Sponge-Gel–Supported Histoculture Drug-Response Assay for Head and Neck Cancer

Correlations With Clinical Response to Cisplatin

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Objective: Sponge-gel–supported histoculture drug-response assay (SSHDRA) represents a promising method to determine chemosensitivity of solid tumors. To determine whether the assay correlates clinically, we compared the in vivo and in vitro effects of cisplatin in 23 of 26 patients with head and neck cancers.

Design: The criterion for in vitro sensitivity to cisplatin was an 84% or greater inhibition by cisplatin of the number of tritiated thymidine–incorporating cells of the histocultured tumors compared with untreated control culture preparations, as measured by means of histologic autoradiography. Comparisons were made with clinical responses, ie, complete response, partial response, or no response.

Patients: The study was carried out in patients with head and neck cancers and comprised 21 patients with squamous-cell carcinoma, three patients with other carcinomas, and two patients with sarcoma.

Results: Ten of 12 patients with in vitro–sensitive tumors had either complete or partial response clinically. The overall accuracy of the SSHDRA was 74% in this correlative clinical trial; the predictive-positive value was 83%, the sensitivity was 71%, and the specificity was 78%. Seven of 11 patients with in vitro–resistant tumors demonstrated no response for a predictive-negative value of 64%.

Conclusions: We conclude that the SSHDRA shows a high correlation for tumors that demonstrate both in vivo drug resistance and sensitivity. The in vitro–like maintenance of three-dimensional tissue architecture of the tumors in histoculture probably contributes to high clinical predictivity of drug response of the SSHDRA. The data support further comparisons to determine the clinical usefulness of the SSHDRA for identifying complete and partial responders to chemotherapy.

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SQUAMOUS CELL carcinomas of the upper aerodigestive tract have a mixed response to cisplatin-based chemotherapy regimens. Whereas some tumors completely regress clinically and pathologically, others show responses that are partial to nearly complete or minimal to none.[1] In a multitude of chemotherapy trials for patients with head and neck cancers, it is only the group of patients who has a complete response where survival is prolonged.[2] Therefore, an important strategy for the successful application of chemotherapy, as part of the multimodality treatment for advanced head and neck cancer, is to determine which patients are likely to have a complete response to the chemotherapeutic regimen. Unfortunately, trial and error results in delayed therapy with other modalities. To date, there have been no reliable pretreatment parameters that indicate which patients will have a complete response to chemotherapy.

Chemosensitivity assays represent one potential method, whereby the response of a patient's tumor to a particular drug might be predicted.[3] Although several types of chemosensitivity assays have been investigated for this purpose, none of them, to date, have been suffi-

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PATIENTS, MATERIALS, AND METHODS

The study comprised 26 patients with cancer involving the head and neck who received cisplatin chemotherapy for advanced untreated or recurrent disease. All patients were assessed and treated under the Head and Neck Oncology Program, University of California, San Diego, which encompasses the University Hospital, San Diego, and the Veterans Administration Hospital, La Jolla, Calif. Fourteen patients had previously untreated disease, and 12 patients had recurrent disease. All patients with previously untreated disease had stage III to IV lesions; 21 patients had squamous cell carcinoma, two patients had adenocarcinoma, two patients had sarcoma, and one patient had a mucoepidermoid carcinoma. Sites of the primary disease included the following: oropharynx (n=9), oral cavity (n=7), paranasal sinuses (n=6), larynx (n=1), parotid gland (n=1), unknown primary site (n=1), and skin (n=1). In four patients, specimens were obtained from cervical lymph node metastases; and in 22 patients, from the primary site.

In 11 patients, specimens for the drug response assay were obtained from biopsy material taken prior to any drug infusions. Fifteen patients were treated with 120 to 200 mg/m^2 of cisplatin every 3 to 4 weeks. Eight patients received 150 to 200 mg/m^2 of cisplatin every 1 to 2 weeks with systemic sodium thiosulfate neutralization. In 12 patients, specimens were obtained from resected tissue following drug therapy. Three specimens were sufficiently contaminated to preclude measurement of drug response in the assay. Thus, correlations of the in vitro growth inhibitory response with the clinical response were possible for the remaining 23 patients.

Clinical response was determined by physical examination for lesions that could be measured by bimanual palpation. Radiologic studies (computed tomography and/or magnetic resonance imaging) were used to measure clinical response for lesions that could not be measured by physical examination. Tumor volume was calculated by multiplying the bidimensional diameter distances. Complete response was indicated by complete disappearance of tumor by clinical examination and/or endoscopic findings. Partial response was indicated by greater than 50% reduction in the size of the target lesion. Tumors that had less than 50% reduction in size, remained stable, or progressed during chemotherapy were considered to be nonresponsive to chemotherapy.

