Drug Response of Head and Neck Tumors in Native-State Histoculture

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• **We describe a chemosensitivity testing of head and neck tumors, in which a native-state histoculture, ie, a three-dimensional culture system that maintains important in vivo properties, including tissue architecture, was used. Fifteen specimens of head and neck tumors were evaluated for sensitivity to the following drugs: cisplatin (DDP) at concentrations of 1.5, 15, and 37.5 μg/mL; fluorouracil at concentrations of 4.0, 40, and 100 μg/mL; and combinations of cisplatin and fluorouracil in corresponding doses. Growth and measurement of drug responses were successfully completed in 10 specimens (five others were contaminated, four of them prior to instituting rigorous antibiotic washes). The results indicated cisplatin sensitivity in five of 10 patients; fluorouracil sensitivity in four of 10 patients; and fluorouracil-cisplatin sensitivity in seven of eight patients. Our preliminary results indicate that the native-state histoculture technique is feasible to test chemosensitivity of head and neck tumors.**

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The development of a reliable and practical assay to test sensitivity of individual tumors to various chemotherapy agents has been the focus of research by several laboratories over the past decade.[1-9] Although many techniques have been developed, disadvantages can be, without exception, identified with each system. The major emphasis has been on perfecting clonogenic assays, which are designed to measure in vitro drug activity on proliferation of stem cells cultured from freshly harvested tumor. In addition to several technical difficulties that these types of assays create, a major conceptual problem of concern is whether the cultured clusters of clonogenic cells derived from the tumor truly represent the complex microenvironment of the tumor. Furthermore, the clonogenic potential of solid tumors harvested from the upper aerodigestive tract is quite low.

A novel approach toward overcoming major disadvantages of clonogenic assays has been the development of a native-state, three-dimensional, gel-supported primary culture system.[10-13] In this assay, important in vivo properties, including tissue architecture, are maintained over relatively long periods. The end point is the proportion of inhibition of tumor-cell proliferation determined by histologic examination using autoradiographic techniques. Preliminary results obtained with the use of this assay in testing neoplasms derived from a variety of non-head and neck organs indicated a high success rate regarding the solid tumors.

The purpose of this study was to determine the applicability of the native-state histoculture for testing the chemosensitivity of head and neck carcinomas. We were interested in knowing the take rate, success in maintaining the culture, and drug response of solid tumors derived from the head and neck. We describe our preliminary findings following 15 consecutive assays performed on tumors freshly harvested from patients with head and neck cancer.

PATIENTS, MATERIALS, AND METHODS

All patients were examined 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. The patients were initially presented to a multidisciplinary treatment planning committee prior to any therapy. Eleven patients had previously untreated disease, and four patients had recurrent disease. Cancer staging for the 11 patients with untreated disease was as follows: stage I, one patient; stage II, no patients; stage III, five patients; and stage IV, five patients. Five specimens were obtained from punch biopsy material, and 10 specimens were obtained from resected tumors submitted to the surgical pathology department. Thirteen specimens were taken from the primary disease site, and two were taken from metastatic lymph nodal disease. The primary sites of disease were as follows: larynx, three; oral tongue, two; unknown primary site, two; piriform sinus, two; base of tongue, one; tonsil, one; soft palate, one; oropharyngeal wall, one; parotid gland, one; and paranasal sinus, one. Thirteen specimens were histologically squamous cell carcinoma, one specimen was a poorly differentiated adenocarcinoma, and another was a poorly differentiated mucoepidermoid carcinoma. Specimens were placed into culture media consisting of Eagle's minimal essential media containing Earle's salts, L-glutamine (0.3 mg/mL), 10% fetal calf serum, nonessential amino acids (1:100 dilution of stock solution from Irvine Scientific, Calif), and gentamicin (0.2 mg/mL). Specimens were immediately delivered to the AntiCancer Inc (San Diego, Calif) laboratories for chemosensitivity testing.

The tumors were minced into pieces 1 to 2 mm in diameter and placed on hydrated pigskin collagen gel matrices (Health Design Industries, Rochester, NY) within 48 hours after removal from the patient. Tissue was 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, 4 μCi/mL of tritiated thymidine (1 Ci = 37 GBq) was added to label replicating cells for 4 additional days.

