An in Vitro Assay Procedure to Test Chemotherapeutic Drugs on Cells from Human Solid Tumors

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ABSTRACT

An in vitro assay was developed to measure the chemotherapeutic drug susceptibility of cells from human tumors. The assay utilized live cells, freshly isolated from tumor tissue, which were incubated for a short period in vitro. The drug-induced inhibition of incorporation of radiolabeled precursor into DNA, RNA, and protein was measured. The assay is sensitive to concentrations of chemotherapeutic drugs in the therapeutic range and is reproducible when tested with replicates of the same tumor cell population.

INTRODUCTION

A sensitive procedure for comparing the effects of chemotherapeutic drugs on the cells of an individual solid tumor prior to initiation of drug treatment would be of great value to the patient. Several in vitro drug testing assays have been developed for solid tumor tissues and cells, which fall into a limited number of experimental approaches: measurement of morphological change in the tumor cells after drug exposure (1, 28); measurement of the inhibition of cell population increase after drug therapy (3, 10, 11, 16, 21-23); measurement of inhibition of cellular respiration after drug exposure (5, 9, 14); and measurement of the inhibition of cell metabolism using radioactively labeled metabolic precursors (2, 4, 8, 13, 20). It is this last approach which has been utilized in the present study. The agents used in this work act in vivo by inhibition of various metabolic activities. The assay measures the inhibition of incorporation of radiolabeled precursors by freshly isolated tumor cells in vitro in the presence of the drugs, by comparison with parallel controls not treated with drugs.

MATERIALS AND METHODS

Drugs. The agents tested were bleomycin sulfate (Blenoxane; Bristol Laboratories, Syracuse, N. Y.), cis-diaminedichloroplatinum (cis-platinum, distributed by the Division of Cancer Treatment, NIH, Bethesda, Md.), cyclophosphamide (Cytoxan; Mead Johnson and Co., Evansville, Ind.), doxorubicin (Adriamycin; Adria Laboratories, Inc., Wilmington, Del.), 5-fluorouracil (Roche Laboratories, Nutley, N. J.), and N,N',N''-triethylenethiophosphoramide (Thio-TEPA; Lederle Laboratories, Pearl River, N. Y.). Three concentrations of each of the drugs to be tested were diluted in Trowell's T8 tissue culture medium supplemented with 20% fetal bovine serum. Aliquots of 50 μl were dispensed on a 96-well Linbro No. ISMVC-96TC microtiter plate. Control wells received equal volumes of medium lacking drugs. Drug concentrations were chosen to correspond approximately to those used in vivo, making the simplified assumption that the drug was distributed through a body water volume of 0.5 liter/kg of body weight. The concentrations used in the assay in vitro were: bleomycin sulfate, 3.75 to 37.5 units/liter; cis-platinum, 0.74 to 7.4 ρg/liter; cyclophosphamide, 2.5 to 25 μg/liter; doxorubicin, 0.25 to 2.5 μg/liter; 5-fluorouracil, 1.25 to 12.5 μg/liter; and Thio-TEPA, 2 to 20 μg/liter. The microtiter plates were then stored at —70° until used.

Tumor Cell Preparation. Samples of tissue from human tumors removed at surgery and from human tumors growing in athymic "nude" mice were placed in serum-free culture medium immediately after excision and transported to the laboratory. The tissue was cut into pieces less than 2 mm in diameter and incubated overnight at 37° in 0.05 to 0.1% collagenase (Worthington, Type I) in complete culture medium. The partially dispersed cells were passed gently through an 80 mesh tissue sieve (E-C Apparatus Corp., St. Petersburg, Fla.), centrifuged at 1500 rpm for 10 min, and washed once with tissue culture medium by resuspension and centrifugation. Live cells were separated from dead ones on a Ficoll-Hypaque density gradient (17). The live-cell layer was aspirated gently, washed with medium by centrifugation, and used for the drug sensitivity assay. The proportions of live cells were determined during cell preparation by trypsin blue exclusion (19).

Drug Sensitivity Assay. One hundred μl of the live-cell suspension (5 to 6 x 10^6 cells/ml) were added to each of the 96 wells. Quadruplicate wells were prepared for each drug concentration and the corresponding controls. Where cyclophosphamide was used, it was activated by microsome treatment as described elsewhere (24). The microtiter plates were then incubated at 37° in a humidified atmosphere of 5% CO2 in air for 72 hr, a time determined to be optimum in preliminary experiments. Fifty μl (1.5 μCi) of tritiated thymidine, uridine, or amino acid mixture (New England Nuclear, Boston, Mass.) were then added to appropriate wells giving a final concentration of 7.5 μCi/ml, and incubation was continued for an additional 12 hr. The assay was terminated by centrifuging the microtiter plate at 1500 rpm for 10 min and aspirating the supernatant medium. The cell pellet was washed once with phosphate-buffered saline (PBS-Dulbecco; Grand Island Biological Co., Grand Island, N. Y.) by centrifugation, and the cells were suspended with the aid of 1 drop of 2.5% trypsin and incubation at 37° for 7 min. One hundred μl of cold 10% trichloroacetic acid were then added to each well, and the plates were held overnight at 4°. The acid-precipitable material was collected on Whatman GF/A glass fiber paper using a
In Vitro Testing of Human Solid-Tumor Cells

