Preliminary Correlations of Clinical Outcome with in Vitro Chemosensitivity of Second Passage Human Breast Cancer Cells

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ABSTRACT

These studies describe the clinical correlations of 63 in vitro chemosensitivity assays on breast cancer cells after short-term monolayer culture. Forty-five of the assays were single agent correlations. Based on cut-off values determined empirically, the test accurately predicted resistance for 36 of 41 patients (88%) who did not respond to the drug. It also predicted sensitivity with a high degree of accuracy: 21 of 22 patients (95%) who responded to the drug tested had a sensitive assay. In five cases, two biopsies were evaluated from the same patient. Whenever assays were performed before and after treatment with a given drug, tumor cells from the second biopsy were more resistant in vitro if the patient failed on therapy. If the patient did not fail, but stopped therapy for other reasons, or if there was no intervening therapy with the tested drug, the two biopsies remained similar in drug sensitivity. These results suggest that in vitro chemosensitivity assays which accurately predict both sensitivity and resistance can be obtained with breast cancer cells after short-term culture and that further prospective trials are warranted.

INTRODUCTION

A number of different in vitro chemosensitivity assays have been described which have proven to be highly reliable in predicting response of a patient's tumor to chemotherapy (for reviews see Refs. 1–3). All of these assays have in common that tumor cells taken directly from the patient are treated with drugs and then cultured. Unfortunately, the small size of many breast cancer biopsies makes these assays unsuitable for 70 to 80% of breast specimens (2). This problem is likely to become even more acute because (a) breast cancer biopsies will be smaller as early diagnostic modalities are more frequently used; and (b) new laboratory tests for predicting prognosis will require additional specimen material.

One solution to the problem of small breast cancer size is to amplify the available tumor cells by in vitro culture prior to testing for drug sensitivity. This report presents preliminary studies evaluating one such system. To obtain sufficient proliferation at first passage in culture, a medium developed specifically for human mammary epithelium (4) was utilized. In this medium, the proliferating cultures are easily trypsinized to single cells which clone with high efficiency when plated on irradiated fibroblasts (5). Analysis of X-ray survival curves excluded the possibility that clumps, rather than single cells, were being plated (6). Furthermore, the number of colonies scored was directly proportional to the number of cells plated (5). With this culture system, the vast majority (>70% of primary breast cancers and hypodermal metastases grow well and clone with high efficiency). In contrast, effusion metastases grow rather slowly in primary culture and usually do not clone (7). Other types of cancers, such as colon or non-small cell lung cancers grow poorly and do not clone.3

The cells growing in this assay have been extensively characterized. All of the cultures were uniformly positive for keratins (8) and other epithelial markers (5), proving that epithelial, rather than stromal cells, were being cultured. Other criteria consistently distinguished cultures derived from malignant and nonmalignant tissues, including amnion invasiveness (9), multinucleation in response to cytochalasin (10), and presence of a tumor-associated glycoprotein (8). Most recently, the assay was used to show that tumor-derived cultures were sensitive to tumor necrosis factor while nonmalignant mammary epitheloid-derived cultures were not (11).

The fact that the cells cultured from tumors differ from those of nonmalignant specimens by so many criteria suggests that at least some bona fide tumor cells are in fact being cultured. However, it is likely that only some, less aggressive, tumor-derived subpopulations are selectively grown in the system. Although cells from hypodermal metastases were more abnormal than those from primary breast cancers, cells from both types of lesions showed only minimal karyotypic changes after culture rather than the gross aneuploidies usually associated with carcinomas. In contrast, effusion metastases, a more aggressive manifestation of the disease which usually grew poorly, were uniformly aneuploid (9, 12, 13).

We have undertaken studies to test the hypothesis that accurate predictive chemosensitivity assays could be obtained with this assay even though (a) the cell cycle kinetics differ from the in vivo state since all of the cells are cycling after culture; and (b) there is less heterogeneity since only selected subpopulations grow in this culture system. In a previous study (14), response to doxorubicin in this system was examined. A broad range of sensitivities in specimens from previously untreated patients was shown, indicating inherent differences in drug sensitivity even in proliferating cell cultures. That these differences might be clinically meaningful was suggested by the fact that specimens from donors who had failed doxorubicin-containing chemotherapy regimens had dose-response curves that clustered at the more resistant end of the spectrum.

