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Chemoprevention of Human Cancer:A Reasonable Strategy?

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Clinical Correlations of Drug Sensitivity in the Human Tumor Stem Cell Assay

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Summary

We have applied an in vitro soft-agar tumor-colony assay (which is now applicable to a variety of human cancers) to measurement of in vitro sensitivity to drugs and prediction of clinical response to cancer chemotherapy. The assay predicts drug resistance with 96% accuracy and sensitivity (in heavily pretreated patients) with 62% accuracy. On a pharmacokinetic basis the zone in vitro sensitivity for any given drug was only 5%–10% of the clinical concentration-time product (Cxt) achievable. This suggests that intratumoral drug concentrations in vivo may be lower than those in the plasma, and/or that > 2 log kills of tumor stem cells (not measurable in the assay) are required for clinical response. Serial in vitro studies showed that acquisition of drug resistance is a common clinical phenomenon which can be directly detected and quantitated in vitro.

Introduction

Of the various cells comprising a malignant tumor, the key replicative units appear to be the small fraction of clonogenic tumor cells or tumor stem cells [10, 14]. Studies of transplantable murine tumors had shown that the chemosensitivity of tumor stem cells was predictive of the in vivo therapeutic response to specific anticancer drugs [1, 9], again suggesting that these cells were highly relevant to the neoplastic process. Tumor stem cells appear to be central to the metastatic process, as they retain the capability to form secondary colonies at distant sites in the body (assuming that they can gain access to the circulation and find "fertile soil" for colonization [12]).

The colony-forming capability of human tumor stem cells has recently been exploited through the development of simple in vitro colony assays in soft agar or other semisolid media. Our group at the University of Arizona Cancer Center initiated studies of human myeloma stem cells in 1975. The program was based on development by Hamburger and Salmon [4, 5] of a simple two-layer agar colony assay. Subsequently, our program was broadened considerably and enlarged in scale as the assay proved suitable to fresh biopsies of a variety of solid tumors as well as myelomas and lymphomas [3–7]. Ovarian carcinoma [6] and melanoma [8] are two solid tumors which have been particularly easy to study with this system, as tissue for biopsy is often readily available, and excellent in vitro tumor-colony growth is obtained frequently. Table 1 summarizes the range of tumors which we have successfully cultured with this assay. Von Hoff et al. at the National Cancer Institute recently reported independent validation of this assay system [15].

Table 1. Human tumor types successfully cultured directly from biopsies with the bioassay for tumor stem cells (of Hamburger and Salmon)[a]

Carcinomas	Sarcomas and other neoplasms
(Adeno, squamous and undifferentiated variants for carcinomas of various sites)	Chronic lymphocytic leukemia
Adrenal	Diffuse lymphomas
Bladder	Ewing's tumor
Breast	Fibrosarcoma
Colon	Hodgkin's disease
Kidney	Liposarcoma
Lung	Macroglobulinemia
Ovary	Melanoma (melanotic and amelanotic)
Pancreas	Multiple myeloma
Prostate	Nephroblastoma (Wilms' tumor)
Thyroid	Neuroblastoma
Upper airways (head and neck)	Nodular lymphomas
Uterus (corpus and cervix)	Rhabdomyosarcoma
Unknown primary (squamous)	

[a] Summary as of May, 1979; more than 500 biopsy samples tested, including primary tumors and metastases

In June 1978, we published our first report on the use of the human tumor assay system for measurement of drug sensitivity in 18 patients with myeloma or ovarian cancer [13]. That report provided preliminary evidence that the assay system might prove useful for prediction of clinical response as well as playing a role in new drug development. The purpose of this report is to update our experience to May 1979. Thus, it includes the 32 clinical correlations reported previously [13].

