Tracking Microchimeric DNA in Plasma to Diagnose and Manage Organ Transplant Rejection

One of the most promising areas of transplantation research is the recent discovery of biomarkers for rejection that are detectable in blood and urine. Biopsy-confirmed rejection, the current gold standard for diagnosis of allograft rejection, is invasive and subject to sampling errors. For example, diagnosis of episodes of rejection of pancreas allografts, which are frequent and can destroy the allograft, depends on conventional percutaneous biopsies that have a diagnostic sensitivity of only 79%–88% (1, 2) and are associated with a 2%–7% risk of serious complications (3). Development of noninvasive assays that detect molecular biomarkers for rejection could revolutionize management of transplant recipients by (a) detecting a prerejection profile that will allow therapeutic interventions before rejection causes graft dysfunction; (b) improving the sensitivity and specificity of rejection diagnosis; (c) developing new classification systems for rejection that will improve prognosis; and (d) providing information for designing individualized immunosuppressive regimens that could prevent rejection while minimizing drug toxicity.

There have been several exciting reports of potential biomarkers for allograft rejection, with the most significant progress occurring in the area of renal transplantation. This work began by studying concentrations of particular mRNAs or proteins that were associated with immune activation or tissue stress (4). These studies have revealed several gene products that have altered expression in blood, urine, and/or biopsy tissue during rejection episodes. In the most recent contribution in this rapidly evolving field, Muthukumar et al. (5) demonstrated that urine concentrations of *FOXP3* mRNA, a member of the forkhead family of cell differentiation genes and a lineage-specific transcript for graft-protecting regulatory T cells, can predict reversal of acute renal allograft rejection with 90% sensitivity and 73% specificity. Although measurement of the products of individual genes such as *FOXP3* will probably not supplant conventional biopsies for diagnosis of rejection, development of panels of informative gene products in blood and urine, in concert with renal function and immune response markers, ultimately should achieve the sensitivities and specificities required for diagnosis and clinical management of kidney rejection (6).

Emerging technologies, such as gene expression profiling (7), proteomics (8), metabolomics (9, 10), and genomics (11), are rapidly advancing the pace of discovery of new biomarkers of rejection. One of the seminal studies used expression profiling of renal biopsy tissue to identify more than 1300 genes that were differentially expressed in kidney allografts (12). Analysis of these genes revealed 3 distinct molecular signatures of acute rejection that were more predictive of allograft survival than was traditional histologic analysis. These data have also generated new hypotheses for the molecular mechanisms of rejection,

such as the striking observation that B-cell infiltration is characteristic of aggressive acute rejections. These approaches are expected to generate improved diagnostic tests as well as knowledge that will lead to more effective therapies.

In the March issue of *Clinical Chemistry*, Gadi et al. (13) used another approach that should complement generic biomarkers: detection of extremely low concentrations of donor-derived DNA (microchimerism) in serum. This is one of many recent applications illustrating the diagnostic potential of cell-free DNA in blood, with applications in prenatal diagnosis and detection; monitoring of a variety of autoimmune, inflammatory, and malignant diseases; and in transplantation (14). Lo's group were the first to report detection of Y-chromosome–specific DNA in plasma from women who had received kidney or liver allografts from male donors (15) and in urine of women receiving kidney allografts (15, 16). Gadi et al. (13) took advantage of sensitive, quantitative real-time PCR assays to show that the concentration of donor-derived DNA in a transplant recipient's serum may be a useful biomarker for rejection after simultaneous pancreas and kidney transplantation. Donor-specific HLA alleles were targeted to demonstrate increased donor DNA in serum in all patients immediately after transplantation, with persistent increases (>8 days posttransplant) only in patients with biopsy-confirmed pancreas rejection. Longitudinal sampling for 1 patient showed increased donor DNA in serum during a rejection episode. These investigators hypothesized that graft-derived DNA increases in the serum soon after transplantation as a result of organ damage resulting from cold ischemia and reperfusion injury, whereas increased concentrations later after the transplant are a result of rejection episodes. Integrating the kinetics of donor microchimerism in peripheral blood along with concentrations of other molecular biomarkers offers an exciting opportunity to refine the molecular signatures of allograft injury during the transplantation process as well as during episodes of rejection and organ toxicity after transplant. However, considerable further investigation is required before this goal can be realized.