In this report, we present our continuing studies to evaluate the clinical relevance of the assay by extending our observations to other drugs besides doxorubicin and to prospective predictions of sensitivity. We show that the assay predicts both sensitivity and resistance with a high degree of accuracy.

MATERIALS AND METHODS

Specimen Material. Specimens were obtained from discarded surgical material or from patients who had excisional biopsies of skin metastases for the purpose of in vitro culture after obtaining appropriate informed consent.

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2 To whom requests for reprints should be addressed, at Geraldine Brush Cancer Research Institute, Pacific Presbyterian Medical Center, 2330 Clay St., San Francisco, CA 94115.
3 H. S. Smith, unpublished observations.
The cell-harvesting process was repeated one or two more times. The clump. Very brief treatment with trypsin at this stage resulted in the combined effect of cell migration and extensive proliferation was a wide area of epithelial cells with extensive mitotic activity surrounding each clump. This process was termed “partial trypsinization.” The dish was then refed and the next morning, tumor cells harvested from primary cultures of clonal growth yielded plating efficiencies ranging from 1 to 2 days later, a somewhat more vigorous trypsinization removed many and began to proliferate extensively. By 6-8 days after plating, the bovine aorta can replace the cell lines as a source of conditioned media. Can be obtained from the American Type Culture Collection (Rockville, MD). Alternately, endothelial cells from human umbilical vein (15) or bovine aorta can replace the cell lines as a source of conditioned media.

Within 24-48 h of plating, tumor cells migrated from the clumps and began to proliferate extensively. By 6-8 days after plating, the combined effect of cell migration and extensive proliferation was a wide area of epithelial cells with extensive mitotic activity surrounding each clump. Very brief treatment with trypsin at this stage resulted in dissociation of contaminating fibroblasts (which also grew poorly in MM) while leaving the tumor epithelial cells attached to the flask. One to 2 days later, a somewhat more vigorous trypsinization removed many of the cells which had migrated from the central clump. This process was termed “partial trypsinization.” The dish was then refed and the cell-harvesting process was repeated one or two more times. The suspended cells were utilized directly in the drug assays.

Clonal Assay for Drug Sensitivity. Breast cancer cells under conditions of clonal growth yielded plating efficiencies ranging from 1 to 10%. As described previously (5, 14), UV-irradiated human fibroblasts were seeded at 1.5 × 10³ cells onto 35-mm tissue culture dishes to provide a feeder layer and all human fibroblast strains tested were equally effective. The next morning, tumor cells harvested from primary cultures were dispersed to a single-cell suspension and plated. Sixteen to 19 h after seeding of tumor cells, the medium was removed and MM with the desired drug concentration was added to each dish. For each drug dose tested, cells are seeded at 350, 1,000, and 35,000 cells/25-mm-diameter culture dish. All dishes with a countable number of surviving colonies are scored. Three drug concentrations were used to establish a dose-response relationship. The drug-containing and control dishes were incubated at 37°C. The medium was removed 4 h later and the dishes were washed once with basal salts. Each dish was then refed, without drug, with MM containing penicillin and streptomycin (100 units/ml each) and 10⁴ freshly prepared irradiated fibroblasts. The dishes were refed twice weekly until readily visible colonies were present (usually within 5–10 days). The dishes were rinsed with phosphate-buffered saline (80 mg NaCl/ml, 0.2 mg KCl/ml, 0.2 mg KH₂PO₄/ml, 0.1 mg CaCl₂/ml, and 0.1 mg MgCl₂·6H₂O/ml), fixed with methanol, and stained with May-Grunwald-Giemsa.

The colonies in each dish were counted, and the number of colonies per 100 plated cells was calculated. The value from the drug-treated cultures was divided by the mean number of colonies per 100 cells plated on control dishes (without drug). The dose-response curves were generated by calculation of the mean and standard deviation for all countable dishes for a given point. There were no differences in dose response at different seeding levels. The relative resistance of the cultures was expressed as the LD₉₀ values. The staff responsible for performing the in vitro assays had no knowledge of the patients’ clinical response until after assay results were reported to the clinicians.

