In Vitro Tests to Predict Human Tumor Sensitivity to Cytotoxic Agents

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ABSTRACT

Current principles and practice of the systemic treatment of cancer rest on the principle of statistics. Based on large, well controlled randomized studies, statistical likelihoods for treatment response and toxicities are derived and applied to individual patients. While this approach has led to great advances in medical oncoloogy, the concept to individually predict antitumor response has remained attractive. The present article summarizes various aspects of this approach as well as potential applications of a variety of assays for anticancer drug development.

INTRODUCTION

Over the past decades, successful chemotherapy regimens have been identified for a number of malignant diseases including testicular cancer and some leukemias, malignant lymphomas, and childhood tumors. These advances have been achieved by carefully designed, prospective clinical studies in large patient populations. Based on the response rates in these trials, an individual patient's probability for response can be estimated. However, for the majority of cancer patients, only marginal treatment is available. There is increasing evidence of defined phenotypic and genotypic diversity within a patient's tumor as well as between different tumors of identical histologic characteristics. If therapies could be based on relevant biologic markers, better outcomes would appear feasible. For this reason, several in vitro and in vivo assays have been investigated for their potential to predict an individual patient's response to chemotherapy. These assays would be helpful in patients with curable disease and effective initial therapy if they could identify the occasional patient with primary resistant disease. At present, there is no convincing evidence that any chemosensitivity assay has such a predictive power.

Irrespective of the experimental system used, in vitro chemosensitivity assays have to cope with several conceptual problems. First, the choice of drug concentrations is arbitrary since it is unclear which concentration best reflects the clinical situation. Second, in vitro chemosensitivity assays offer only a limited ability to study intratumor and intertumor heterogeneity. Third, experimental conditions interfere with the usual physiologic microenvironment of a patient's tumor cells. Finally, the selection pressure on tumor cells by the experimental system used remains a variable that is poorly controlled.

Table 1 summarizes in vitro systems that have been used to predict an individual patient's response or lack of response to chemotherapeutic agents. Clonogenic tests can be distinguished from nonclonogenic tests. Details on these assays are provided below.

DYE TECHNIQUES

Of the many tests evaluated, none has convincingly demonstrated its predictive value. A combination of fast green dye, hematoxylin and eosin had promising results in retrospective studies.[1,2] However, final conclusions have to wait until a prospective evaluation is performed.

The tetrazolium assay, another well-known dye technique, has been used by the National Cancer Institute to perform mass screening of potential anticancer agents.[3,4] This assay is based on the ability of vital cells to reduce the compound to a blue formazan product. Thus, the metabolic activity of cancer cells can be determined by photometry in a semiautomated fashion. This assay is simple, rapid, relatively inexpensive, and can be used conveniently in cancer cell lines. However, insufficient data are available with regard to its predictive value if freshly explanted tumor specimens are used.

Recently, a more novel combination of fast green dye and hematoxylin-eosin staining, the differential staining cytotoxicity (DiSC) assay, has been reported to improve treatment outcome in chronic lymphocytic leukemia.[5]

TABLE 1. IN VITRO TECHNIQUES USED TO PREDICT PATIENT RESPONSE TO CHEMOTHERAPY

Nonclonogenic assays
- Dye exclusion
- Fluorescence
- Explant (organoid) cultures
- Precursor incorporation
- Intracellular drug concentrations
- Determination of specific biochemical and molecular markers

Clonogenic assays
- Human tumor cloning assay

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FLUORESCENCE

Fluorescent dyes may be used to determine the in vitro chemosensitivity of freshly explanted tumor biopsies. In a recently proposed test, the tissue is not completely disaggregated into single cells. Instead, cell clusters, termed "microorgans," are prepared by mechanical or enzymatic techniques to preserve cell-cell interactions.[6] At present, insufficient information is available on this assay's predictive value.

In another approach, propidium iodide has been used in conjunction with flow cytometric DNA determinations to quantify drug effects on tumor cells.[7] However, technical difficulties limit this method's application to primary tumor specimens, and sufficient data are not yet available to determine a definitive predictive value for clinical response or resistance.