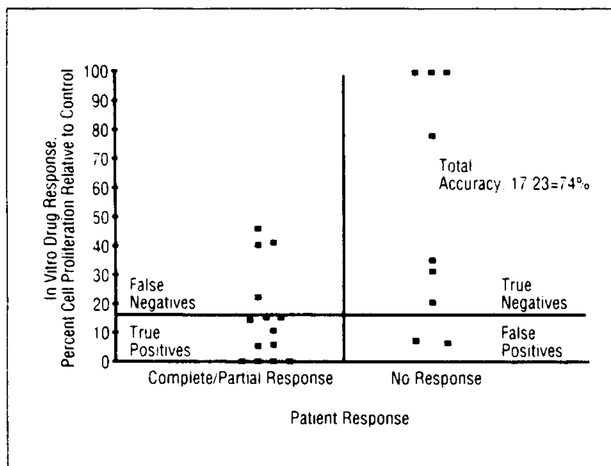
Biopsy specimens were obtained using 5-mm punch forceps taken from an accessible area of the visible tumor using local anesthesia. Adjacent tissue was checked histologically in all patients to ensure that the material submitted for assay was representative. Specimens were immediately placed in transport media consisting of Hanks' balanced salt solution, L-glutamine (0.3 mg/mL), 10% fetal calf serum, nonessential amino acids (1:100 dilution of stock solution [obtained from Irvine Scientific, Irvine, Calif]), and gentamicin (0.2 mg/mL). Specimens were immediately delivered to the AntiCancer, Inc (San Diego, Calif) laboratories for further chemosensitivity testing.[3-15]

The tumors were minced into pieces of 1 to 2 mm in diameter and placed on hydrated pigskin collagen-gel matrices (Health Design Industries, Rochester, NY) within 48 hours of removal from the patient. Tissue specimens were exposed to the drugs dissolved in the culture media for 24 hours on day 1. After allowing tumor cell growth in culture for 3 to 11 days, tritiated thymidine (4 μCi/mL; [1 Ci=37 gbq; 1 gbq=37$\times 10^9$ disintegrations per second]) was added to label the nuclei of replicating cells for an additional 3 to 4 days.[3-15] The quality of the cultured biopsy specimen was controlled by examination of the histologic autoradiograms where tumor cells are readily distinguishable from stroma and necrotic areas.

The cultures were then washed with isotonic phosphate-buffered saline, placed in histologic capsules, and fixed in 10% (volume/volume) formaldehyde solution. The histocultures were then dehydrated, embedded in paraffin, sectioned, and placed onto slides. The slides were deparaffinized and then coated with emulsion (Kodak NTB2, Eastman Kodak, Rochester, NY) in a darkroom, and exposed for 5 days at 4°C before developing. After rinsing, the slides were stained with hematoxylin-eosin. The slides were then analyzed with a polarizing microscope at ×400 magnification in a blinded fashion. Replicating cells were easily determined by the presence of bright green reflecting silver grains over the cell nuclei. Benign stromal cells were excluded by their morphologic appearance. The number of cells undergoing DNA synthesis was determined in at least three visual fields with the maximum labeling for each tumor piece being used to determine the drug response. The drug-response values were calculated by dividing the maximum number of labeled tumor cells on the drug-treated slides by the maximum number of labeled tumor cells on the control slides that are derived from non–drug-treated culture preparations that are used in every case analyzed.[3-15]

Rigorous antibiotic washes were performed in all cases. This consisted of streptomycin, amikacin, penicillin, gentamicin, amphotericin β (Fungizone), chloramphenicol and tetracycline. A minimum of two washes were done. Cisplatin was added to the culture media in concentrations of 1.5 μg/mL (equivalent to the therapeutic plasma level), 15 μg/mL (×10), and 37.5 μg/mL (×25). The drug was added after 24 hours following the establishment of the tissue on the sponge gel. Total drug exposure time was 24 hours.