The filters were washed repeatedly with water and dried, and the radioactivity was determined in a scintillation fluid of 4 g of PPO and 0.1 g of POPOP per liter of toluene. The inhibition of incorporation is expressed as the difference in incorporation between drug-treated and control cells divided by the incorporation in control cells, stated as percentage.

RESULTS

Sensitivity of Tumor Cells to Drugs. In most cases, the range of drug concentrations found to be effective in vitro correlates well with the doses usually used clinically, as judged by the simple estimate of in vivo concentration described above. The fact that in vitro assay made use of a 10-fold range of drug concentrations allowed a comparison of the relative sensitivity of cells from individual tissues to the various drugs examined. This assay system clearly showed the different sensitivities of different tumors to a given drug (Chart 1), as well as the different sensitivities of a given tumor to different drugs (Chart 2).

Both the percentage of inhibition of DNA synthesis by different levels of chemotherapeutic drugs and the maximum extent of inhibition were different for different tumors, cells from the 2 kidney tumors differing substantially from one another (Chart 1). When human kidney tumor (SS78) and counterpart nontumorous kidney from the same patient were tested in the in vitro assay, the nontumorous kidney cells exhibited a greater inhibition of thymidine incorporation than did kidney tumors. This reflected the fact that the nontumorous kidney cells divided more rapidly in vitro than did the isolated kidney tumor cells, thus becoming more sensitive to the action of doxorubicin, which intercalates the adjacent base pairs of a replicating DNA strand. On the contrary, the protein and RNA synthesis of nontumorous kidney cells were less affected by doxorubicin than cells from kidney tumors and other tumors tested, indicating a differential toxicity to malignant and normal tissue. Chart 2 shows a variation in sensitivity of the human prostate carcinoma cells (SS78) to various chemotherapeutic drugs in the in vitro assay. The concentrations of the various drugs giving 50% inhibition can be computed from such an assay yielding information of potential clinical value.

Response of Cells Isolated from Solid Tumor and Counterpart Tissue Culture Cells to Doxorubicin and Cyclophosphamide. Cells freshly isolated from a human kidney tumor (SS78) were tested against doxorubicin and cyclophosphamide by the in vitro assay. A part of the same cell suspension was used to establish the cells in tissue culture (25). The cultured cells were used at passages 5 and 7 to perform parallel assays. Due to rapid growth during the assay, the tissue culture cells at passages 5 and 7 were used at a lower cell concentration than that of cells isolated from solid tumor or in culture passage 1. Results of these assays are shown in Chart 3. Examination of the inhibition of incorporation of tritiated thymidine by doxorubicin (which intercalates DNA) and cyclophosphamide (which alkylates DNA) showed that the rapidly dividing cells established in tissue culture were more sensitive (p < 0.001) to these 2 drugs, both of which require replicating DNA to exert their cytotoxic action. Cells isolated from solid tumor showed less inhibition by doxorubicin and cyclophosphamide of the incorporation of tritiated thymidine,
These results indicate that the initial chemotherapy profile the sensitivity to inhibition of uridine and amino acid incorporation in cells freshly isolated from the tumor and cells from the same tumor after growth in culture showed little difference. These results indicate that the initial chemotherapy profile obtained by testing freshly isolated tumor cells was retained by the same population of cells grown in culture, although the latter had become more sensitive with respect to thymidine incorporation.

To determine whether the test results were influenced by the environment in which the cells were growing before the test, a comparison was made between cells of a prostatic cancer cell line [PC-3 (12)] in tissue culture and cells from a nude mouse-supported tumor derived from the same cell line. The responses of the cells from the 2 environments were closely similar.

**Tissue Required.** The amount of tissue required for a single assay using one 96-well microtiter plate varied according to the proportion of viable cells in the tumor and the proportion of cellular to noncellular material. The cells were dispensed at 5 to 6 x 10^4 per well, so a total of at least 5 x 10^5 viable cells was required to seed a complete microtiter plate. Allowing for 50% viability and a 50% loss during isolation and dispersion, 2 x 10^5 cells were required for one chemotherapeutic assay. Thus, a minimum of 20 mg of tumor tissue was required if there were 10^6 cells per mg of tissue (7). In practice, 100 mg to 1 g of tumor was used as starting material.