EXPLANT (ORGANOID) CULTURES

Short-term explant cultures were investigated during the early years of chemosensitivity testing but have been abandoned due to technical problems and lack of standardization. As mentioned earlier, a recent method uses staining of tumor cell clusters with fluorescein. In a series of 50 patients, the sensitivity was reported to be 100% and the specificity 84%.[6] These results need to be confirmed in larger prospective trials.

PRECURSOR INCORPORATION

Precursor incorporation techniques fulfill a number of requirements for chemosensitivity assays. They are relatively inexpensive, rapid, and feasible in many tumor types. They also allow for determination of cell growth kinetic parameters. However, they cannot differentiate between normal tissue and tumor and may also give false-negative results if lethally damaged cells continue synthesis of macromolecules (eg, DNA) during the assay period.

Most commonly, [3H]thymidine incorporation has been used to directly determine the extent of DNA synthesis. This can be determined by liquid scintillation counting or by autoradiography (thymidine labeling index). The latter method is too time-consuming for general use but is more specific for malignant cells, and it also allows for estimates of tumor growth kinetics. The predictive value of [3H]thymidine incorporation as determined by liquid scintillation counting has been heavily debated.[8-11] No definitive prospective trial has yet been conducted to confirm the encouraging results from retrospective studies.

CELLULAR ADHESIVE MATRIX

Both two-dimensional and three-dimensional matrix models have been developed but only the two-dimensional matrix model has undergone clinical studies. Fibronectin- and fibrinopeptide-coated plastic surfaces provide a selective environment for the culture of tumor cells. After fixation, cells are stained and the number of cells in the treated plates is compared with the number of cells in the control plates. In one retrospective study, clinical outcome was related to assay results.[12] However, no large prospective trial has confirmed these results.

INTRACELLULAR DRUG CONCENTRATIONS

Determination of intracellular drug concentrations requires sophisticated techniques that are specific for each compound and its metabolites under investigation. This approach may be particularly difficult if combination chemotherapy is used since tumor response and

TABLE 2. SPECIFIC BIOCHEMICAL AND MOLECULAR MARKERS FOR DRUG RESISTANCE

Molecular Alteration	Mechanism	Agent Affected
Alteration of drug transport		
Expression of P-170 glycoprotein	Increased drug efflux ("Pleiotropic drug resistance")	Miscellaneous[17]
Increased enzyme activity		
Glutathion-S-transferase	Drug inactivation	Alkylating agents[18]
Aldehyde dehydrogenase	Drug inactivation	Cyclophosphamide[19]
Guanin-O6-alkyl transferase	DNA repair	Nitrosoureas[20]
Ribonucleotide reductase	Increase binding sites urea21	
Decreased enzyme activity		
Deoxycytidine Kinase	Drug activation	
Pyrimidine salvage pathways	Drug activation	5-Fluorouracil
Topoisomerase II	Decrease of binding sites	Anthracyclines[24]
	Epipodophyllotoxins[25]	
Gene amplification		
Dihydrofolate reductase	Increase of binding sites	Methotrexate[19]
Ribonucleotide reductase	Increase binding sites	Hydroxyurea[21]

patient survival may be influenced by other components of the regimen.

Cellular retention of arabinosyl cytidine triphosphate (Ara-CTP) has been reported to correlate with remission duration in patients with previously untreated acute nonlymphocytic leukemia.[13] However, other investigators have not found a correlation between clinical response or remission duration and formation of Ara-CTP.[14-16]

There is no definitive evidence that determination of intracellular drug concentrations has any predictive value for other antitumor agents.

SPECIFIC BIOCHEMICAL AND MOLECULAR MARKERS

Rapid progress has been made to better understand the mechanisms responsible for drug resistance. Initially, receptors to female hormones (estrogen and progesterone) were identified to predict outcome of hormonal treatment in breast cancer. Increased understanding of molecular mechanisms has guided the development of further markers such as cerB-B2 to predict treatment outcome in certain subsets of patients undergoing marker-directed treatments such as trastuzumab.