The cultures were then washed with isotonic phosphate-buffered saline, placed in histology capsules, and fixed in 10% (vol/vol) formaldehyde solution. The histocultures were then dehydrated, embedded in paraffin, sectioned, and placed onto slides. The slides were deparaffinized and then coated with an emulsion (Kodak NTB2) 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 power) in a blinded fashion. Replicating cells were easily determined by the presence of bright-green-reflecting silver grains over the cell

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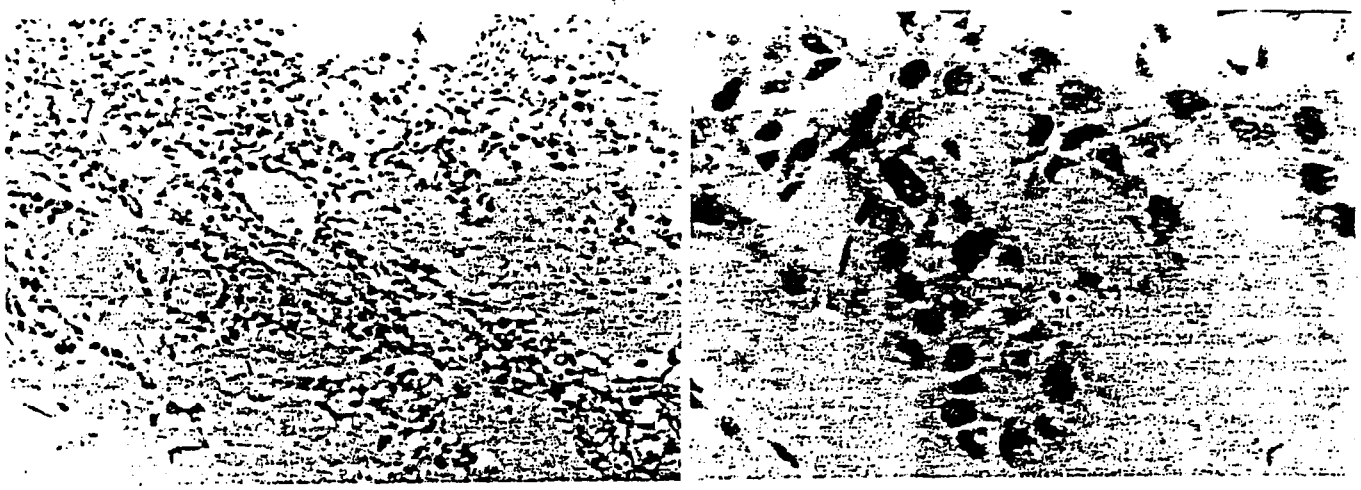

Fig 1.—Histologic appearance of squamous cell carcinoma of oral cavity after 7 days in tissue culture. Black granules indicate cell proliferation as demonstrated by tritiated thymidine uptake (hematoxylin-eosin, ×10 [left] and ×40 [right]).

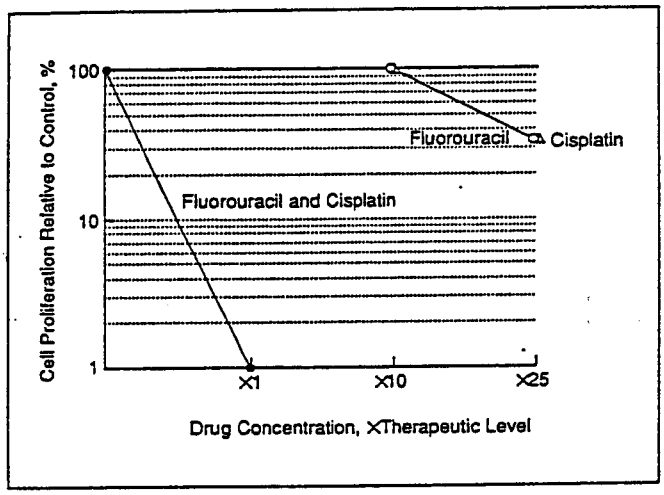

Fig 2.—In vitro drug response of squamous cell carcinoma to cisplatin, fluorouracil, and combination of cisplatin and fluorouracil. See "Patients, Materials, and Methods" section for explanation of drug concentration levels.