Drug Preparation. Stock solutions of each drug at 1 mg/ml were divided into aliquots and frozen at −70°C. For each experiment, a new ampoule was used. Drugs and stock solutions were as follows: doxorubicin (Adria Laboratories, Columbus, OH) diluted with sterile physiological saline; cisplatinum (Bristol, Syracuse, NY) diluted with sterile water, vinblastine sulfate (Eli Lilly, Indianapolis, IN) diluted with sterile physiological saline; 4-epidoxorubicin and idarubicinol (a generous gift of Dr. S. Ganzina, Farmitalia, Milan, Italy) were diluted with sterile physiological saline. Idarubicinol is the active metabolite of 4-demethoxydaunorubicin, a new derivative of daunorubicin synthesized by Farmitalia Carlo Erba (termed idarubicin, or MI30, IMI30, NSC 256439). After i.v. and p.o. administration in humans, 4-demethoxydaunorubicin is rapidly metabolized to its 13-dihydroderivative, idarubicinol, which has been shown to be an active metabolite in experimental models.

Patient Correlations. To obtain clinical correlations, all patients were clinically evaluated by two different clinicians who were not aware of the in vitro results. The following criteria were used. Complete response, the disappearance of all known disease determined by two observations separated by at least 4 weeks. None of the cases in this study had a complete response. Partial response, at least a 50% reduction in the size of all measurable tumor areas as measured by the sum of the products of the greatest length and maximum width. No lesions may progress and no new lesions may appear. These parameters must have been present for at least 2 measurement periods separated by at least 4 weeks. Stable disease, when a patient’s status fails to qualify for either a response or progressive disease. Progressive disease, an increase of >25% and at least 2 cm² of original lesions and of the development of any new lesion.

RESULTS

Three anthracycline derivatives were evaluated: doxorubicin, 4′-epidoxorubicin, and idarubicinol (the active metabolite of 4-demethoxydaunorubicin). Dose-response curves representing sensitive and resistant assays for each analogue are presented in Fig. 1. All of the anthracycline data accumulated for specimens from patients with no prior therapy are illustrated in Fig. 2.

To obtain clinical correlations, specimens were divided into two categories, those clinically sensitive and those clinically resistant to chemotherapy. Patients were considered clinically resistant with either “progressive” or “stable” disease, and...
sensitive with either "partial" or "complete" remissions. Samples from clinical sensitive patients were obtained by biopsy immediately prior to a chemotherapy regimen where sensitivity is defined as a partial or complete response to that therapy. For in vitro predictions of sensitivity, only single agent therapies were included in the study since one cannot ascertain which drug or drugs in a combination regimen are responsible for a patient’s response to multiagent combinations. Samples from clinically resistant patients were obtained in 2 ways: (a) those biopsied immediately prior to treatment with chemotherapy when the patient subsequently showed either stable or progressive disease on therapy (prospective correlation); or (b) those biopsied at the time of therapeutic failure following treatment (retrospective correlation). We evaluated specimens from clinically resistant patients treated with either single or multidrug regimens since patients failing a given combination regimen would be resistant to all drugs in that regimen.

For the anthracycline derivatives, Fig. 3 compares LD₉₀ values for samples from previously untreated patients with LD₉₀ values for specimens from patients for whom clinical correlations are available. In Fig. 3, the closed symbols indicated primary breast cancers while the open symbols represent metastases. One metastatic specimen was to lung (indicated in Fig. 3 by **); all others were hypodermal lesions. As defined in Fig. 3 legend, the different symbols indicate whether the correlations were prospective or retrospective and whether the patients were treated with single or multidrug regimens.

For each of the analogues, there was a broad range of LD₉₀ values for specimens from patients with no prior therapy. Most of these were primary breast cancers; however, samples of hypodermal metastases from patients with no prior therapy, although fewer in number, showed the same broad range of LD₉₀ values. Those samples classified as clinically resistant in vivo tended to cluster with LD₉₀ values at the resistant end of the spectrum when tested in vitro. In contrast, tumors from patients who responded with either complete or partial responses clustered with more sensitive LD₉₀ values.

