Protocol Transplant Biopsies: An Underutilized Tool in Kidney Transplantation

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Discussion on the use of protocol biopsies in renal transplantation requires that important information that has come to light in recent years be taken into consideration. First, the reduction in the incidence of clinical rejections and increased graft survival at 1 yr has not translated into improved long-term outcomes. Second, the serum creatinine, the most widely used test to monitor the function of the graft, has been shown to be insensitive for the detection of early graft pathology and cannot be relied on for the assessment of adequacy of immunosuppression. Third, there has been a proliferation of immunosuppressive drug regimens based on a reduction in net immunosuppression, which requires a rigorous assessment of their safety. Finally, advances in genomics, proteomics, and metabolomics will usher in a new era of understanding of the biology of renal transplantation and, by extension, of fundamental processes in other areas. Protocol biopsies (the "standard of science") are a requirement in this new era, as they allow for the unbiased study of the correlations between allograft histology and function under different immunosuppressive regimens.

Protocol biopsies have been useful for the detection of unexpected acute pathology (*e.g.*, subclinical acute rejection) and for the diagnosis of early chronic changes, primarily interstitial fibrosis and tubular atrophy. An emerging literature suggests that subclinical acute rejection, interstitial fibrosis, and tubular atrophy may be surrogates for subsequent graft dysfunction and loss. The timely recognition of these early histologic changes may result in optimization of the immunosuppressive regimen and an improvement in both short- and long-term patient and graft survival.

The potential benefits of protocol biopsies discussed above must be balanced against their risk. Two recent studies from Europe are reassuring, as they report a low major complication rate (including transfusion requirement and catheterization) of between 0.4 and 1%, with only one graft lost in approximately 2500 biopsies (1,2).

The most used system for the scoring of renal allograft histopathology is the Banff schema (3), which requires that at least 10 glomeruli and two arteries be obtained for sample adequacy. Two cores are usually procured, as an early study showed that between 10 and 25% of acute rejections would be missed if only one core were obtained (4). More recently, a study that examined the progression of chronic histologic changes in serial biopsies concluded that inaccuracies as a result of sampling occur in up to 25% of cases (5). Thus, sampling error, in the diagnosis of both acute and chronic pathology, is a limitation of the biopsy in renal allografts.

This review discusses the use of protocol biopsies for the diagnosis of acute and chronic pathology in turn and then deals with future directions in this area. Of necessity, not all subjects that relate to protocol biopsies are discussed.

Protocol Biopsies for Detection of Acute Subclinical Rejection

Subclinical Rejection: Prevalence, Risk Factors, and Significance

Our group was the first to report that acute rejection, as defined in the Banff schema (3), was present in up to one third of well-functioning renal allografts in the first three mo post-transplantation, and the term "subclinical rejection" was coined (6). Our current definition of subclinical rejection requires that the serum creatinine be increased by <10% 2 wk before the protocol biopsy and that the histologic Banff score is "ai2at2" (type IA acute rejection) or greater. Numerous groups since have confirmed the occurrence of subclinical rejection as defined above, in both adults (7–14) and children (15,16). Some investigators include "borderline" rejection (Banff score <ai2at2) in the subclinical rejection category (7,9–11,14). In a series of 330 consecutive protocol biopsies in patients who were on cyclosporine-based therapy and had a prevalence of subclinical rejection of 15%, we found that 75% of subclinical rejection was Banff type IA and 24% was type 1B; arteritis (Banff IIA or greater) was found in a minority of cases (17).

Prevalence of Subclinical Rejection

The prevalence of early subclinical rejection is influenced by the use of antibody induction and by the type of maintenance immunosuppression. The first reports describing subclinical rejection were in patients who did not receive antibody induction and were treated with cyclosporine-based immunosuppressive regimens (6,8,9,18–20) with (20) or without (6,9,18,19) mycophenolate mofetil (MMF). The prevalence of subclinical rejection between months 1 and 3 was approximately 25 to 30%.

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In some studies, the use of cyclosporine microemulsion (21,22) and MMF (14,21,22) reduced the prevalence of subclinical rejection. However, this was not observed in all studies (20).