Although Gadi et al. (13) observed substantial differences in the mean concentrations of donor-derived DNA in sera of patients with and without rejection, the predictive value was poor. This may reflect, in part, variability caused by analytical factors. Donor DNA in the serum may be derived from the allograft as well as from passenger leukocytes. In a study of microchimerism after transfusion, Lee et al. (17) reported that serum samples had concentrations of cell-free DNA that were ~20-fold higher than the concentrations in fresh plasma samples. If clotted blood tubes were stored at 4 °C for 4 to 5 days, the concentrations of cell-free DNA increased 100-fold. These observations suggest that most of the cell-free DNA in

serum samples is generated during the clotting process and that the concentration of cell-free DNA in serum may be influenced considerably by sample-processing conditions. In addition to the possible contribution of DNA from passenger donor peripheral blood mononuclear cells (PBMCs), recipient PBMCs are also lysed during blood clotting, producing high concentrations of recipient DNA in serum, which could impact the sensitivity and/or specificity of detection of low-concentration donor DNA derived from the organ. Further studies are needed, therefore, to evaluate the relative utility of serum vs plasma for detection of chimeric DNA in the setting of organ rejection monitoring as well as for other diagnostic applications targeting cell-free genomic DNA in blood.

Detection of microchimerism by real-time PCR is technically challenging. Rigorous precautions need to be taken, from sample collection through PCR setup, to prevent contamination with minute quantities of allogeneic DNA. This can be particularly difficult in a laboratory environment where amplified DNA from earlier PCR assays is prevalent. DNA preparations and certain lots of reagents may contain inhibitors of the PCR that diminish sensitivity for some assays. Subtle differences in thermal cycling can influence these assays.

Efforts to standardize real-time PCR assays are beginning. For example, Lai et al. (18) recently examined several aspects of assay quality control, including the number of calibrators, the optimum number of replicates for each calibrator, and the frequency of calibration. These investigators demonstrated that the design of calibration curves can contribute considerable variability to real-time PCR assays. Before real-time PCR assays can be used for reliable diagnosis, considerable collaborative effort will be required to standardize the assays and to demonstrate proficiency across multiple laboratories.

The multiple tissue sources of donor DNA can complicate the interpretation of the measured concentrations of donor DNA in recipient serum. Gadi et al. (13) assumed that the donor DNA in serum is the result of organ damage, but long-term donor leukocyte microchimerism can occur from passenger leukocytes in the organ graft (19). These donor-derived leukocytes may contribute appreciable concentrations of donor DNA to the serum in the absence of organ damage. Because donor leukocyte microchimerism has been associated with both tolerance (19) and rejection (20), the presence of donor-derived DNA in serum may not always be deleterious. In addition, assays for donor microchimerism may detect DNA derived from third-party cells derived from blood transfusion, maternal–fetal exchange, or previous allografts (21). Additional sources of microchimerism have been suggested by a study of women without sons who had detectable Y-chromosome DNA in their blood (22), presumably from pregnancies that did not progress to live births. Although the use of HLA alleles increases the specificity for detecting cells from a particular donor, use of common HLA types might fail to differentiate DNA derived from 2 or more individuals. Targeting HLA allelic differences could also bias the capacity to detect microchimerism because these gene products are involved in immunologic mechanisms underlying tolerance and rejection. Recent development of real-time PCR assays for microchimerism that target a battery of insertion and deletion polymorphisms that have no functional relevance could allow sensitive, nonbiased detection of chimerism for this and other applications (23).

After some of these barriers have been overcome, it is likely that measurement of donor-specific DNA along with other biomarkers will substantially advance diagnostic capabilities for all solid-organ transplant recipients. The importance and enthusiasm for this approach was recently reviewed in a research report from the American Society of Nephrology (24), which made the discovery and standardization of biomarkers for prediction, diagnosis, and prognosis of rejection the highest research priority. Their recommendations for advancing the field include creating banks of samples with associated clinical data and developing a network of laboratories for assay standardization. Given the wealth of information that could be derived from preserved samples in the near future, every clinical trial should preserve samples that could be used to study genetic polymorphisms and molecular biomarkers under a variety of conditions, including different immunosuppressive regimens.

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