Treatment of CD20-overexpressing lymphoma cells with rituximab, another monoclonal antibody specifically directed against a cell surface antigen, has also been successful. In addition, determining the resistance of specific molecular markers may allow one to predict whether a given agent will be inactive clinically and whether interference with these mechanisms may be helpful to overcome drug resistance. However, this approach would tell the clinician only what drugs to exclude from treatment regimens. Table 2 summarizes important markers of drug resistance.[17-25] To date, no definitive trials are available to determine how accurately these markers predict clinical resistance.

HUMAN TUMOR CLONING ASSAY

Clonogenic assays have been most thoroughly studied for their potential to predict an individual patient's response to chemotherapy. The endpoint of these assays is drug-mediated inhibition of cellular proliferation of malignant cells in vitro.[26-28] In principle, tumor tissue is disaggregated to yield single cells. The cell suspension is then exposed to antitumor agents. After removal of the drugs, the cell suspension is seeded into a semisolid medium (agar or methylcellulose) to selectively inhibit proliferation of nonmalignant cells. After 2 to 4 weeks, clonogenic tumor cells will have undergone several divisions and formed tumor cell colonies that can be counted. Colony formation by drug-treated cells is expressed relative to colony formation of untreated cells in controls.

More than 2,000 patients have been studied in retrospective and prospective trials using clonogenic assays. Table 3 summarizes the cumulative results of 2,300 clinical correlations.[29] According to these data, there is a 69% probability that a patient will have a clinical response if the tumor specimen is sensitive in vitro. If the tumor is resistant in vitro, the patient has only a 9% chance for a clinical response. Although these correlations are far from optimal, they are in the same range as other clinically accepted assays, such as the determination of estrogen- or progesterone-receptor status in breast cancer patients to predict clinical response to endocrine therapy. A recently published, prospective, randomized correlative trial compared assay-guided therapy with clinician's choice of drugs, and results indicate that assay-guided chemotherapy may lead to higher response rates.[30] Although there was no difference in survival between these groups, this study provides encouraging leads for future clinical trials.

Applicability of clonogenic assays on a larger scale is limited. These techniques are time-consuming and not yet optimized. Twenty to fifty percent of tumor specimens (depending on tumor type) will not grow in vitro to give sufficient numbers of colonies for chemosensitivity testing. Furthermore, no definitive clinical trial has yet been performed to demonstrate prolonged patient survival by assay-guided chemotherapy.

Clonogenic assays have been combined with [3H]thymidine incorporation to increase the number of evaluable specimens and to decrease incubation time.[31] However, the experimental endpoint in this assay is no longer direct visualization of clonal proliferation. Instead, the amount of DNA-bound radioactivity is determined and considered representative of cell growth. The relationship between colony formation and [3H]thymidine incorporation is nonlinear and an algorithm has been developed for

TABLE 3. SUMMARY OF 2,300 CLINICAL CORRELATIONS USING CLONING ASSAYS TO PREDICT CLINICAL RESPONSE OR LACK OF RESPONSE FOR AN INDIVIDUAL PATIENT

	N	%
True Positive	512	69
False Positive	226	31
True Negative	1,427	91
False Negative	135	9
TOTAL	2,300	
Sensitivity*		79
Specificity†		86
Positive Predictive Value‡		69
Negative Predictive Value§		91

* Sensitivity = $\frac{\text{True Positives}}{\text{True Positives + False Negatives}}$

† Specificity = $\frac{\text{True Negatives}}{\text{True Negatives + False Positives}}$

‡ Positive Predictive Value = $\frac{\text{True Positives}}{\text{True Positives + False Positives}}$

§ Negative Predictive Value = $\frac{\text{True Negatives}}{\text{True Negatives + False Negatives}}$

conversion.[32] No prospective trial has been performed to study the predictive value of this improved system.

SUMMARY

Over the past 40 years, numerous attempts have been made to develop in vitro systems to predict an individual patient's response to chemotherapeutic agents. An ideal system would be rapid, reliable, inexpensive, simple, and applicable to all tumors. At present, no such assay is available, and even the most extensively studied assays will identify agents that do not work more often than agents that will. The lack of clinically active agents is an important factor for the evaluation of any predictive assay, and there clearly is an urgent need for more active compounds. Here, chemosensitivity and molecular marker assays may be of great value. With a few exceptions for specific molecular markers, routine use of global assays in the clinical setting is still premature.

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