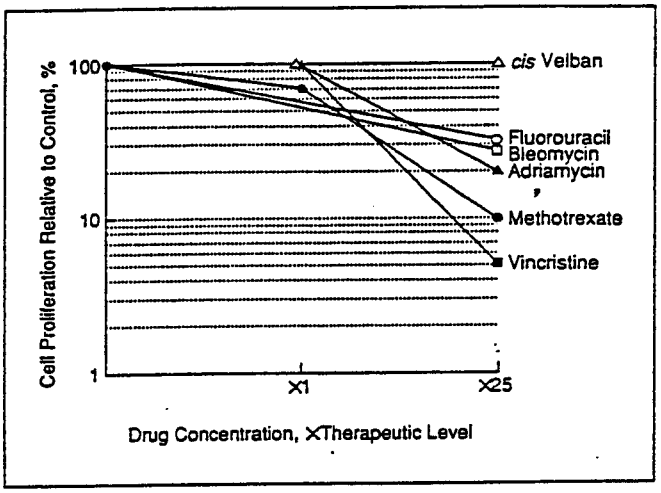

Fig 3.—In vitro drug response of poorly differentiated metastatic adenocarcinoma to the six agents tested. See "Patients, Materials, and Methods" section for explanation of drug concentration levels.

nuclei. Benign stromal cells were excluded by their morphologic appearance. The percentage of cells undergoing DNA synthesis was determined in at least three visual fields with the heaviest labeling for each tumor piece. The growth fraction index was calculated by dividing the number of labeled tumor cells by the number of unlabeled tumor cells (Fig 1).

Rigorous antibiotic washes (that consisted of streptomycin, penicillin, gentamicin, fungazone, chloramphenicol, and tetracycline) were instituted after four of the first seven specimens became contaminated in tissue culture. A minimum of two washes was done. Using this regimen, only one of the subsequent eight specimens cultured was contaminated.

There were 10 samples that remained viable to test for chemosensitivity. Nine of these samples were tested for sensitivity to cisplatin, fluorouracil, and combinations of cisplatin and fluorouracil. Cisplatin was added to the culture media at concentrations of 1.5 μg/mL (equivalent to the therapeutic plasma level), 15 μg/mL (10-fold the therapeutic plasma level), and 37.5 μg/mL) (25-fold the therapeutic plasma level). Fluorouracil was added at concentrations of 4 μg/mL (therapeutic plasma level), 40 μg/mL) (10-fold the therapeutic plasma level), and 100 μg/mL (25-fold the therapeutic plasma level). Combinations of cisplatin and fluorouracil were added in doses corresponding to the therapeutic level and 10-fold the respective individual doses for each agent. The specimen obtained from the patient with adenocarcinoma was tested with a wider spectrum of drugs, comprising Adriamycin (29 and 290 ng/mL), bleomycin (2100 ng/mL), cisplatin (1.5 and 15 μg/mL), fluorouracil (40 μg/mL), Velban (73 ng/mL), vincristine (23 and 230 ng/mL), and methotrexate (2.25 and 22.5 μg/mL). Figures 2 and 3 show the drug responses in two of the samples tested.

Tumor specimens were considered to be *completely sensitive* to the drug if 100% cell kill was identified at the dose corresponding to the therapeutic plasma level, and *partially sensitive* to the drug if 90% cell kill was identified.

RESULTS

Growth and measurement of drug responses were successfully completed in 10 specimens (five others were contaminated, four of them prior to instituting rigorous antibiotic washes). Confirmation of the original tissue his-

tology was made in all of the specimens.