Tumor specimens were considered to be sensitive to the drug if an 84% or greater inhibition of tritiated thymidine incorporation into tumor cells was identified after exposure to the 15-μg/mL concentration of cisplatin in the culture media. The cutoff determinations were based on the cumulative experiences with the SSHDRA.[3,5-7]



Sponge-gel–supported histoculture drug-response assay for head and neck cancer. Correlation of in vitro and in vivo responses to cisplatin. For in vitro response, tritiated thymidine incorporation measured by histologic autoradiography was used as the end point. In vivo clinical response was determined either by bimanual palpation or radiologically. In vitro, the cutoff point for sensitivity was an 84% or greater reduction in the number of tritiated thymidine–incorporating cells treated with cisplatin compared with untreated culture samples. Cisplatin was used at 15 μg/mL concentration in vitro. In vivo, the cutoff for at least a partial response was greater than 50% reduction in the size of the target lesion. Complete and partial responders were grouped together. Fifteen patients were treated with 120 to 200 mg/m² cisplatin every 3 to 4 weeks. Eight patients received 150 to 200 mg/m² cisplatin every 1 to 2 weeks with systemic sodium thiosulfate neutralization.

ciently successful to become adopted for clinical use.[3] Most of these assays are based on measuring the proliferation of stem cells cultured from freshly harvested tumor.[3] These so-called clonogenic assays are technically difficult to perform, and, for solid tumors (particularly head and neck cancers), only a small percentage of tumors can be successfully assayed.[3] In addition, a major conceptual problem of concern is whether cultured clusters of clonogenic cells derived from the tumor truly represent the complex microenvironment in which malignant cells grow.[4]

We have attempted to overcome the major problems of clonogenic assays by developing a sponge-gel–supported histoculture drug response assay (SSHDRA) for solid tumors.[3-15] With this system, important in vivo properties, including three-dimensional tissue architecture, are maintained over relatively long time periods.[3-15] The end point used in this study is the percentage inhibition of tumor cell proliferation determined by histologic examination by means of autoradiographic techniques.[5-7] We previously reported on a high rate of maintaining tissue viability for head and neck tumors and the ability to measure drug responses.[8] In this article, we have correlated the in vitro and in vivo effects of cisplatin in 23 patients who received cisplatin chemotherapy. The purpose of the correlation was to determine the accuracy of the SSHDRA as a potential tool for predicting drug response for patients with this disease.

RESULTS

The **Figure** indicates that if an 84% reduction in the number of cells incorporating tritiated thymidine in drug-treated histoculture samples of head and neck cancer compared with control samples is used as the cutoff for sensitivity in vitro, the in vitro results correlate with clinical response, partial responses and complete responses, in 17 (74%) of 23 cases. The Figure indicates that, with the 84% reduction cutoff, 10 cases were true positive and only two cases were false negative, for a very high 83% predictive-positive value. There were seven nonresponders accurately correlated and four cases with false-negative results for a predictive-negative value of 7/11 or 64%. The sensitivity of the SSHDRA in this clinical trial correlation was 71%, and the specificity was 78%. Thus, the SSHDRA correlates well with both clinical sensitivity and clinical response for head and neck cancer. It should be noted that in 12 of the patients, the biopsy specimens were taken after the patients had been treated with cisplatin. However, when the results for the pretreated patients and nonpretreated patients were compared, the overall accuracy of the SSHDRA was almost exactly the same, ie, 75% vs 73%, respectively.

In this study, 81% of the patients had squamous cell carcinoma, with three patients having other variants of carcinoma and two patients with sarcomas. In this regard, it should be noted that the SSHDRA has been used successfully for over 25 different tumor types.[3] The use of the different histologic methods allowed us to study head and neck cancer in general.[3,5-7] Thus, we were able to have a comprehensive study with both untreated and treated patients with no apparent effect on accuracy.

COMMENT

These data are consistent with previously reported preclinical correlative trials with the SSHDRA where in vitro response of human tumors to chemotherapeutic drugs was correlated to in vivo response of the tumors growing subcutaneously in nude mice.[9] In this preclinical study, the overall correlation rate of the SSHDRA to in vivo response was 89.8%, with 90% true-positive results and with 81.8% sensitivity and 94.6% specificity. The correlative rates are somewhat higher in the preclinical study than in this clinical study, possibly because in the preclinical study, a tetrazolium dye (MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) end point was used instead of the tritiated thymidine histologic autoradiographic end point used in the clinical study. In addition, as Phillips et al[16] have pointed out, often preclinical studies with animals offer more controlled experimental conditions than studies with patients. Given the fact that the study described here was in patients, the high accuracy, especially the predictive-positive rate, suggests that the tritiated thymidine-incorporation end point with the SSHDRA is clinically useful.