Materials and Methods

Detailed descriptions of the methods of cell culture and measurement of drug sensitivity have been reported previously [3, 4, 6, 13]. In brief, a single cell suspension is prepared from the tumor biopsy using mechanical dissociation techniques. Aliquots of cells are exposed for 1 h at 37° C to at least three concentrations of each of a series of 6–10 anticancer drugs. Drugs are studied in vitro only at low concentrations generally ranging up to 1.0 μg/ml, with emphasis on concentration-time exposures (Cxt) which are in a range which would be pharmacologically achievable in vivo. Subsequently, the cells are washed twice by centrifugation, and suspended at a concentration of 500,000 cells/ml in an enriched tissue culture medium containing 0.3% molten agar; 1 ml of this mixture is plated in each 35-ml plastic Petri dish on top of a 0.5% agar feeder layer containing various nutrients and growth stimulants. All drug assay points are plated in triplicate and incubated at 37° C in a humidified CO_2 incubator for 2–3 weeks and evaluated serially by inverted phase microscopy and counted when a sufficient number of colonies (consisting of > 30 cells) have developed to permit measurement of a 1–2 log reduction in survival of colony-forming units. A sensitivity index is computed from the area under survival-concentration curves using a linear scale out to an upper limit which is defined by clinically achievable dosage exposures. For any given drug,

patients are ranked with respect to the in vitro sensitivity index (area under the curve) and the initial spread of clinical responses used to create a training set to determine boundaries between sensitivity and resistance. Proof of the neoplastic nature of the colonies is routinely obtained using a newly developed dried-slide technique [11] which provides exellent morphology for pathology review.

While such drug sensitivity assays have been carried out on more than 200 biopsy samples, the current report relates only to studies in 66 patients who (a) had sensitivity to multiple agents measured in vitro and (b) had retrospective or prospective data available for independent clinical evaluation of therapeutic response in vivo. Drugs studies in vitro included melphalan, doxorubicin, BCNU, methotrexate, vinblastine, cisplatinum, bleomycin, fluorouracil, actinomycin, dacarbazine, and m-AMSA. Clinical trials generally included either single-agent chemotherapy or simple two-drug combinations. Prospective selection of specific agents for clinical trial on the basis of marked in vitro sensitivity proved feasible in ten instances. Aside from these instances, prospective trials were initiated independently of the in vitro assay results. Standard criteria of response were employed as reported previously [13].

Results and Discussion

A total of 148 clinical correlations of in vitro and in vivo sensitivity or resistance could be made in the 66 patients reported. Many of these patients could be analyzed for one retrospective correlation and one or more prospective correlations in relation to clinical trials carried out subsequent to the in vitro assay. Each correlation was based on single clinical trial (generally 6 weeks in duration) with a single agent or simple drug combination. The overall results of these studies are summarized in Table 2. The predominant tumor categories included were myeloma, ovarian carcinoma, and melanoma, with the miscellaneous category consisting of patients with diffuse histiocytic lymphoma, oat cell carcinoma of the lung, or hypernephroma.

Despite the fact that many of these patients had been heavily pretreated, a total of 42 correlations could be made when the in vitro assay showed sensitivity. In 26 (62%) of these instances, the patient also showed a clinical response to treatment. Drugs uncommonly used for certain tumors were sometimes identified and proved effective. Examples include cis-platinum or actinomycin for melanoma, and bleomycin or vinblastine for ovarian cancer. All responses were at least partial responses except for two of the four melanoma patients who had mixed responses (one to BCNU-dacarbazine, and the second to m-AMSA). This suggests that more clonal heterogeneity of metastases might be present in melanoma, and necessitate multiple biopsies for assay when feasible. Patients who achieved clinical responses with the agents to which they showed sensitivity in vitro uniformly manifested exquisite in vitro sensitivity. Thus, the Cxt required in vitro to fall in the sensitive zone on the sensitivity index rankings was only 5%–10% of the pharmacologically achievable Cxt or peak concentration achievable in vivo.

