Ex Vivo Programmed Cell Death and the Prediction of Response to Chemotherapy

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Opinion statement

Since the earliest introduction of cytotoxic chemotherapy, investigators have pursued laboratory techniques designed to match patients to available drugs. Most of the work, published through the 1980s, reflected the prevailing view of cancer as a disease of dysregulated cell proliferation. Noteworthy, the description of apoptosis and programmed cell death, fundamental to our modern understanding of human tumor biology, did not occur until well after the heyday of in vitro chemosensitivity testing. By incorporating the modern tenets of carcinogenesis associated with perturbations in cell survival we can now re-examine laboratory assays of drug response in the context of drug-induced programmed cell death. Although there is interest in the use of genomic analyses for the prediction of chemotherapy response, the painful recognition that genotype does not equal phenotype will continue to limit broad application of these platforms. Biosystematics instructs that biological pathways rarely follow predicted routes. Efforts to force human biology to behave according to preconceived scientific dictates have proven costly and unsuccessful. Whole-cell experimental models with the capacity to evaluate all the operative mechanisms of cellular response to injury, acting in concert, provide valid tools for the study of human cancer. Educated by cellular behavior, we can expeditiously examine molecular processes of interest. This article briefly reviews the history of whole-cell experimental models of in vitro chemosensitivity testing then focuses on cell-death measures as the most robust predictors of clinical outcome in human cancer.

Introduction

The development of cytotoxic drugs for cancer in the 1940s enabled physicians, for the first time, to provide objective responses in disseminated malignancies, albeit at significant toxicity. By the early 1950s, despite the small number of drugs available, investigators began to develop techniques to match patients to the available treatments. The first paper, published in 1954 [1], used the reduction of tetrazolium in tumor explants to examine drug effects. A wide variety of techniques ensued, including the measurement of DNA synthesis with tritiated thymidine (or uridine), proliferation of cells in monolayer, elaboration of radioactive CO_2 from C_{14}-radiolabeled glucose, and colorimetric or radiolabeled measurements of protein synthesis. These culminated in the description of the human tumor colony formation or clonogenic assay, published to acclaim in 1978 [2]. Despite the efforts of dedicated investigators, no laboratory test to predict response to cancer chemotherapy gained wide clinical use, leading to nihilism on the part of academics and uncertainty among clinical oncologists.

Although there are many reasons why predictive assays based on the study of human tumor explants in primary (nonpassaged) culture failed to gain acceptance, the most frequent argument marshaled against their use was their inability to identify clinically active drugs. The oft-repeated adage, that drugs found "sensitive" in vitro rarely worked, whereas drugs found "resistant" in vitro were truly ineffective, became dogma—an entire industry has arisen that provides these services, not for the desperately needed purpose of

chemosensitivity testing, but instead for the provision of the marginally useful chemo-resistance testing, one form of which is dubbed extreme drug resistance.

Recognizing the consistent failure of proliferation-based methods to identify active drugs in vitro begs a fundamental question. Is it simply impossible to predict clinical outcomes using fresh specimens of human tumors, or have prior methods misjudged the biological principles that underlie the prediction of response to chemotherapy? This paper examines the concepts of cancer biology, mechanisms of response to noxious stimuli, and the tenets of tumor behavior in vitro.

Treatment

Biological principles

- The centuries-old concept of cancer as a disease of increased cellular proliferation was dealt a blow by the groundbreaking description of apoptosis [3••]. The recognition that observed geometric tumor growth curves could be equally well explained by the failure of cellular senescence and death represented, in the truest sense, a paradigm shift. Indeed, the failure of contemporary drugs to cure malignancies was at once explained by their function as inhibitors of cell replication at the DNA synthesis and mitotic level. In accordance with apoptotic dysregulation, measures of cell growth inhibition become tangential to the prediction of clinical response unless they are directly associated with apoptosis induction. In a review of the topic, Reed [4••] observed that "essentially all traditional anticancer drugs use apoptosis pathways to exert their cytotoxic actions." This forces us to question whether the objective responses observed, to date, must be attributed to serendipity. By deconstructing the errors of modern oncology that focused exclusively on cellular proliferation we now refocus attention on the dysregulation of cell death. In so doing, we are for the first time developing strategies that "target" those features unique to tumor cells that confer survival advantage. As we enter the era of targeted therapies, it is increasingly evident that survival pathways and not cellular proliferation pathways will be the focus of the next generation of chemotherapeutics.
- When we examine assays designed to test cancer chemotherapy drugs in vitro, the widely recognized failure of proliferation-based endpoints (ie, clonogenic, H3*-thymidine incorporation, monolayer growth to confluence) to predict clinical outcomes is easily explained. Because tumor cells are not distinguished by their proliferative capacities, growth inhibitory endpoints, by definition, cannot distinguish active from inactive drugs. Put simply, the measurement of chemotherapy-induced growth inhibition is irrelevant to clinical prediction. Furthermore, by only measuring growing cells and leaving temporarily dormant Go/Gx cells invisible to pulse-chase, growth to confluence, or colony formation assessments, these endpoints fail to address a crucial subset of tumor cells fully capable of repopulating the entire tumor.
- Only assay methods that gauge drug-induced programmed cell death, apoptotic or other, have the capacity to predict the sterilization of all viable tumor elements. Cell death endpoints as surrogates for programmed cell death can examine nuances of drug response, allowing for the assessment of drug synergy, dose response, drug sequencing, mechanistic evaluations, and molecular correlates. Growth-based endpoints lacking the capacity to identify active drugs have been relegated to the elimination of inactive drugs or "chemo-resistance" testing.