It should be emphasized, however, that the results of the present clinical study demonstrate the clinical usefulness of the SSHDRA for identifying both resistant and sensitive patients. In addition, the SSHDRA was able to evaluate 23 (88%) of 26 patients. Thus, the SSHDRA demonstrates a significant advantage over the various clonogenic assays in that clinical sensitivity can be highly correlative with an 83% predictive-positive rate compared with 69% predictive-positive rate in the clonogenic assay in a large series.[3] Another important advantage is the high evaluability rate in the SSHDRA, which, in this study, was 88% and which matches histoculture data for other tumor types,[3] compared with the usual low evaluability rate in the clonogenic assays.[3]

The clonogenic assay technique has other drawbacks. It was found that a very low percentage of disaggregated tumor cells, on the order of 1 in 10^4 or lower, would grow in agar-based medium. The cells that did grow were termed *tumor stem cells*,[17] although it was never made clear what the relationship was of cells that could grow in agar to particular malignant cell types. As Von Hoff[18] points out, in his laboratory in San Antonio, Calif, of approximately 14 000 patient tumors attempted to be grown, only 30% of the cases have yielded evaluable results. In addition, the assay takes a number of weeks to yield useful data.

Cloning assays also have the problem of the use of multiple drugs in the testing process, since it is difficult to add drugs sequentially to the culture system. Quality control has also been a problem with the cloning assays, in particular difficulties of distinguishing clones from cell clumps. However, these problems seem to have been somewhat reduced.[18] Weisenthal and Lippman[19] argue, however, that aggregates that seem to be colonies pose a large problem in the clonogenic assay. Weisenthal and Lippman[19] state that the clumps may create a bias in assays that predict drug inactivity in that the clumping could produce an artifactual, but coincidentally accurate, true-negative rate in clinical correlation studies in which most patients have tumors that are chemotherapy-resistant. In this light, Weisenthal et al[20] noted that, in the clonogenic assays, 60% to 65% of specimens were not sensitive to spirogermanium, sangivamycin, and homoharringtonine. Other assays have shown that essentially all cell types, normal and neoplastic, are sensitive to these drugs, indicating that the clonogenic assay results were really false-negative and, according to the authors, probably due to clumping artifacts.[3]

Weisenthal and Lippman[19] note, with regard to theoretical problems of the clonogenic assay, that cell disaggregation necessary for plating in the clonogenic assay results in a loss of normal cell-cell interactions and three-dimensionality that may be critical in drug response.[21,22] Spheroids[23-25] can be constructed from disaggregated cells to form three-dimensional structures. Similar to solid tumors, the interior may be hypoxic and at low pH, and interior cells may not be accessible to metabolites and drugs as the exterior cells. Of critical importance when drug response was compared by Bhuyan's group[24] in spheroids to single-cell suspension, the spheroids were much more resistant to antimetabolites such as cytosine arabinoside and methotrexate, greater than 11-fold and greater than 125-fold, respectively, and somewhat more resistant to vincristine, doxorubicin, and other antibiotics. Alkylating agents affected both cellular configurations equally. These results emphasize the importance of three-dimensionality. Indeed, in recent experiments, Smith et al[26] have seen for certain drugs such as piercidin C that colon carcinoma cells are sensitive as single-cell cultures but are resistant in vivo and, importantly, are resistant as three-dimensional sections in vitro. These results emphasize the importance of three-dimensional intact-tissue culture[4,27] to allow accurate in vivo drug-response predictions.

Kern and Weisenthal[28] have significantly modified the clonogenic assay. The main modifications are, in addition to measuring colony formation, to measure tritiated thymidine incorporation in the cells incubated in the agar-based medium. In addition, Kern and Weisenthal used products of drug concentration times duration of exposure that were approximately 100-fold higher than those achievable clinically. They found for patients whose tumors showed a less than 40% thymidine-incorporation inhibition or less than 15% colony-formation inhibition that the assay is 99.2% specific and 43% sensitive in identifying the patients as drug resistant. Only one of 127 patients with tumors showing extreme drug resistance responded to chemotherapy. Kern and Weisenthal believe that a high specificity for drug resistance was possible because the drugs were tested at very high concentration products (concentration times duration of exposure) in vitro. However, this assay could not identify patients who were drug sensitive.

With regard to head and neck cancer, the SSHDRA now can be used to identify regimens for cisplatin, as in this study, or other agents, that induce clinical responses. This is most critical since patients who have complete responses in this disease, indeed, have a greater probability of survival. In addition, patients with partial responses may have a greater probability of resectability and organ preservation. Future technical developments will focus on increasing the accuracy of the drug-response assay to even a greater extent.

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