More recent studies have reported on early subclinical rejection in patients who received tacrolimus, either with (10,11,13,21,22) or without (7) MMF. Jurewicz (7) reported a prevalence of subclinical rejection (that included "borderline") of 18% at 3 mo in patients who received kidneys from deceased donors and were treated with tacrolimus, azathioprine, and prednisone. Nankivell *et al.* (22) reported a prevalence of subclinical rejection (including borderline) of >50% at 1 mo that was virtually abolished at 3, 6, and 12 mo in patients who were on tacrolimus and MMF. Gloor *et al.* (11) reported a similar prevalence of subclinical rejection of only 2.6% at 3 mo in patients who were on tacrolimus and MMF. In this latter study, however, >60% of patients were recipients of living-related grafts, and >50% of patients had received induction therapy with antilymphocyte agents. In very early protocol biopsies (mean time 8 d posttransplantation) in patients who received tacrolimus and steroids plus MMF in two thirds, Shapiro *et al.* (10) reported a 21% prevalence of borderline rejection and a prevalence of 25% of type I or type II rejection, despite stable or improving function. Overall, however, the reported prevalence of subclinical rejection after the first month is much less in patients who are treated with tacrolimus than in patients who are treated with cyclosporine, ranging between 3 and 25%, even when borderline rejections are included.

There are very few data on early subclinical rejection in patients who are on sirolimus. In one study (12), protocol biopsies were performed at 3 and 12 mo in patients who were randomly assigned to sirolimus and low-dose tacrolimus (calcineurin inhibitor [CNI] sparing) or sirolimus and MMF (CNI-free). Subclinical rejection was found in 6 and 15% of patients and chronic allograft nephropathy (CAN) was found in 53 and 15% of patients in the CNI-sparing and CNI-free groups, respectively. A recent study in 40 patients who had HIV and received basiliximab induction, sirolimus, cyclosporine, and prednisone included protocol biopsies at 1, 6, 12, and 24 mo posttransplantation. The prevalence of subclinical rejection was 29% at the combined biopsy time points (23).

The prevalence of subclinical rejection decreases in protocol biopsies that are performed at later time points. In adults, after the first year, the overall prevalence of subclinical rejection was approximately 18% (22), and at 2 yr, it was 8.9% in patients who were on tacrolimus and 9.2% in patients who were on cyclosporine (24). In children, the prevalence of subclinical rejection also declined over time, albeit less markedly. In children who were treated with cyclosporine plus azathioprine or mizoribine, the prevalence of subclinical rejection was 50, 32, 19, and 16% at 1, 2, 3, and 5 yr posttransplantation, respectively (15).

Risk Factors for Subclinical Rejection

Subclinical rejection is associated with histoincompatibility between the donor and the recipient. In the Winnipeg studies, the prevalence of subclinical rejection in protocol biopsies that were performed at 1, 2, and 3 mo was 0, 25, and 20%; 32, 32, and 30%; and 30, 37, and 63% in zero, one, and two HLA-DR mismatched patients, respectively (25). A correlation between HLA mismatches and the prevalence of subclinical rejection has been reported also by others (10,14). Moreover, our group found that subclinical rejection is more prevalent in patients who are presensitized to class I or class II HLA antigens as detected by flow cytometry (26). Therefore, early subclinical rejection seems to be an alloimmune response triggered by either mismatching of or presensitization to the major histocompatibility antigens.

Significance of Subclinical Rejection Detected in Protocol Biopsies

The importance of treatment of subclinical rejection was suggested by the only randomized study done to date that showed that treatment of subclinical rejection in months 1, 2, and 3 was associated with a reduction in interstitial fibrosis and tubular atrophy at month 6 and with the preservation of graft function at 2 yr as compared with a control group in whom protocol biopsies were not done (18). Others have shown that interstitial fibrosis and tubular atrophy develop in patients in whom subclinical rejection is diagnosed but not treated (8,9,21,27). The above outcomes for undiagnosed or untreated subclinical rejection were reported for adult recipients of deceased donor kidneys (8,9,18,27). In a cohort of adult patients who received living donor grafts and were followed for 10 yr, the diagnosis of subclinical rejection at 14 d posttransplantation was associated with a significantly worse graft survival even when treated (14). In this study, an increased incidence of clinical rejections was reported in the patients that had subclinical rejection at 14 d, and protocol biopsies were not done beyond 14 d posttransplantation. Finally, in children, the finding of subclinical rejection in serial protocol biopsies (done at 1, 2, 3, and 5 yr) was associated with progression of CAN as scored by the chronic allograft damage index (CADI) (28), as well as with decreased renal function and lower graft survival (15).

The pathogenicity of subclinical rejection is supported by studies in which the immunohistochemical characterization of the graft-infiltrating cells and the transcriptional analysis of the genes present in the graft have been compared across the range of acute inflammation observed on renal biopsy. In the most recent and complete of such studies, Hoffman *et al.* (29) concluded that subclinical rejection and clinical rejection are probably different stages of the same process, as the differences in the immunophenotype of the infiltrating cells and the gene transcriptional findings that were observed between the two were quantitative more than qualitative. In clinical rejection, however, one novel finding was the increased expression of T-bet, a maturation factor for cytotoxic T cells.

