Diagnostic Tools for Monitoring Kidney Transplant Recipients

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Summary: Recent advancements in immunobiology have introduced several new diagnostic tools for monitoring kidney transplant recipients. These have been added to more established tests that, although imperfect, remain important benchmarks of diagnostic utility. Both new and old tests can be characterized with regard to their practicality, and as to whether they detect aberrant function or define the cause of dysfunction. Unfortunately, no current test is both practical and specific to a particular disease entity. Accordingly, the diagnosis of graft dysfunction remains dependent on the proper use and interpretation of many studies. This article reviews the current assays that have been evaluated in the clinic for the diagnosis of renal allograft–related diseases. These are limited to assays based on routinely obtainable samples such as blood, biopsy tissue, and urine. Newer studies are presented, along with more mundane assays, to highlight the practical use of studies regardless of their degree of mechanistic sophistication.
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Renal allograft function is determined by the combined effects of many factors. Systemic disorders such as cardiopulmonary and vascular disease, dehydration, sepsis, and malignancy converge with alloimmunity, drug toxicity, primary renal diseases, urogenital infections, and technical complications to manifest a patient's composite renal health. Unfortunately, similar phenotypes of renal dysfunction can result from widely disparate causes, making it difficult to intervene with therapeutic precision, and, importantly, all of these conditions are dynamic. Environmental exposures to infectious pathogens modulate heterologous alloimmunity, continuously challenging and modifying the immune response. Immunosuppressive drug absorption and compliance vary with time. Indolent conditions become apparent with time mimicking more acute processes. The clinician's job thus demands timely systemic appraisal of multiple physiologic and pathologic conditions. Accordingly, an array of biologically precise tools is required for the ideal care of an allograft recipient.

Standard monitoring methods include clinical assessment, serum and urine parameters of renal function, immunosuppressive drug levels, and histopathologic evaluation of the renal allograft biopsy. All have limited specificity, but when combined with clinical acumen yield reasonable diagnostic precision. In recent years, these tools have been supplemented by an increasing number of biologically based assays with promise for disease-specific readouts. As these diagnostic methods become more specific to the biologic processes mediating graft dysfunction, they should guide more appropriate therapies and ideally identify key mediators of allograft dysfunction before irreversible graft damage occurs. In addition, these methods may contribute to a better characterization of the mechanisms of allograft dysfunction and rejection. This article reviews new diagnostic tools

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that add mechanistic, causative, and/or prognostic information to the data obtained from current established tests. The focus is on assays that have been evaluated in clinical trials.

PRACTICAL CONSIDERATIONS: WHERE AND WHY TO LOOK?

The complete assessment of a renal allograft recipient involves a systematic appraisal of the patient. Indeed, the primary causes of alloimmunity involve many sequestered organs including the thymus, bone marrow, secondary lymphoid organs, and lymphatics. However, in clinical practice, one is limited by practicality to only 3 sites for routine diagnostic study: the peripheral blood, the allograft, and the urine; and all of these have relative advantages and disadvantages.

The peripheral blood is easily accessible, allowing for serial monitoring. However, the specificity of tests on the peripheral blood suffers from the fact that its serum reflects pathology throughout the body, not just the allograft, and its cells typically are those that are not mediating disease, rather cells that are in transit, or those that have not been drawn as effectors to a site of inflammation. The urine also is easily accessible and through its origin likely reflects modifications occurring in the allograft more directly than the peripheral blood. However, even in the urine systemic interference cannot be excluded, and in severe cases of dysfunction, urine is not available. The allograft biopsy, on the other hand, provides highly specific information, but is invasive and not suitable for frequent serial monitoring.

In assessing novel assays, distinction should be made between tests with diagnostic value, those that provide mechanistic insight, and those that serve both these roles. Similarly, assays of dysfunction should be discriminated from those that address etiology. In recent years an increasing number of sensitive tests have been described suggesting diagnostic importance, but being more appropriately characterized as mechanistic. For example, tests correlating with the concurrent presence of rejection that is clinically overt may not aid in diagnosis, but rather may be important adjuncts adding causative or prognostic information. Thus, when surveying new analytic methods, one should recognize that correlation and prediction are valuable but substantially distinct entities.

Table 1. Peripheral Blood Assays That Have Been Studied in Clinical Transplantation

Functional parameters
Protein markers of inflammation
Gene transcripts
Lymphocyte frequency and activation
Alloantibodies
Immunosuppressive drug levels and pharmacogenomics
Endogenous viral replication

Peripheral Blood Assays

Several parameters can be measured in the peripheral blood (Table 1). All of these are limited by their peripheral relationship to the graft and other sites of allosensitization, but have the advantage of accessibility.