In order to verify that the cells which behaved in the Ficoll-Hypaque density gradient centrifugation as dead cells were indeed dead by criteria other than uptake of trypan blue, parallel chemotherapy experiments were carried out using the dead and the live cell populations. Incorporation of labeled precursors by the dead cell fraction, expressed as a percentage of that in the live cell fraction was: DNA, 11%; RNA, 17%; and protein, 11%. A small proportion of live cells was trapped in the dead cell pellet, which could account for the limited amount of isotope incorporation in that fraction. Monitoring the proportion of live cells by trypan blue exclusion during preparation of the cell suspensions showed that most of the dead cells came from the tumor itself and were not a product of the preparation procedure.

**Reproducibility.** It was not possible to perform repeated assays on human surgical specimens, but this comparison was made on a nude mouse-supported human prostatic carcinoma, PC-3, and on human bladder tumor cells growing in tissue culture. The inhibition of incorporation of the metabolic precursors by doxorubicin tested on different days was very reproducible (Chart 4), using either tissue culture cells or cells from the solid tumor.

**DISCUSSION**

This assay for in vitro testing of the effects of chemotherapeutic drugs on cells from solid tumors allows dispensing of cells in replicate samples; it is rapid, is reproducible, and accurately determines the sensitivity of the cells to the drugs. It is already useful for research. In order to develop fully the usefulness of this assay, for clinical application, it will be necessary to compare its results with the effects of treating the same tumors in vivo, with a view toward developing a screening assay capable of predicting the best chemotherapeutic regimen for individual patients.

For dispersing the tumors and recovering viable cells, overnight digestion with collagenase (6) followed by very gentle
sievig (15) was found to be the most generally applicable procedure. While most of the tumors used in developing the assay were from urogenital tissues, the method should be applicable to other tissues as well. Analysis of tissue from benign prostatic hyperplasia was not possible, since the epithelial cells could not be adequately dispersed (27). Testing of low-grade prostatic carcinoma may present the same problem, but anaplastic prostate tumors showed much less intercellular attachment and could be dispersed and analyzed with this system.

The question of the nature and origin of the cells being tested is of great importance, since the sensitivity of the tumor cells themselves is probably the critical factor in response to chemotherapy. In addition to the neoplastic epithelial cells, the tumors being studied also contained supporting stroma with fibroblastic cells; in the nude mouse-supported tumors, there was evidence that the stroma was of mouse origin, rather than human origin (18). Light microscopy showed that the cells which attached to the microtiter wells were chiefly of epithelioid morphology. There may be some selective adverse effects of the collagenase treatment on fibroblastic cells, as suggested by observation that epithelial cell lines derived from kidney carcinoma (SS78 (25)) and prostate carcinoma (DU145 (26)) could be subcultured easily in 0.1% collagenase in culture medium, while the fibroblastic cells from a rhabdomyosarcoma (16574) could not. Without adequate markers, however, it was not possible to establish beyond doubt the origin and nature of cells present in the viable cell fraction obtained from the collagenase-treated human tumors.

In a search for a test procedure which would be faster and technically more satisfactory than in vitro colony formation method (21), we chose the inhibition of incorporation of metabolic precursors into macromolecules because of its relative rapidity and simplicity and because it was directly relevant to the action of the chemotherapeutic drugs in vivo. Roper and Drewinko (21) rejected the uptake of radiolabeled thymidine as a measure because there was no dose-dependent effect on the tissue culture cells in their system, with any of the 3 drugs that they tested. That was clearly not a problem in the present assay, in which there was a strong dose-dependent response with a variety of drugs, including 2 of those tested by Roper and Drewinko. While thymidine incorporation is probably a more promising measure of the effectiveness of a chemotherapeutic drug in vivo than the corresponding measure with uridine or amino acid, we have included the latter 2 to allow more comprehensive examination of the effect of the drugs. Obviously, the assay could be easily modified to use any one or combination of the precursors as the particular needs of the study warrant. Normal cells from the same patient could also be tested by the same procedure, to allow determination of the differential effect of drugs under consideration, and drug combinations could also be examined.

The assay provides a reproducible, sensitive, and relatively rapid and convenient procedure for determining the effects of drugs on biosynthesis in cells freshly isolated from solid human tumors, and, as such, already has useful research potential. Comparison with results in vivo will determine whether such factors as type of cell isolated, differential survival of cells through isolation and culture, tumor cell heterogeneity and the nature of the property measured will allow the assay to achieve clinical applicability. Such studies are now in progress, comparing results of the in vitro assay with clinical effects. In the hope of obtaining a more rapid estimate of the comparability of the in vitro test with in vivo results, assay results are being compared also with the effects of treatment of human tumors grown in the athymic nude mouse.

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S. Shrivastav et al.


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