Phospho-specific Flow: Fixating on the Target

Mark Levis

In this issue of Clinical Cancer Research, Perl and colleagues (1) detail the successful use of phospho-specific flow cytometry to assess real-time mTOR inhibition by sirolimus in a clinical trial of patients with acute myelogenous leukemia (AML). The technique they describe has the potential to be an effective method of determining target inhibition for any number of agents used to inhibit kinase pathways in clinical cancer trials.

At the center of this report is a clinical trial in which patients with relapsed, refractory, or otherwise high-risk AML were treated with a combination of chemotherapy and a targeted agent, sirolimus. Like many such endeavors in oncology, the trial was based on a substantial body of preclinical data. mTOR is postulated to play a critical role in chemotherapy resistance. Previous work from this group indicated that the combination of mTOR inhibitors with chemotherapy results in synergistic cytotoxicity in AML cells in vitro (2). Therefore, the design of the trial is relatively simple: Administer intensive chemotherapy after loading the patient with sirolimus (rapamycin). Of importance, however, the central hypothesis being tested in this trial is not that sirolimus will be of benefit, but rather that inhibition of mTOR will be of benefit. It is easy enough to measure the plasma drug level of sirolimus (or any other targeted agent), but how do the investigators know that mTOR is actually being inhibited in the malignant cells of the patient receiving the therapy?

Ever since the advent of modern targeted therapies, the need to determine the efficacy of target inhibition in vivo has been apparent. The concept is easy in principle, but daunting from a technical standpoint. Direct biochemical measurement of the targeted protein or cellular function in malignant cells from the patient undergoing the treatment could be put forth as a gold standard, but that standard is rarely, if ever, achieved, particularly in trials of solid tumors. This has necessitated the use of surrogate tissues, such as skin biopsies for epidermal growth factor receptor inhibitors (3). Researchers conducting trials in leukemia patients often have an advantage in this regard because the targeted tissue can be sampled with the relatively minimal invasiveness of a blood draw. For example, in one study (4), inhibition of BCR-ABL by imatinib in patients with chronic myelogenous leukemia (CML) was tracked with dephosphorylation of Crk-L. CML, however, is somewhat unique in that patients with this disease typically have a large tumor burden available for sampling. Other leukemias are less accommodating: AML, for example, can present with a relatively low blast count in the peripheral blood. To make matters more difficult, when a targeted therapy is combined with chemotherapy, the blasts are cleared from the blood quickly, yet maintaining target inhibition in the marrow blasts (the real target) is of paramount importance. In such cases, a surrogate measurement, such as the plasma inhibitory activity assay, may be employed (5). However, a drawback of this approach is that it measures the target inhibition in a surrogate tumor-cell type rather than in the actual malignant cells being treated in the patient.

In what may represent an important new advance in translational oncology, Perl and colleagues (1) have devised a means of tracking target inhibition in just a few thousand cells isolated in real time from patients in a clinical trial. Patients with relapsed or otherwise high-risk AML were loaded with sirolimus and then treated with a conventional salvage chemotherapy regimen (mitoxantrone, etoposide, and cytarabine) while still receiving sirolimus. Whole-blood samples were collected at baseline, day 4, and day 7 of treatment. Within 1 hour of collection, a small volume of formaldehyde was added to the blood to fix the cells (Fig. 1). In formaldehyde fixation, proteins are cross-linked, typically at lysine residues (6). In this case, that means the signaling effects of sirolimus are essentially “frozen” in time immediately after collection. The red blood cells can be removed from the sample by lysis, and then the fixed white blood cells can be analyzed immediately or stored frozen. One can then easily identify blasts by conducting a flow cytometric analysis of the white blood cells using conventional cell surface markers such as CD45 and CD33. The tricky part of this assay is to analyze the intracellular signaling proteins within the gated blasts. Sirolimus inhibits

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the mTORC1 subunit of mTOR, leading to loss of phosphorylation of the ribosomal protein S6 kinase (7). Therefore, this assay is predicated on the investigator’s ability to measure the level of S6 phosphorylation accurately in these fixed cells. Permeabilizing the fixed cells with methanol allows an anti-phospho-S6 antibody to access the interior of the cell. Direct target inhibition within the blasts can thus be quantified in a reproducible fashion.

The advantages of this technique are immediately obvious. This is real-time measurement of target inhibition within the patient’s actual cancer cells. Only a small number of malignant cells are needed, so patients with low blast counts can be included in these studies. The reagents used are common, and flow cytometry is used by any center conducting clinical trials. Moreover, the samples can be collected, fixed, and then shipped to a central laboratory for the phospho-flow, so multicenter trials can easily accommodate this approach for their correlative studies.

This innovative study actually represents a step in a logical progression that began with the development of phospho-specific flow (8, 9), which has thus far been limited to examining fluctuations in a select group of phosphoproteins (10). However, multiparameter flow cytometry, as practiced now, remains constrained by the number of wavelengths available on the machine. The group that spearheaded the development of phospho-specific flow cytometry is now exploring a much more powerful approach to analyze signaling changes in single cells: mass cytometry (11). In this exciting new technique (which seems destined to be applied to a clinical trial setting soon), antibodies to a wide array of signaling proteins are labeled with stable isotopes, each of which represents a unique signature that is detectable by mass spectrometry analysis. The only limiting issue remains the specificity and avidity of the antibodies.

There are, of course, drawbacks. The approach as it stands now is probably only applicable to the various types of leukemia. Cross-linking of proteins by formaldehyde may actually obscure or alter the ability of an antibody to bind to an epitope, potentially introducing a significant artifact into this system. The antibody to the phosphorylated epitope may lack sensitivity or (worse) specificity, rendering the results useless. Certainly, anyone planning to apply this approach to clinical correlative studies will first need to validate it carefully for the target in question before beginning the sample-accrual process.

Nonetheless, these are encouraging results. Phospho-flow analysis of intracellular signaling has been under development for several years now (12), but the work by Perl and colleagues represents one of the first successful applications of the technique to a translational oncology clinical trial. It seems likely that others will soon follow.

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References


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