The accumulated correlations for Vinca alkaloids are illustrated in Fig. 4. Preliminary studies in culture indicated that all specimens gave similar LD₉₀ values when assayed with vincristine or vinblastine (data not shown). All of the in vitro assays were done with one compound, vinblastine. Most of the patients were also treated with vinblastine; however, two received vincristine not vinblastine. Both of these patients were nonresponders; assays of their tumor yielded LD₉₀ values of 0.043 µg/ml and >0.075 µg/ml. Similar to the anthracyclines, there was a broad range of LD₉₀ values for both primary breast cancers and hypodermal metastases from previously untreated patients. All of the cases where patients responded to therapy with Vinca alkaloids clustered at the sensitive end of the LD₉₀ value spectrum. Most of the specimens from nonresponders had LD₉₀ values more resistant than those of the responders. In three cases, the cells were sensitive to vinblastine, but the patients did not show either an objective partial or complete remission. These three cases are considered negative correlations for the assay. Two of these patients progressed on therapy with vinblastine; however, the third had stable disease for 5 months. When this third patient relapsed, a second biopsy was evaluated and found to have a much higher LD₉₀ value (>0.075 µg/ml) than the first sample (0.013 µg/ml).

Although cis-platinum is not usually used to treat breast cancers, response to this drug was also evaluated (Fig. 5). Again, there was the characteristic range of sensitivities among specimens from previously untreated donors. One patient whose tumor showed a marked sensitivity to cis-platinum was treated.
with this drug and showed a partial response which lasted for 2 months, at which time therapy was changed to carboplatin, a platinum analogue, for 3 cycles with no change for 4 months. This was done because of neurotoxicity from a's-platinum. The second biopsy showed a partial response which lasted for 2 months, at which time therapy was changed to carboplatin, a platinum analogue, for 3 cycles with no change for 4 months. This was done because of neurotoxicity from a's-platinum. The second biopsy more resistant than first.

Further evidence supporting the clinical relevance of the assay is summarized in Table 1 which describes cases where two biopsies from the same individual were performed before and after treatment with a given drug. In all 7 cases examined, the results support the conclusion that the in vitro assay accurately reflects the in vivo response. Whenever assays were performed before and after treatment with a given drug, the LD90 values always increased if the patient failed on the therapy. If the patient did not fail, but stopped therapy for other reasons, or if there was no intervening therapy with the tested drug, LD90 values always increased if the patient failed on the therapy. If the patient did not fail, but stopped therapy for other reasons, or if there was no intervening therapy with the tested drug, LD90 values always increased if the patient failed on the therapy.

In Figs. 3 to 5, a threshold is drawn to separate LD90 values that correspond to in vitro sensitivity and resistance. These thresholds were picked empirically in order to distinguish optimally between clinical responders and nonresponders. As additional data accumulate, it will be possible to define these values more precisely. Using these values, Table 2 summarizes the data.

Among 41 patients who did not respond to therapy, 36 had assays in the resistant range. In contrast, among 22 patients who responded with at least a partial remission, only 1 patient had a resistant in vitro assay. These differences were highly significant (P < 0.01) by the χ² test. Included in Table 2 are the results for anthracyclines and vinblastine analyzed separately. In each case, χ² analysis indicated significance. However, the fact that patients who were resistant to multidrug regimens were compared to patients sensitive to single agents might bias the results by artificially increasing the differences between sensitive and resistant. Therefore, Table 2 also summarizes the data comparing only cases treated with the single agent tested in vivo. Although the number of cases is smaller, the conclusions remain the same.

**DISCUSSION**

These studies describe in vitro chemosensitivity assays on breast cancer cells after short-term culture. Using empirically defined values, the test accurately predicted resistance for 36 of 41 patients whose tumors did not respond in vivo. The test also predicted sensitivity with a high degree of accuracy: 21 of 22 patients, 95% of whom responded to the drug treated, had a sensitive assay. These results suggest that in vitro chemosensitivity assays which accurately predict both sensitivity and resistance can be obtained with breast cancer cells after short-term culture even though (a) the cell cycle kinetics differ from the in vivo state, since all of the cells are cycling after culture; and (b) there is less heterogeneity because only selected subpopulations grow in this culture system.

