive response; r, resistance.

found *in vivo* and *in vitro*. Mitomycin C must first be chemically or enzymatically reduced to be activated to crosslink DNA. Due to the less than optimal reducing conditions in the *in vitro* system, the effectiveness of mitomycin C may have been compromised (15, 16).

The experiments with 5-FU and mitomycin indicate the native-state system, being similar in histology to the *in vivo* system, may be used to study the mechanism of drug actions itself by varying the conditions under which the histocultures are exposed to the drugs.

Although there has been a number of attempts to identify specific forms of drug resistance that do not require tumor culture for their identification, it seems that drug resistance is multifactorial in most cases (3). Specific tests based on P-glycoprotein or glutathione transferase may have limited predictive capability with cells that can become resistant due to many parameters with many mechanisms resulting in great variation between different tumors in different patients. Thereby, the net result of all factors involved, being measured by tumor culture seemingly is the most efficacious means of identifying drug resistance at this time (3).

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