The implications of programmed cell death

- After the description of apoptosis, a series of observations clarified its molecular basis. The ced genes found in the *Caenorhabditis elegans* roundworm [5] were shown to have structural homology with the human BCL2 gene family [6], establishing biological preservation of these crucial processes over

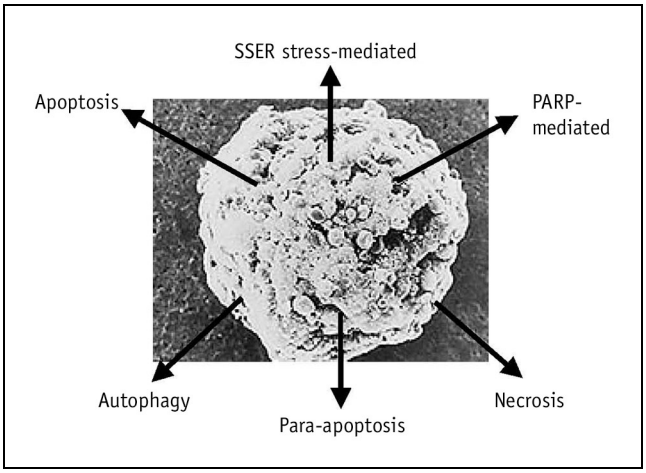

Figure 1. Mechanisms of programmed cell death. PARP—poly-ADP-ribose-polymerase; SSER—smooth surface endoplasmic reticulum.

hundreds of millions of years. However, it is crucial to recognize that apoptosis is but one of a panoply of death options available to a cell. Indeed apoptotic, para-apoptotic, autophagic, smooth surface endoplasmic reticulum stress-mediated, poly-ADP-ribose-polymerase–mediated, and necrosis-like cell death events all participate in cell involution after appropriate endogenous or exogenous stimuli (Fig. 1). The simple measurement of caspase activation or expression of phosphatidyl serine residues on the cell membrane may grossly underestimate the complexity and redundancy of cell death/survival pathways. It has been shown that cells committed to death will overcome exposure to potent caspase inhibitors to die via pathways that mimic necrosis [7••]. Fresh explants of human tumors in culture have provided significant insights, including the first observation of activity for chlorodeoxyadenosine in hairy cell leukemia, synergy between purines and mustard alkylators in lymphoid malignancies [8], the activity and synergy for platins plus gemcitabine in breast and ovarian cancers [9,10], characterization of topotecan combinations [11], and the accurate selection of disease targets for gefitinib [12,13], all of which have been subsequently confirmed in clinical trials. By measuring cell death in primary culture spheroids, the cumulative effect of all of the operative survival and death cascades can be gauged at once. Regardless of the specific pathway, the end result (the death of the tumor cell) provides the needed insight to allow for improved drug selection.

Cell death–based laboratory analyses

- A variety of cell death assays have been applied. The specific endpoints differ, yet the underlying principles are largely congruent. First, tumor cells are not propagated or stimulated. Second, the endpoints assess valid cellular functions associated with cell survival. Among these are measures of energy metabolism, membrane integrity, respiration, protein synthesis, enzymatic activity, or ATP content. Cells maintained in a "native" state avoid the disruption in cell-cell communication that can lead to artifactual death by anoikis.