Functional Parameters

Measurements of renal function indicate effect rather than cause. As such, their strength is in detecting pathology that clearly has reached clinical significance. However, they are not able to completely define pathologic processes.

Serum creatinine is the most commonly used functional parameter because it is inexpensive and universally available. It is well known that multiple factors impact on the accuracy of serum creatinine as an indicator of the glomerular filtration rate (GFR), including sex, age, muscle mass, and dietary protein intake. In addition, analytic factors such as blood glucose concentration, bilirubin level, and drugs interfere with its measurement. Nonetheless, serum creatinine is recommended as a screening test for changes in allograft function in renal transplant recipients.[1] Although mundane, it has definitive utility that more sophisticated tests often lack, and it remains a standard against which other tests need to be compared for true diagnostic utility.

Importantly, creatinine is best used as a dynamic rather than static measurement. Incremental increases in serum creatinine level during the first year posttransplant are associated with a progressive decline of graft half-life. A change in serum creatinine between 6 and 12 months greater than 0.5 mg/dL relates to a relative hazard of 2.26 for graft failure.[2] Similarly, a decrease of 30% or more of the reciprocal serum creatinine over the first 3 years posttransplant is predictive of late graft failure.[3] Even changes within the first 48 hours of implantation accurately assess early graft function[4] and correlate with allograft function at 1 year, independent of acute rejection.[5] Conversely, single-point cut-off values of serum creatinine, rather than serial monitoring, fail to predict graft loss.[6]

Several functional parameters are derived from the serum creatinine in an attempt to standardize for muscle mass and other confounding variables.[7] Generally, correlation between formulas for calculated GFR and radionuclide clearance in kidney transplant recipients varies considerably. Most formulas tend to overestimate the GFR, with a mean error between 3% and 30%.[8-10]

Given the variations in calculated and measured GFR with creatinine-based assays, other markers have been pursued. Serum cystatin-C is a 13-kd cysteine protease inhibitor present in nucleated cells that is filtered freely, almost completely reabsorbed, and metabolized by the proximal tubule with no tubular secretion.[11] Cystatin-C serum levels are independent of sex and muscle mass,[12] but remain variable with age.[13] The accuracy of cystatin-C–based calculation of GFR is comparable with creatinine clearance measured by ^{125}I-iothalamate[14] and, in other studies, higher than creatinine-based equations[15,16] or the Modification of Diet in Renal Disease formula.[17] Of note, however, methylprednisolone increases, and cyclosporine decreases, serum levels of cystatin C.[18,19]

Protein Markers of Inflammation

Allograft rejection increasingly is being recognized as a systemic inflammatory response involving secondary lymphoid tissue, cell trafficking, and chemotaxis, as well as local events initiated at the time of reperfusion and propagated by effector cell infiltration. Thus, many molecules including chemotactic factors, markers of cell injury, and effector molecules have been investigated as potential correlates for allograft rejection (Table 2). Not unexpectedly, many studies have reported on different levels of inflammatory markers between rejectors and nonrejectors.[20-26] However, in general, these factors have suffered from an inability to distinguish allograft rejection from other physiologic inflammatory states.

Table 2. Markers of Inflammation That Have Been Studied in Clinical Transplantation

β-2microglobulin
C-reactive protein, serum-amyloid protein
TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-15, IL-18, soluble CD30, soluble CD40L
Perforin, granzyme B, FasL
Aminoterminal propeptide procollagen III
Neopterin

Despite low specificity, some markers have been shown to be useful in combination with other diagnostic studies. Most common among these is serum β-2 microglobulin. β-2 microglobulin is a non–covalently bound portion of the major histocompatibility complex (MHC) class I molecule that is shed during cell division and death. It increases during states of high cell turnover, such as infection and malignancy. Importantly, it also is excreted, reabsorbed, and processed by the renal tubules, and as such serves as a marker of tubular damage and general inflammation.[27-29] A significantly higher serum β-2 microglobulin level has been reported in patients with irreversible rejection compared with reversible rejection, and in patients with a normal serum creatinine level as a marker of viral infection.[30] In addition, the simultaneous levels of serum and urine β-2 microglobulin may differentiate rejection (increase in serum β-2 microglobulin and only modest increase in the urine) from drug toxicity (increased urine levels and decreased serum levels).[31] More recent studies using proteomic technology (see later) report on urine β-2 microglobulin and its cleavage forms as noninvasive markers of subtle

degrees of acute tubular injury not reflected by serum creatinine.[29,32]

Other soluble MHC-related molecules are increased in the serum after immune activation.[33] CD30 is a transmembrane glycoprotein of T cells and a member of the tumor necrosis factor (TNF)-receptor superfamily,[34] originally described (Ki-1) in Hodgkin's disease[35] and subsequently identified as a marker for a subset of interferon (IFN)-γ and interleukin (IL)-5–producing T cells.[36] Soluble CD30 is released into the circulation by activated T cells and therefore can be a useful noninvasive parameter of immune activation. Indeed, pretransplant[37-39] and posttransplant,[40-42] increased soluble CD30 levels have been associated with an increased risk of rejection and graft loss.