Five of the 10 specimens that tested positive for cisplatin sensitivity, including one specimen with poorly differentiated adenocarcinoma and one specimen with poorly differentiated mucoepidermoid carcinoma, were completely or partially sensitive to this drug. Four of the nine specimens tested for fluorouracil sensitivity were completely (two specimens) or partially (two specimens) sensitive. Eight specimens were tested for sensitivity to the combination of cisplatin and fluorouracil. The dose of each drug was equivalent to the respective therapeutic level, when tested individually. Three specimens were completely sensitive to the combination of cisplatin and fluorouracil, four were partially sensitive, and one specimen was resistant.

The adenocarcinoma specimen was resistant to all of the six agents tested (Fig 3).

COMMENT

The results of this study indicate that the native-state three-dimensional histoculture assay may be feasible to determine drug sensitivity for head and neck cancers. We were successful in maintaining the cultures to study the proliferative effects of a variety of agents on 10 of the 15 specimens (eight of the nine specimens after instituting rigorous antibiotic washes) submitted. Since chemotherapy has become an important investigational treatment modality for patients with advanced head and neck cancer, the development of assays predictive of chemosensitivity could provide useful guidelines for this purpose. Obviously, clinical correlations will provide the most important indicator for the applicability of the native-state histoculture assay in therapy for head and neck cancer.

The development of sensitivity assays to predict tumor response to various agents, including radiation, has been the subject of investigation for several research laboratories over the past three decades. The initial efforts were directed toward radiosensitivity assays, but there have been intensive investigations over the past 10 years focused on the development of assays predictive of chemotherapy responses.

Table 1.—Types of In Vitro Predictive Assays

Types of Assay	Source, y
Clonogenic	
Thymidine incorporation assay	Sondak et al,[3] 1988
Agar diffusion chamber	Selby and Steel,[4] 1982
Capillary cloning system	Maurer and Ali-Osman,[5] 1981; Von Hoff et al,[6] 1986
Cellular adhesive matrix	Baker et al,[7] 1986
Nonclonogenic	
Thymidine incorporation assay	Daidone et al,[8] 1985
Vital dye exclusion	Weisenthal et al,[9] 1983
Organoid culture	Smith et al,[14] 1981
ATP assay	Garewal et al,[15] 1986
Native-state histoculture	Freeman and Hoffman,[10] 1986; Vescio et al,[11] 1987; Hoffman et al,[12] 1989; Vescio et al,[16] 1990
Fluorescence viability	Rotman,[17] 1989

Table 2.—Comparisons of Chemosensitivity Assays

Assay	Advantages	Disadvantages
Hamburger-Salmon clonogenic assay	Most correlations with clinical trials	Low tumor growth (30%), testing drug combinations difficult
National Cancer Institute Screening Group Modification	Good reproducibility	Low tumor growth
Von Hoff capillary tube modification	80% of tumors can be assayed	Technically difficult
Kern Radionuclide Incorporation assay	Rapid availability of results	Fewer clinical correlations, cannot distinguish nontumor cell uptake
Silvestrini Radionuclide Incorporation assay	Many clinical correlations, tumor architecture preserved	Cannot distinguish nontumor cell uptake, poor predictor of cycle-specific drugs, 30% false-negative rate for some tumors
Weisenthal Disc assay	Morphological end point	Cannot evaluate cytostatic drugs, application limited to hematologic tumors
Bogden subrenal capsule assay	In vivo assay, useful for new drug evaluation	Costly, large-team effort required
Baker life-trac assay	80% colony formation, high assessability, good correlates with radiation survival curves, measures total tumor cell proliferation in long-term culture	Time consuming, cannot distinguish nontumor cell growth
P170 glycoprotein assays	Theoretical advantage of measuring the drug resistant gene P170	Many other mechanisms for drug resistance
Native-state histoculture assay	Uses morphological end points, distinguishes proliferation or viability of nontumor cells, very high rate of evaluation for all tumor types, multiple agents can be tested sequentially, uses relatively small amounts of tissue, low frequency of false-negative results	Labor intensive
Rotman fluorescein-binding assay	Rapid assay	Unable to distinguish nontumor cells, measures cell death rather than proliferation, not clear that tumor cells can enter the cell cycle under conditions used

This most recent phase of sensitivity assay research has been led by Hamburger and Salmon[1] and Courtenay et al[2] who were the first to report on in vitro techniques to grow human tumors in soft agar. In 1977, Hamburger and Salmon introduced the use of the soft agar to the human tumor cloning assay as a possible predictor of response of patients' tumors to chemo-

therapy. Modifications of the cloning assay technique as well as other nonclonogenic assays were subsequently introduced (Table 1).