MTT

- The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay method uses the flavoprotein succinate dehydrogenase, an intermediate in the Krebs cycle, to generate a signal. This enzyme removes two hydrogen atoms from the methylene carbon of succinate to form fumarate. Its capacity to

act upon other substrates with the appropriate dicarboxylate structure enables succinate dehydrogenase to convert tetrazolium to the darkly colored pigment formazan, providing a colorimetric measurement of cell viability. The MTT assay has been applied in many tumor types, among the most successful being childhood acute lymphoblastic leukemia [14]. These investigators used the MTT results to distinguish sensitive from resistant ALL cells and applied genomic analyses to explore the molecular basis of drug resistance [15]. Although biologically valid, the assay is limited by its requirement for relatively pure tumor cell populations. Greater concern surrounds its reliance on succinate dehydrogenase itself. The function of this enzyme is recognized in the enzymology literature as a classic example of competitive inhibition. It is well recognized that numerous chemical species can compete at the active site, potentially inhibiting signal. Recently, Chinese investigators revealed that bioflavonoid species could reduce tetrazolium to formazan in an acellular environment [16], with significant impact on the interpretation of results. Therefore, it is essential that MTT analyses be conducted by experienced investigators and that data be interpreted cautiously to avoid confounding variables, particularly in developmental work focused on novel compounds.

Membrane integrity

- Several approaches using membrane integrity to assess cell response to injury have been developed. The long-established use of trypan blue exclusion was modified to a fast green endpoint by Weisenthal *et al*. [17], who introduced glutaraldehyde-fixed avian erythrocytes as an internal standard. The differential staining cytotoxicity assay has an established track record in hematologic and solid tumors. Its capacity to distinguish tumor cells from nontumorous elements morphologically enables this assay to avoid the signal-to-noise quandary that plagues many endpoints. However, it is labor-intensive and more subjective, requiring skilled technologists to score results. Nonetheless, delayed loss-of-membrane integrity proved to be a robust measure of apoptotic phenomena in a comparative study conducted by Australian investigators [18].
- A different membrane-based assay uses cellular retention of fluoroscein to gauge viability in a process known as fluorochromasia. Fluorochromasia reflects the selective retention of fluoroscein by viable cells after the enzymatic cleavage of fluoroscein monoacetate by esterase. Viability is scored by the intensity of fluorescence provided by tumor cells harvested after they have been exposed to drugs. However, the laudable use of membrane integrity is limited in this assay by its inability to discriminate benign from malignant elements. This signal-to-noise pitfall shared by some of the other assay endpoints may have contributed to its failure to gain wide use, despite prior efforts at commercialization.

Cellular ATP content

- Adenosine triphosphate, the primary energy source in all living cells, is principally the product of mitochondrial oxidative phosphorylation. Loss of viability results in the virtual instantaneous dissipation of all intracellular ATP. The measurement of ATP after drug exposure as a surrogate for survival has been accomplished through the use of the enzyme luciferase. This enzyme, found in fireflies, uses ATP to generate light. Thus, the viability of tumor cells can be gauged after drug exposure by measuring the intensity of photon signals with a very sensitive luminometer. This assay again exemplifies a viability endpoint, because it reflects metabolic survival with no

relationship to proliferative state. It has been successfully applied in numerous tumor types, with particular focus on ovarian cancer. However, similar to other endpoints, it is limited by a lack of discrimination between benign and malignant elements in the tissue culture environment. On average, tumor populations must constitute 70% of the sample to provide an interpretable signal not contaminated by the ATP content of benign elements. The ATP endpoint is a good measure of viability and when appropriately applied has correlated well with clinical outcome.

Protein synthesis

- Protein synthesis has been used in the study of human tumor response to chemotherapy. Two basic approaches have been pursued. The SRB assay uses sulforhodamine B, a dye that binds basic amino acid residues following trichloroacetic acid fixation after cytotoxic drug exposure to gauge tumoricidal effect. The assay has proven useful in the preclinical setting and has been the subject of head-to-head comparisons with the MTT assay in cell lines. A second assay method uses the incorporation of tritiated [H_3*] leucine to gauge the effect of drugs on cells in culture. The crucial role of protein synthesis for cell survival renders these approaches appropriate as measures of cellular viability. The requirement for radioisotopes may have diminished the attractiveness of the [H_3*] leucine endpoint over colorimetric measures. However, like the MTT and related endpoints, signal-to-noise ratios in heterogeneous specimens remain a hurdle to accurate clinical prediction for protein synthesis assays. Neither technique has been actively explored in the clinical setting.
- Additional endpoints, including the measurement of radiolabeled CO_2 borrowed from the bacteriology literature [19], have been explored. Although these assays have legitimate biological bases, many lack the capacity to discriminate benign from malignant elements found in human tumor primary cultures, thereby limiting their utility to cell line systems that are comprised of tumor cells exclusively.