Perforin, a pore-forming protein, and granzyme B, a serine protease, are released by degranulation from cytotoxic T cells and cause DNA fragmentation and cell death after activation of caspase 3; Fas ligand (FasL) is a transmembrane protein of the TNF family mediating apoptosis of target cells. These molecules are recognized effectors of T-cell–mediated allograft rejection, as documented by several studies on allograft biopsy and urine (see later). In the peripheral blood, one study reported increased levels of granzyme B and perforin during allograft rejection.[43] They have been more extensively studied transcriptionally (see later).

The inflammatory response associated with brain death has been recognized increasingly as significant and interrogated by diagnostic tests. Higher levels of serum neopterin, a protein released by activated macrophages[44] and considered a marker of cellular immune activation, and lower levels of serum IL-1–receptor antagonist have been reported during the first 10 days after deceased donor kidney transplantation compared with living donor.[45] These assays have been suggested to indicate pretransplant immune activation in the donor[46,47] and have potential prognostic significance in the recipient.[48]

Gene Transcripts

Gene transcripts (messenger RNA) of relevant cytokines including IL-2, IL-4, IL-5, IL-6, IL-10, IL-15, IFN-γ, and effector molecules such as perforin, granzyme B, and FasL have been investigated as diagnostic markers in the peripheral blood in several studies.[43,45,49-53] Peripheral blood messenger RNA analysis is dependent on cellular presence because extracellular RNA is degraded rapidly. Thus, peripheral blood transcriptional studies are a reflection of cell traffic more than engaged effectors. In addition, their usefulness in lymphocyte-depleted patients potentially is limited. As with most peripheral blood studies, transcriptional markers can be expected to have a specificity that is impaired by concomitant infection or systemic inflammatory events unrelated to the graft.

Although many gene transcripts clearly are increased during allograft rejection, this is a transient phenomenon and nonspecific to the allograft: other causes of systemic inflammatory response, similar to viral infections,[54,55] produce increased expression of proinflammatory molecules. In related studies, polymorphism of genes regulating cytokine production in the donor[56] and in the recipient[57,58] have been shown to be associated with the risk for rejection. However, these associations are weak and are not useful for acute diagnosis.

Lymphocyte Frequency and Activation

Activated allospecific T cells play a key role in allograft rejection, and their frequency has long been recognized as a determinant of rejection risk.[59-61] Several methods have been developed to estimate a patient's allospecific T-cell precursor frequency. Hirschhorn et al[62] originally introduced the mixed lymphocyte reaction as a method to assess the compatibility between 2 individuals: when lymphocytes from the peripheral blood of 2 unrelated individuals were cultured together, they "were stimulated to enlarge and divide." This since has been shown to be largely a factor of MHC class II mismatch, and thus it is not a direct measure of pan-alloreactivity. It also measures proliferation, not effector function. Accordingly, the mixed lymphocyte reaction has not been shown to be clinically useful in predicting rejection in the era of modern immunosuppression. Other assays for estimating allospecific precursor frequency such as limiting dilution analysis[63,64] or

alloantigen-stimulated cloning have been technically impractical or difficult to reproduce.

Flow cytometry with labeled MHC tetramers has emerged recently as an accurate and quantitative method for detecting T cells with a T-cell receptor (TCR) that binds to a specific MHC molecule.[65] The phenotypic characterization of low-frequency T-cell populations is possible with this assay, allowing the identification of antigen-specific T cells with a frequency of less than 1:100,000.[66] However, it remains limited by an incompletely representative array of MHC tetramers such that pan evaluation of outbred mismatches is difficult. Even so, MHC binding does not assess the functionality of T cells that bind to the tetramer, and effectors will appear the same as cells with potential regulatory function.

Elispot is a recent derivative of enzyme-linked immunosorbent assay used to detect cytokine-producing T cells at single-cell resolution.[61,67-69] By using Elispot, IFN-γ production has been recognized as a potential marker of T cells primed to prior antigen exposure (infection, blood transfusion, pregnancy). Although in its infancy as a technique, Elispot does have the advantage of being able to detect varying response after antigen engagement such that, for example, T_H-1 cells could be differentiated from T_H-2 cells, and nonproliferative effectors could be evaluated. The frequency of IFN-γ–producing T cells, used as a marker of alloreactivity, has been shown to correlate with the risk of allograft rejection in small pilot studies.[68,70,71] However, this test is likely to be useful in assessing risk, but not in making acute diagnoses.