The clonogenic assays are attempted to measure only the dividing cells with unlimited dividing potential. This is based on the premise that the cells most critical to the process of tumor progression are the so-called *tumor stem cells* or *clonogenic cells*. Single-cell suspensions prepared from solid-tumor samples are plated on soft agar and cultured. Colonies of cells (30 cell aggregates) are apparent within 7 to 12 days. Drugs are subsequently added, usually for a 1-hour exposure interval. Modifications of the Hamburger and Salmon clonogenic assay include the National Cancer Institute Screening Group Modification, the Von Hoff capillary tube modification,[6] and the Baker life-trac assay.[7] The NCI Screening Group modified the assay by including positive controls with the use of tetrazolium to distinguish viable features from nonviable and, ultimately, by using radionuclide tritiated thymidine uptake for the end point to eliminate all of the false-negative reactions. The Von Hoff capillary tube modification basically involves culturing the tumor cells in capillary tubes, rather than in 35-mm Petri dishes, which has resulted in an increase in assessable colony growth from 30% to 80% and in a log increase in the cloning efficiency. The Baker life-trac assay uses plates coated with a material called *cellular adhesive matrix*. Greater than 80% of tumors planted form colonies on the cellular adhesive matrix. The advantages and disadvantages of the clonogenic assays in comparison with other techniques are outlined in Table 2.

Nonclonogenic assays include the Silvestrini Radionuclide Incorporation assay,[8] Weisenthal Disc assay,[9] Organoid Culture,[14] ATP assay,[15] native-state histoculture assay,[10-12,16] and Rotman fluorescence binding assay[17] (Table 1). The Silvestrini assay uses thymidine incorporation after a short exposure (3 hours) of small chunks of tumors to the drug. The Weisenthal Disc assay refers to the differential staining cytotoxicity, in which the end point results in damage to the total tumor cell population assessed after 4 days in culture, following drug exposure. Conceptually different from the clonogenic assays, it measures the total tumor cell population, dividing and nondividing. In the Rotman assay, fragments of tumor cells are grown in small chambers and treated with drugs. Fluorogenic substrate is then added to identify the fluorescent viable cells. The advantages and disadvantages of the nonclonogenic assays are outlined in Table 2.

The potential strength of the native-state histoculture assay lies in its ability to overcome many of the problems associated with other techniques. Its major advantage is its morphological end point, one that uses autoradiography and histology to determine whether the proliferating cells are actually tumor cells or other nontumor cells, such as lymphocytes or fibroblasts. The native-state histoculture assay is also capable of measuring drug effects on nonproliferating tumor cells. Although clonogenic cells are thought to be the most critical for tumor growth, there is evidence that resting cells can be recruited at some later time to become replicating or dividing cells. Another theoretical advantage of the histoculture assay is the preservation of the tissue architecture in a three-dimensional configuration. The tumor cell–stromal cell interface, recently recognized to be important for control of cell growth and differentiation through autocrine and paracrine pathways, is, thereby, maintained by the culture configuration.

Finally, one must consider the reliability of chemosensitivity assays. Ideally, an assay should be accurate in predicting both sensitivity and resistance to chemotherapy agents. In a review of the literature by Von Hoff,[18] regarding 54 different in vitro–in vivo clinical correlation trials performed in 35 institutions over the last 12 years, there were 69% true-positive results and 91% true-negative results. Analyzed another way, the overall sensitivity of the tests, calculated as true-positive results/(true-positive results + false-negative results), was 79%, and the overall specificity of the tests, calculated as true-negative results/(true-negative results + false-positive results), was 86%. The reliability of the native-state histoculture assay has yet to be determined. High correlation of chemosensitivity between human tumors explanted in the native-state histoculture and implanted into athymic mice has recently been found.[13] Studies are also under way to correlate the assay results with the clinical response.

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