Clinical results

- Table 1 provides the accumulated results of 21 clinical trials that compared patient outcomes with the results of cell-death assays conducted on their own tumor tissues in parallel analyses [20–39]. The selected studies reflect several common solid tumor malignancies encountered in clinical practice identified in Medline searches. All studies applied valid cell-death endpoints and documented objective clinical responses (complete or partial) or nonresponses according to established criteria. The studies were not designed as comparators of assay-directed versus standard therapy trials. Instead, they are correlative analyses that, in aggregate, examine the validity of cell death measures as predictors of clinical response.
- Table 2 is a representation of predicted clinical outcomes for patients treated with an assay, with performance characteristics borrowed from the studies described earlier. These have a sensitivity of 78.4% and specificity of 90.1%. The left column provides clinical expectation of response encountered in clinical practice (pre-test expectation). These range from 10% for malignancies such as melanoma or renal cell carcinoma, up to 70% reflective of diseases such as untreated ovarian cancer or breast cancers. The two columns to the right reflect the likelihood of clinical response for patients found sensitive or resistant (post-test expectation). The fold advantage of active drugs versus inactive dugs is provided on the right. As can be seen, the use of assay-active drugs confers superior likelihoods of response for any given pre-test expectation.

Table 1. Published results of cell death assays in common human solid tumor malignancies

Disease	*n*	Overall response rate	Positive predictive accuracy	Negative predictive accuracy	Study
Breast	194	64.9%	82.9%	88.9%	[19–24]
Colon	54	16.6%	80%	97.7%	[23,25,26]
NSCLC	47	29.7%	66.7%	93.1%	[23,27–29]
Gynecologic	345	56.2%	77%	87.9%	[10,19,23,30–37]
Small cell lung cancer	19	26%	50%	84.6%	[19,23,28]
Total	659	50.6%	78.4%	90.1%	—

NSCLC—non–small cell lung cancer.

Table 2. Clinical application of assay-directed therapy*

Literature reported response rate	Response rate for assay (+) patients	Response rate for assay (-) patients	Fold advantage, assay (+) vs assay (-)
10%	46%	2.6%	17.6
30%	77%	9.3%	8.3
50%	88.9%	19.3%	4.6
70%	94.8%	35.8%	2.6

*Performance characteristics calculated from the aggregate results cited in Table 1 [10,19,23–37].

Future directions

- Medical practice resists change. The discovery of *Helicobacter pylori* and description of its role in peptic ulcer disease, viewed as heretical by many in academia, led to the Nobel Prize in medicine 25 years later. Paradigm shifts demand that accepted norms be revised as new explanations for established phenomena come to the fore. We are witness to a paradigm shift in human tumor biology predicated upon the recognition that dysregulated cell death and not alterations in cell proliferation is causative in human malignancy. Although many investigators have embraced this concept, these same investigators have not or will not apply this new understanding to in vitro assays for the study of human cancer. Ex vivo techniques that measure drug-induced cell death are valid surrogates for programmed cell death in vivo. The simple application of these techniques allows patients of average response likelihood to be dichotomized into sensitive and resistant subgroups with significantly different likelihoods of response. The consistent observation that cell death and not cell growth events correlate with clinical response reflects the primacy of cell survival in human carcinogenesis. Although the scientific community aggressively pursues molecular platforms to predict response to chemotherapy, clinicians are forced to apply the blunt instrument of clinical trials to answer questions amenable to ex vivo analyses available today. Recent unsuccessful efforts with an ATP endpoint in ovarian cancer [40] had little to do with the assay's validity. Instead, failure on the part of these investigators to define a comparator arm for power analysis doomed the trial from its inception. Cooperative groups have the opportunity to conduct definitive tests of the assay methods based on the modern concept of programmed cell death, yet they remain wedded to outdated methodologies of cell growth inhibition. In a review of the topic, Cortazar and Johnson [41••] stated, "We believe that the appropriate design for testing a tumor would be one that is resected routinely where the tumor can be cultured consistently." They went on to

suggest ovarian, locally advanced breast, or stage II/III non–small cell lung cancers as candidate diseases. We have the ability to design and implement appropriate clinical tests of these hypotheses. Patients and practitioners should demand that fair trials testing these important methodologies be conducted immediately.

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