The function of immune cells also can be assessed by measuring increases in intracellular adenosine triphosphate in T cells after activation (Cylex Immune Cell Function Assay, Cylex Inc., Columbia, MD).[72,73] A low adenosine triphosphate concentration (<225 ng/mL) in a blood sample after lysis of CD4+ T lymphocytes is interpreted as low energy production by CD4+ cells and therefore as low immune response, compared with moderate (226-524 ng/mL) and strong immune responses (>525 ng/mL). This assay may be useful in assessing an overall degree of composite immunosuppression that correlates with risk of rejection, and, as such, could be useful in avoiding overimmunosuppression. As an acute diagnostic tool, however, this study, at best, will be supportive in assessing the cause of acute renal dysfunction.

Alloantibodies

Donor-specific alloantibodies (DSAs) have long been recognized to cause graft injury including hyperacute rejection and early graft loss.[74,75] More recently, the de novo production of DSAs posttransplant has been reported in association with steroid-resistant acute rejection early posttransplant (<3 mo),[76] with an increased risk of graft loss at 6 months,[77] and a doubling of the risk of late rejection.[78] Antibody-mediated rejection increasingly is being recognized as a significant and distinct process.[77,79,80] The Banff 1997 classification of renal allograft rejection has been updated to include "antibody-mediated allograft rejection" in addition to other categories of rejection.[81] Thus, alloantibody presence is a clear sign of donor-directed immunity that carries important prognostic weight.

Non–human leukocyte antigen (HLA) antibodies (MHC I chain-related antigen), expressed on epithelial, endothelial, and monocytic cells,[82] also have been reported recently in patients with graft failure and absent HLA antibodies.[83-86] Several techniques have been developed that have made the detection of DSAs rapid, quantitative, and specific to individual antigens. In addition to the established complement-dependent lymphocytotoxic methods and panel reactive antibody assays, solid-phase MHC-labeled bead arrays such as the Luminex assay (Luminex Corporation, Austin, TX) now permit daily quantitative analysis of individual allospecificities. In one recent study, DSAs were detected pretransplant by Luminex in 36% of sensitized patients and correlated with delayed graft function and lower 6-month graft survival.[87] The detection of low-level DSAs in nonsensitized patients may represent an additional tool for risk stratification and immunosuppression management. In general, the literature is growing in support of DSA assessment as a sign of progressive graft injury warranting clinical action.

Immunosuppression Monitoring and Pharmacogenomics

Current immunosuppressive drugs have a narrow, and at times nonexistent, therapeutic index. The avoidance of overimmunosuppression and underimmunosuppression is a major challenge in the clinic, and the degree of immunosuppression can be used as supportive evidence in assessing the differential diagnosis of graft dysfunction. Given the absence of a test for adequate immunosuppression, patients are monitored routinely for signs of drug toxicity using serum drug levels. However, the correlation between the measured drug level and the actual drug exposure is imprecise as a result of factors related to assay variability, interactions with food and concomitant medications, and the degree to which the drug is concentrated in blood cells versus being free in the serum. The pharmacologic effect of a drug also is modified by the presence of circulating active but often undetected drug metabolites,[88] free or sequestered intracellularly.[89] In general, extreme drug levels are supportive but not definitive in the diagnostic process. Importantly, there is no test to assess the biological activity of the drugs used in transplantation and dosing remains rather empiric.

Increasing attention has been devoted in recent years to pharmacogenomics as a means of objectifying therapeutic drug management (reviewed by Thervet et al[90] and Fredericks et al[91]). Based on the genetic profile of a relevant drug-metabolizing enzyme pattern, patients may be classified as high or low metabolizers relative to a specific drug. Cyclosporine and tacrolimus, similar to many other drugs, are metabolized by enzymes of the cytochrome P450 family (CYP).[92,93] Gene polymorphism for these enzymes is found in the vast majority of the population, whereas the frequency of those who are homozygous for the wild-type allele is very low.[94,95] Among others, 2 predominant CYP isoenzymes are active mainly in the liver (CYP3A4) and in the intestines and kidney (CYP3A5).[96,97] Expressers of CYP3A5 show lower tacrolimus levels early posttransplant and delayed achievement of target tacrolimus levels compared with nonexpressers. They also tend to reject earlier than nonexpressers.[98]

P-glycoprotein is the product of the multidrug resistance gene (MDR1) and is expressed as a transporter on the surface of epithelial cells in the intestine, kidney, adrenal gland, biliary tract, and pancreas.[99] A reduced expression of this transporter limits the cellular uptake of drugs (reviewed by Zhang and Benet[97]). Recent studies have reported on the different impacts of gene polymorphism for CYP3A5 and MDR1 on calcineurin inhibitor levels in kidney transplant recipients.[94,100] In 1 study glucocorticoids increased the expression and activity of CYP3A enzymes, at least in the homozygous mutant. Sirolimus did not appear to be as affected by CYP3EA5 and MDR1 polymorphism as did the calcineurin inhibitors.[95] From these studies it seems likely that pharmacogenomics will offer new tools to aid in therapeutic drug monitoring and individualized immunosuppression.