Our group has also studied the phenotypic and activation marker profile of graft-infiltrating cells by immunochemical methods (30) as well as the transcripts for a more limited number of proinflammatory and cytotoxic genes (31). Our conclusions from these studies were essentially the same as those of Hoffman *et al.* (29), namely that subclinical and clinical rejection likely are different stages of the same potentially damaging alloimmune process. Indeed and as stated by Hoffman *et al.* (29), there are no data to suggest that subclinical

tubulointerstitial inflammation is regulatory or in any way beneficial to the graft.

Our current view is that a reduction in the prevalence of subclinical rejection can be achieved satisfactorily with a baseline immunosuppressive regimen that includes tacrolimus and MMF in patients with low immunologic risk. We do not think that antibody induction is necessary in these patients because of the concern for excessive immunosuppression and infection with polyoma. In such low-risk patients, the performance of protocol biopsies for the purposes of diagnosing early subclinical rejection may be unwarranted. However, protocol biopsies should be considered in patients who are sensitized to their donors, as their risk for subclinical rejection and the development of early graft fibrosis are greater than normal.

Protocol Biopsies for Detection of Interstitial Fibrosis and Tubular Atrophy

Interstitial Fibrosis and Tubular Atrophy: Prevalence, Risk Factors, and Significance

The more frequent sampling of the tubulointerstitial space in small biopsy cores makes the scoring of interstitial fibrosis and tubular atrophy more useful as an outcome measure than the scoring of glomerular and vascular lesions. Interstitial fibrosis and tubular atrophy are scored semiquantitatively in the two most frequently used classifications of renal allograft pathology, the Banff (3) and CADI systems (28). However, more precise quantification of the volume of interstitial fibrosis can be obtained by histomorphometry (32) and with the use of collagen-specific stains such as Sirius Red and image analysis techniques (33,34).

Protocol biopsies that are obtained in the first year posttransplantation show a rapid increase in the prevalence of interstitial fibrosis and tubular atrophy. Nankivell *et al.* (27) in the largest study of protocol biopsies to date showed that, compared with biopsies that were performed at implantation or at 1 or 2 wk, both interstitial fibrosis and tubular atrophy increased by a factor of 10 during the first posttransplantation year, with less of an increase during the subsequent 9 yr. In a similar study, of shorter duration, with biopsies done at 1, 2, 3, and 6 mo, our group reported negligible interstitial fibrosis and tubular atrophy (and total "chronic score") at 1 and 2 mo with a fivefold increase at 6 mo (19). The prevalence of interstitial fibrosis and tubular atrophy at different times posttransplantation using Banff scores for "chronic/sclerosing allograft nephropathy" (CAN) (3) shows an approximately similar progression. Thus, the prevalence of CAN in protocol biopsies that are performed at 3 to 4 mo is between 24 and 42% (8,9,35), at 6 months is approximately 40% (13,36), at 1 yr is approximately 50% (5,15), and at 2 yr is between 50 and 90% (8,21,24). All of the above studies were performed in patients who were under cyclosporine-based immunosuppression. However, one of these studies compared tacrolimus and cyclosporine-treated patients, in both of whom the prevalence of CAN was approximately 70% at 2 yr (24). Finally, two recent studies reported the prevalence of CAN in protocol biopsies in patients who were on sirolimus (37,38). In one study (37), approximately 32% of patients who switched to sirolimus at 3 mo had new-onset CAN at 1 yr, as compared with 65% of those who were maintained on cyclosporine. In the other study (38), patients who were randomly assigned to sirolimus had a 34% prevalence of CAN at 2 yr as compared with almost 80% for those who were on cyclosporine. Of additional interest, in the latter study, DNA microarrays showed enhanced expression for immunity/inflammation and fibrosis/tissue remodeling genes in the cyclosporine as compared with the sirolimus cohort.

Other studies have used the CADI system for the scoring of chronic pathology (39,40). In the latter study (40), protocol biopsies were obtained at 1, 2, and 3 yr in patients who were treated with cyclosporine, MMF, and prednisone. In this study, the CADI score more than doubled between baseline and 12 mo and more than tripled by 36 mo.

Risk Factors for Interstitial Fibrosis and Tubular Atrophy

A number of independent risk factors have been correlated with the development of interstitial fibrosis and tubular atrophy in the first 6 to 12 mo posttransplantation. These include ischemia-reperfusion, acute rejection (clinical or subclinical), early nephrocalcinosis, and donor age (9,18,36,40). Fibrosis beyond 1 yr has been correlated with rejection (clinical and subclinical), donor age, and cyclosporine nephrotoxicity in some studies (15,21,24,38) but not others (40). The data on sirolimus, although preliminary, are intriguing and suggest that a maintenance drug regimen that includes a CNI may be a risk factor for late-onset interstitial fibrosis (38).