The negative correlations may be the result of an inherent limitation of in vitro drug sensitivity assays, since there may be pharmacological reasons for a patient's drug response that cannot be measured in vitro. For example, sufficient drug concentrations may not reach the tumor cells in vivo because the tumor has a poor blood supply or the patient may metabolize and excrete the drug at a rapid rate. If the tumor cells themselves are very resistant, even good drug delivery will not help; therefore, in vitro assays may be best for predicting resistance. Predicting sensitivity may be more difficult because such pharmacological issues may prevent response to the drug in some patients with very sensitive tumor cells.

Other studies have defined these cut-off values as the drug levels achievable in vivo (16) rather than empirically defining the cut-off values for sensitivity and resistance. The latter approach does not consider the fact that effective drug concentrations may vary, depending on factors such as drug bound to culture fluid proteins or plastic substrates, or to drug uptake being different in cultured cells compared to cells in vivo. Therefore, we have relied upon the fact that whatever the effects of culture may be, they will be constant; hence, the relative LD90 values, regardless of the absolute values, will reflect the in vivo situation.

The simplest way to obtain clinical correlations is to treat patients with the single agent assayed in vitro. However, very few breast cancer patients are treated with single agents. Therefore, patients treated with multidrug regimens were included in clinical correlations, but only if they did not respond or if they responded but subsequently failed prior to biopsy. Unlike other studies (17), patients who responded to combination regimens...
were not included. Our rationale is that patients would fail a multidrug regimen only if their tumors were resistant to every drug in the combination. Hence, a resistant assay in vitro would be a positive correlation while a sensitive assay would be a negative correlation. If a patient responded to combination chemotherapy, a resistant assay in vitro for one of the drugs could not be scored as a negative correlation because the patient might be responding to other drugs in the combination. Therefore, considering a sensitive in vitro assay for one of the drugs as a positive correlation overestimates the accuracy of the assay. Furthermore, there is no proof that the patient actually responded to the drug tested.

For all drugs tested, there was a broad range of sensitivities among specimens from previously untreated patients, suggesting that some cancers are inherently resistant to a given drug. Included among these specimens were both primary breast cancers and hypodermal metastases. Since skin metastases showed the same range of sensitivity as primary breast cancers, it may be possible to use data obtained with primary breast cancers to indicate sensitivity of subsequent lesions if there is no intervening therapy with the same drug. Further studies with primary lesions and metastases from the same patient are needed to test this hypothesis.

One advantage of the assay for use in adjuvant trials is that the success rate for cultivating primary breast cancers is greater than 70% (5). A similar high success rate has been found for hypodermal metastases. In contrast, although the enriched medium grows effusions and some lymph node metastases in primary culture, we have found that these cultures tend to grow more slowly than the primary tumors and usually do not clone at second passage (7). Hence, further work to improve the culture system is needed if these advanced tumors also are to be utilized for predictive assays. It is noteworthy that the agar clonogenic assay is most successful with effusion metastases and least effective for primary breast cancers and hypodermal metastases (18). Hence, the two assays may complement each other.

The fact that the assay predicts both sensitivity and resistance for breast cancers is an important first step in using the assay clinically. The correlations in the study were found in patients with metastatic breast cancer and the responses seen were all partial responses. Further studies will be required to determine whether utilization of this assay can modify morbidity and survival of patients with metastatic breast cancer and whether the cost versus benefit ratio is sufficiently high to warrant routine clinical application.

Even if it proves useful to predict which drugs will be most effective against metastatic breast cancer, the ability of results from in vitro assays to change the natural history of advanced breast cancer, using currently available drugs, will be limited. Adjuvant therapy has, however, altered the natural history of breast cancer. Unfortunately, only about 20% of the women treated with adjuvant therapy show a prolongation of overall survival (19). It is highly likely that many of those women who recur after adjuvant therapy do so because of the inherent resistance of their tumors to the drugs used. Thus, a long-range goal of these studies is to develop an in vitro assay which can be used to select the optimal drug regimen when other prognostic tests indicate that adjuvant therapy is warranted. The success of the assay in predicting sensitivity suggests that an appropriate study question to consider would be to use this assay to choose between the standard of care alternatives for adjuvant therapy (i.e., anthracycline versus nonanthracycline-based regimens).

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