Endogenous Virus Replication

The ability to monitor the endogenous replication of virus such as the Epstein-Barr virus (EBV), cytomegalovirus, polyoma/BK virus, hepatitis C virus, and others has greatly improved in recent years. Indeed, viral copy number now can be determined rapidly in peripheral blood via polymerase chain reaction (PCR)-based techniques and this may be a novel approach to evaluate the aggregate degree of immunosuppression. This approach is based on the assumption that the immune system allows the replication of viruses proportionate with its degree of aggregate dysfunction and that the effector mechanisms controlling viral replication are similar to those mediating allograft rejection. This has the advantage of detecting pathogenic processes before clinical disease while at the same time identifying overimmunosuppression regardless of its cause. In its most blunt use, EBV levels have been used to assess response to immunosuppressive withdrawal during EBV-driven posttransplant lymphoproliferative disorders. In a recent pilot study, EBV levels were increased in most transplant recipients and the degree of increase was associated significantly with the proximity to induction therapy, the presence of biopsy-proven acute allograft rejection, and success in empiric drug withdrawal.[101] If confirmed by larger studies,

Table 3. Assays on Kidney Allograft Tissue and Imaging Techniques

Histology (Banff[81])
Immunohistochemistry
PCR, reverse-transcription PCR
Microarray
Arterial resistive index
Infrared imaging
Positron emission tomography, magnetic resonance imaging

the monitoring of viral replication may provide an additional tool for determining the appropriate level of posttransplant immunosuppression.

Assays on Kidney Tissue

Histology

The kidney allograft is arguably the most relevant place to evaluate the cause of allograft dysfunction, particularly in assessing the presence or absence of rejection. As such, it is a rich target for diagnostic tools (Table 3), and the allograft biopsy is the gold standard procedure for the diagnosis of rejection. Although limited by sampling error, and associated with a low risk of complications ranging from pain, hematuria, arteriovenous fistula, and, rarely, graft loss, the allograft biopsy provides essential standardized information for the management of transplant recipients.[81,102] Nevertheless, discrepancy between pathologic findings and the clinical scenario frequently is observed in the clinic. In particular, in the absence of histologic rejection, the biopsy is not infrequently unable to discern the cause of allograft dysfunction. Conversely, biopsy specimens of patients with preserved graft function may show various degrees of inflammatory infiltrate whose functional and prognostic significance remains unclear.[103] Although immunohistochemical analysis for cell phenotype or complement activation is standard in many centers and has proven value,[25,104-106] the allograft histology has to date been predominantly morphologic and not functional. This is changing and many mechanistically relevant assays are now available to supplement the histologic diagnosis.

The timing of allograft biopsy increasingly is recognized as a key factor in the management of kidney transplant recipients. Several studies have documented the role of protocol biopsy, compared with biopsy performed to investigate the cause of graft dysfunction, in detecting subclinical rejection.[107-109] A recent large study documented the development of fibrosis and atrophy in the majority of kidney transplant recipients within the first 2 years and emphasized the need for an early identification of risk factors and markers of fibrosis to improve the long-term outcomes.[110]

PCR–Based Assays

The reverse-transcription PCR is used commonly to characterize the gene transcript levels and has been studied extensively using renal allograft biopsies. Numerous studies have reported intragraft transcript profiles associated with rejection, response to treatment, or progression to chronic damage. Among the transcripts associated with acute rejection are IL-2,[111] IFN-γ, IL-6 and IL-8,[112] IL-2 and INF-γ,[113] Granzyme B, perforin, and FasL,[114-116] the chemokines IP-10, RANTES, MIP-1α, MIP-1β, lymphotactin, and their receptors CCR2 and CCR5,[117] adhesion molecules ICAM-1,[118] ICAM-1 and VCAM-1,[119] and profibrotic growth factors transforming growth factor (TGF)β-1,[120,121] TGF-β, thrombospondin, and fibronectin.[122] All of these clearly fit into the current understanding of alloimmune biology, but in general these studies have shown that PCR can confirm a biopsy result.[123] What is of more importance is its use to expand beyond information already gleaned from histologic examination.