We would propose that two separate explanations may be related to this requirement for exquisite in vitro sensitivity. Firstly, the intratumoral drug concentrations achieved in vivo may be far lower than those measurable in the plasma. Secondly, while the in vitro assay has a sensitivity limit of 1–2 logs in vitro (based on assay design), clinical response may require a 3 or more log reduction in survival of tumor stem cells, and hence require a Cxt of the drug in vivo which is ten times that which can be measured in

Table 2. Update of correlations of in vitro and in vivo sensitivity to anticancer drugs (May 1979)

Tumor type	No. of points	No. of clinical trials for correlations	Tumor sensitive both in vitro and in vivo	Tumor sensitive in vitro and resistant in vivo	Tumor resistant in vitro and sensitive in vivo	Tumor resistant both in vitro and in vivo
Ovarian	25	72	9	7	1	55
Myeloma	20	48	12	6	1	29
Melanoma	18	24	4[a]	3	2	15
Misc.[b]	3	4	1	0	0	3
Total	66	148	26 (62% true positive)	16	4	102 (96% true negative)

[a] Mixed responses

[b] The miscellaneous category includes one patient each with oat cell carcinoma (sensitive in vitro and in vivo) and one each with hypernephroma and lymphoma both of whom were resistant. With the Fisher exact test [2] the association of in vitro and in vivo results was highly significant ($P < 0.000001$)

vitro with this assay. Both explanations may well apply in many instance. A total of 106 correlations were obtained where in vitro resistance was observed. In 96% of the correlations where in vitro resistance was manifest, the patients also failed to respond to this treatment in vivo. Thus, this assay has extraordinary power to predict which drugs will only cause toxicity, and to indicate that they can be deleted from clinical trial. Patients who failed to respond to agents in vivo sometimes had in vitro survival-concentration curves showing resistance to levels of drug which exceeded the clinically achievable Cxt by a factor of 10 or more with no evidence of drug-induced lethality over the entire dose range tested. More frequently, however, the in vitro response was one suggesting an admixture of sensitive and resistant tumor stem cells within the biopsy sample. Such in vitro survival curves showed an initial steep slope with lethality to 40%–60% of the tumor colony-forming units at low doses of the drug, but with a plateau of resistant cells whose survival was not decreased even at drug doses above the normal range (10–100 μg/ml). Such curves were observed even with cycle-nonspecific drugs such as melphalan, doxorubicin, and cis-platinum. Our overall experience with prediction of sensitivity or resistance with the assay is extremely good. Using the Fisher exact test [2] the probability that the correlations shown in Table 1 could be due to chance alone is less than one in one million ($P < 0.000001$).

Serial studies of in vitro drug sensitivity proved feasible in seven patients who recieved treatment with the drug tested between the two serial assays. These results are summarized in Table 3. Twelve individual comparisons could be made. In six instances the in vitro sensitivity index (expressed as area under the curve) did not change between assays. Two of these were patients who were sensitive and responded to treatment, relapsed of treatment and could be subsequently reinduced into remission again (e.g., myeloma with melphalan). Four patients who were initially resistant in vitro remained so when retested in vitro after failing to respond to the same agent in vivo.

Table 3. Serial in vitro sensitivity studies in seven patients

Sensitivity index[a]	No. of instances	Circumstance
No change	6	2 s → s (sensitive on both tests) 4 r → r (resistant on both tests)
Increase	6	3 s → r (conversion from sensitive to resistant) 3 r → R (increasing resistance)
Decrease	0	

[a] Area under the in vitro survival concentration curve

In six instances, the sensitivity index (area under the curve) increased by at least 50%. In three of these, the patients converted from sensitive to resistant in vitro after having had an initial response followed by a relapse on treatment as well. Three patients who failed to respond to treatment also had an increased area under the curve as well indicating increasing drug resistance of the tumor stem cells. In no instance did a patient show evidence of increasing in vitro sensitivity (decreased sensitivity index) on serial testing. Based on this relatively small experience to date with serial testing, the general pattern appears to be one of progressive acquisition of increasing drug resistance to single agents with which the patients were treated. Thus, the acquisition of drug resistance is a common phenomenon which can be directly detected and quantitated in vitro.

While the focus of this report has been on clinical drug testing and prediction of response, it is clear to us from our various studies that the in vitro assay may have many other uses. Not only should such a system be useful for a variety of investigations of cancer biology [e.g., 12]. It also could greatly simplify preclinical screening of cytotoxic, hormonal, and immunotherapeutic agents as well as for studying potential new and innovative treatment modalities. We are currently working on automated technique for tumor-colony counting which should greatly facilitate such applications of the assay system.

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