The introduction of real-time quantitative PCR has increased the accuracy of standard PCR and improved its speed, reliability, and diagnostic prognostic value. Indeed, transcript profiles have been developed that markedly differentiate recently reperfused allografts and stable transplants from normal kidneys despite minimal histologic differences: gene expression profiles on postreperfusion biopsies showed up-regulation of inflammatory cytokines (IL-6, IL-10, and TNF-α), mediators of tissue injury and migration factors (endothelin-converting

enzyme, renin, nuclear factor κ B, IL-8, M-CSF, and G-CSF), whereas stable allografts with normal function 1 month posttransplant showed increased T-cell surface markers and costimulatory molecules (CD3, HLA-DR, CD 86, and CD154).[124]

Avihingsanon et al[125] recently reported the role of early immune activation (TNF-α, TGF-β, CD25 expression within 15 minutes of reperfusion) as predictive of delayed graft function and acute rejection, and decreased expression of antiapoptotic Bcl-XI as a predictor of poor graft function at 6 months. Increased expression of genes of epithelial-mesenchymal transformation and of fibrogenesis also recently were expressed differentially before the development of histologic graft fibrosis.[126] Similarly, recent studies have shown that quantitative differences in T_H-1–related transcripts such as the transcription factor T-bet can distinguish between subclinical rejection and rejection leading to clinical dysfunction.[127,128] Interestingly, the transcript profile in BK nephropathy recently was shown to closely mimic that of acute rejection, indicating that viral-specific transcripts will be required to differentiate viral-driven inflammation from allo-specific inflammation.[129]

These studies clearly have shown that large amounts of clinically relevant data can be derived from the allograft biopsy in the same amount of time as it takes to process standard histology. Advances in microfluidic technology now make routine analysis of hundreds of genes possible using a single consumable.[127] Real-time PCR thus stands poised to be used as a supplement to graft histology in routine diagnosis and awaits the appropriate validating trials.

Microarrays

Microarray technology has developed extensively in the past 5 years and has expanded transcriptional analysis from hundreds of genes to thousands within a relevant time course (reviewed by Lockhart and Winzeler,[130] Mansfield and Sarwal,[131] Hyatt et al,[132] and Sarwal[133]). This technique is based on the hybridization of the complementary DNA from a tissue sample to a high-density array of oligonucleotides representing several thousands of known or undefined human genes with the intent of identifying differential gene expression in comparison with a known standard. Because of the large number of genes tested simultaneously, this method typically is considered hypothesis-generating rather than hypothesis-driven. Nevertheless, this technique is superb for identifying genes to be used subsequently on PCR low-density microfluidic arrays.

An increasing number of reports have been published on microarray analysis in allograft rejection. Akalin et al[134] identified up-regulation of 11 genes and down-regulation of 2 genes (of >7,000 genes) in patients with rejection. Sarwal et al[135] identified different molecular profiles associated with 3 subgroups of rejectors among 67 pediatric kidney transplant recipients with similar histologic findings. In particular, markers for B cells (CD20, CD74, immunoglobulin heavy and light chains) were associated with steroid-resistance rejection and graft failure. This later finding has been validated by immunohistochemical detection of B cells in rejections with poor prognoses.[136] The pattern of combined expression of 8 genes among all the detected genes by microarray on biopsy at 6 months posttransplant was reported by Scherer et al[137] to correlate with the subsequent development of chronic allograft nephropathy (CAN) at 12 months.

Other studies reported on microarray profiles associated with CAN.[138,139] Microarray on peripheral blood, in addition to allograft biopsy, documented distinct patterns of gene up-regulation between rejectors, nonrejectors, and patients with normal graft function.[140] More importantly, this study documented up-regulation of genes of the inflammatory and immune response in patients with preserved graft function, indicating that normal graft function does not imply inflammatory and immune quiescence. In addition, the list of up-regulated genes in graft injury/dysfunction was longer than the list of genes of acute rejection, suggesting that rejection is not the exclusive process involved in graft dysfunction. This and other studies highlight the redundancy of the alloimmune response and the molecular heterogeneity of allograft rejection. Serial genomic

monitoring of peripheral blood and allograft biopsy may provide useful tools for a more accurate assessment and management of allograft dysfunction.

Allograft Imaging

Despite the exceptional sophistication of biopsy-based assays, they remain limited by their invasiveness and sampling error. Whole-organ analysis clearly is ideal, and several whole-organ imaging techniques exist that are growing in their ability to derive clinically relevant information. Among imaging techniques, the calculation of the renal segmental arterial resistance index (the percentage reduction of the end-diastolic flow compared with the systolic flow) during follow-up Doppler ultrasound provides a measure of the vascular resistance within the allograft. A resistance index of 0.80 or higher has been shown to be predictive of subsequent allograft failure.[141]

Infrared imaging has been applied to assess the intraoperative perfusion of the graft in real time and it was found to correlate directly with ischemic time and posttransplant allograft function.[142] Although not applicable as a routine diagnostic tool, it may be useful in the research setting, targeted at quantifying the ischemic injury to the graft.[143] Other imaging techniques, including magnetic resonance imaging and positron emission tomography scan (reviewed by Szabo et al[144] and Grenier et al[145]), offer an increasingly accurate assessment of both morphology and function of the renal parenchyma and may provide additional information on the state of the kidney allograft in selected patients.

Urine-Based Assays

Urine represents a whole-kidney approach to renal assessment. Although potentially limiting in situations of severe dysfunction, it typically is available and generally is expendable (Table 4). Monitoring the urine is not a novel strategy in transplantation. Studies from 2 decades ago documented increased levels of adenosine-deaminase binding protein, a glycoprotein on the brush border of proximal tubule cells, as a marker of tubular damage in patients with acute rejection, calcineurin inhibitor–related toxicity, and chronic damage.[146,147] Urine assays are limited to some extent by native kidney excretion and thus may vary based on the degree and cause of native renal impairment. Moreover, these measures of tubular injury are not specific for immunologically mediated injury and may represent injury from other causes including ischemic damage and calcineurin inhibitor therapy. Because urine concentration varies dramatically in health and disease, all urine tests require standardization of collection and storage techniques. Nevertheless, a reliable urine-based assay is a much-sought-after commodity to markedly improve the ability for patients to undergo serial noninvasive monitoring.

Table 4. Assays on Urine

Creatinine clearance
Proteinuria
Cytology
BK polyoma virus markers
Markers of immune activation
Proteomics

The calculation of creatinine clearance and the measurement of total creatinine excretion are standard functional parameters evaluated on 24-hour urine collection as in nontransplant recipients and already have been discussed previously.[1] In addition, urine collection to assess proteinuria also is important as an indicator of allograft dysfunction associated with increased patient mortality.[148] Between 15% and 25% of recipients will have significant proteinuria of 0.5 to 1.0 g excreted per day, persisting for at least 3 months.[1,148] Proteinuria can be screened simply by urine dipstick testing, which is influenced not only by the amount of protein excreted but also by the urine concentration. Thus, false-negative results may occur in patients with large urine volumes. A more accurate method to assess the extent of proteinuria is the determination of the protein/creatinine ratio, and values greater than 200 mg protein/g creatinine are considered abnormal.[149] In such cases, further quantitation with 24-hour urine collection is appropriate. Individuals with native kidney function should have a baseline value obtained before transplantation, particu-

larly in the setting of pre-existing glomerular disease, although native proteinuria often decreases after transplantation.[150] The presence of proteinuria can signal pathologic changes including recurrent or de novo glomerular disease, calcineurin inhibitor toxicity, or tubular atrophy/interstitial fibrosis.[151] Early detection should trigger biopsy evaluation and may suggest ongoing alloantibody-mediated injury.[152] A regular interval screening every 3 to 6 months after transplant has been suggested.[1]

Cytology

Urine cytology is an established adjunctive test in the evaluation of any renal disease. Just as histology has been supplemented by immunohistochemistry, so too has cytology been aided by urine flow cytometry. Increased markers of immune activation in the urine (HLA-DR, CD3, CD14, CD54, and IL-2R) have been reported to differentiate allograft rejection from stable function[153] and other causes of graft dysfunction[154,155] and to characterize resistance to antirejection treatment.[156]

The presence of BK virus in the urine and in biopsy specimens of kidney transplant recipients (reviewed by Randhawa and Brennan[157]) has been investigated increasingly over the past decade as a cause of graft dysfunction. The cytopathic changes, inflammatory infiltrate, tubular atrophy, interstitial fibrosis, and gene expression profiles associated with BK infection overlaps with those of cellular rejection. The ability to differentiate viral-specific from allospecific inflammation remains a challenge.

Inflammatory Proteins

As in the peripheral blood, several studies have reported on urinary cytokine, chemokine, and other parameters as markers of allograft ischemic injury,[158] allograft rejection,[24,25,159] and CAN.[160] The major limitation of these assays is their low specificity.[161] To date, no test has been evaluated in a setting that includes a broad array of inflammatory and noninflammatory diseases combined with biopsy analysis to detect nonovert disease.

The combination of urine with blood or biopsy tests may increase the accuracy of urine markers. Correspondence between urinary monocyte chemotactic peptide-1 (MCP-1) excretion and MCP-1 gene expression on biopsy has been reported in patients with rejection, followed by decrease in urine MCP-1 after treatment of rejection.[162]

Other studies monitored urine and serum concentrations of macrophage migration inhibitory factor as an early marker of rejection and as a discriminator from cyclosporine nephrotoxicity.[163] In a recent study, neutrophil gelatinase–associated lipocalin (a part of a heterodimer of gelatinase present in granules of activated neutrophils) has been reported as an early marker of acute kidney allograft ischemic injury in a urine sample collected less than 24 hours posttransplant, allowing for a timely (4 h) and accurate (sensitivity, 90%; specificity, 83%) prediction of delayed graft function.[164]

Urinary Transcript Assessment

Methods are now emerging to procure nondegraded RNA from urine samples and transcript-based assays are being expanded to urinalysis. Many transcripts recognized as relevant in biopsy studies also have been found to be potentially useful in urine. Increased levels of messenger RNA encoding for perforin, granzyme B,[165] granulysin, CD3 and RANTES,[166] sICAM-1,[167] IP-10,[168] and FOXP3 (a transcription factor specific for Treg)[169] have been reported in recent studies to correlate with overt clinical rejection. Furthermore, FOXP3 has been suggested to provide prognostic value in the ease of reversing a rejection.

Other studies have investigated adhesion molecules sICAM-1 and sVCAM-1, and sC4d as markers of steroid-resistant rejection,[170,171] cleaved β-2microglobulin[32] and retinol-binding protein[172] as markers of tubular injury and fibrosis,[173] and α1-microglobulin and TGF-β1[174] as predictors of CAN.

These studies require strict attention to standardization. Specifically, increased transcript number also can be seen with increased cell number and be indicative of lymphocyturia or oliguria, merely concentrating the cells in the urine. Studies now require that these assays be applied in diverse clinical settings, correlated with biopsy analysis, evaluated in subclinical and preclinical disease, and compared with

more mundane but established methods of urinalysis.

Urine Proteomics

The application of proteomics to urine samples of transplant recipients is an attractive option, likely to significantly expand the pool and the accuracy of current diagnostic tests (reviewed by O'Riordan et al[175]). The development of a protein chip is based on the same principle of microarray for DNA: known antibodies on a chip bind and detect proteins with specific chemicophysical characteristics. However, the urine is a complex biological fluid with a large number of proteins in a wide range of concentrations. Because abundant proteins can obscure the detection of the rare, it is necessary to reduce the complexity of the proteome in the sample to increase the detectability of low-abundance proteins thought to be implicated in a specific disease process. In addition, the identification of a protein associated with a disease process is only the first step toward the determination of its pathogenetic and prognostic significance.

A recent study reported on the high accuracy of urine proteomic profile in differentiating early posttransplant graft-versus-host disease and sepsis after bone marrow transplantation.[176] Three recent studies have reported on urine proteomic profiles obtained by mass spectrometry in kidney transplant recipients.[32,177,178] All 3 studies identified a distinct urine profile associated with acute rejection, although different in each study. The functional significance of the proteins identified in these profiles remains to be determined.

FUTURE DIAGNOSTIC TOOLS

The rapid progress in immunobiology and biotechnology continues to expand the number of promising diagnostic tools. A selected and incomplete list of candidates includes protein chip technology,[179] characterization of small (<1,000 d) organic molecules (metabolomics) in urine,[133] tissue arrays for the combined monitoring of genomics, proteomics, and metabolomics in a sample of tissue, and dynamic imaging of the immune response by direct visualization of cell interactions using in situ fluorescent-labeled light microscopy.[180] All of these assays have great appeal for the comprehensiveness and mechanistic sophistication. However, to date, they have not been applied in clinical transplantation.

CONCLUSIONS

A single test enabling a timely and accurate diagnosis of graft dysfunction in kidney transplant recipients still is lacking, and given the diversity of ailments afflicting an allograft, unlikely to exist. Predictably, a combination of new diagnostic tools will be required to monitor the complex interaction between the recipient and the allograft. Protocol biopsies reveal that a normally functioning allograft is not always a normal kidney, and emphasize the need for serial and aggressive monitoring of transplant recipients to detect early causes of allograft dysfunction and prevent graft loss. The *-omic* approach provides a large and increasing amount of data with a potentially big yield in diagnostic accuracy. However, the functional and clinical relevance of this information will have to be determined. As new immunosuppressive agents and treatment protocols continue to develop, a parallel refinement of diagnostic tools is required to set the end points and to validate the outcomes of clinical trials. A constant dialogue between the laboratory and the clinic is likely to result in the effective application of new diagnostic tools to improve the care of transplant recipients.

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