Significance of Interstitial Fibrosis and Tubular Atrophy in Protocol Biopsies

Interstitial fibrosis and tubular atrophy that are detected as early as 3 to 6 mo posttransplantation in well-functioning transplants are correlates of later allograft dysfunction and loss (9,19,27,35,41). Similarly, increased chronic scores reported with the CADI system at 1 yr (40) or 2 yr (39) have been correlated with graft losses at 3 and 6 yr, respectively. Using more rigorous quantitative assessments of interstitial fibrosis, the extent of this lesion in a 6-mo protocol biopsy was shown to correlate with graft survival (32) and with time to graft failure (33). Patients with concomitant subclinical rejection and CAN may have a greater risk for graft dysfunction and loss, as has been reported in adults (19,33) and in children (15). Although a single protocol biopsy that demonstrates interstitial fibrosis may provide some indication of the subsequent risk for graft dysfunction or loss, it is obvious that many events subsequent to the biopsy can modify the course of the transplant. Ideally, a baseline biopsy should be obtained before implantation, thus allowing for the more reliable determination of new-onset fibrosis at subsequent time points. This is particularly important given the increasing use of extended criteria donors, in whom a significant amount of interstitial fibrosis is observed in the implantation biopsy. The detection of interstitial fibrosis and tubular atrophy remains a useful end point for clinical trials (42), and quantitative methods of interstitial fibrosis assessment likely will improve on its predictive value (32–34). Finally, secondary prevention trials that enter patients with interstitial

fibrosis should markedly reduce the number of patients required to detect a change in the rate of graft loss (33,40,41).

Protocol Biopsies in Renal Transplant Patients: Future Directions

Immunologic risk factors are undoubtedly important in determining graft survival, as demonstrated by the correlation between graft survival and HLA matching (43). However, immunosuppressive regimens that have markedly reduced the incidence of clinical rejection episodes have not resulted in an improvement in long-term graft outcome (44). The current data suggest two possibilities that might explain this seeming paradox. First, it is possible that immunosuppressive regimens in use are deleterious to the graft through direct nephrotoxicity, through metabolic alterations in the host (*e.g.*, causing vascular disease), or by favoring graft-damaging infections, such as polyoma. Alternatively, it is possible that insufficient immunosuppression that results in subclinical alloreactivity underlies late graft dysfunction and loss. These two major pathways that lead to allograft loss are shown in Figure 1.

The nephrotoxicity of CNI and their unfavorable cardiovascular profile are documented amply in the literature. Early results with sirolimus are encouraging but inconclusive. For example, is the interstitial fibrosis observed in cyclosporine-treated patients in the study reported by Flechner *et al.* (38) the result of ongoing subclinical inflammation, a possibility suggested by the increased expression of immunity/inflammation genes? Would increased early exposure to cyclosporine and replacement of the CNI with azathioprine or MMF achieve the same results as those achieved with sirolimus?

Conversely, subclinical inflammation, by histologic criteria, seems to be reduced with tacrolimus and MMF, but will "whole graft" readouts, *e.g.*, using proteomic or metabolomic analysis of the urine, or the more precise transcriptome analysis of tissue concur with the histologic diagnosis? We need to be reminded that the biopsy represents an exceedingly small sample of the whole organ and that sampling error is likely a major limitation of this technique (Figure 2). Unfortunately, there are no subtle

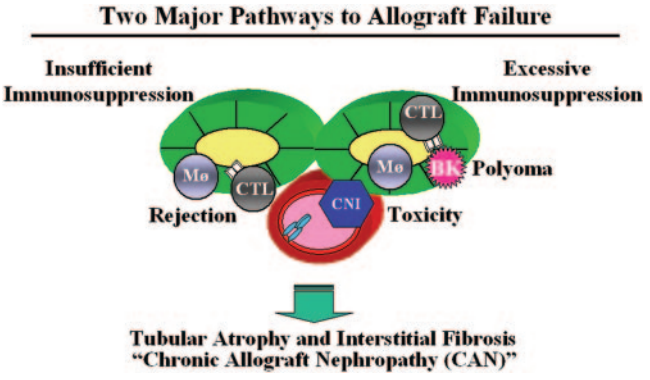

Figure 1. Two pathways to chronic allograft nephropathy (CAN) and allograft failure. Insufficient immunosuppression (left) may lead to CAN *via* clinical and subclinical rejection. Excessive immunosuppression (right) may lead to CAN *via* drug toxicity or polyoma.

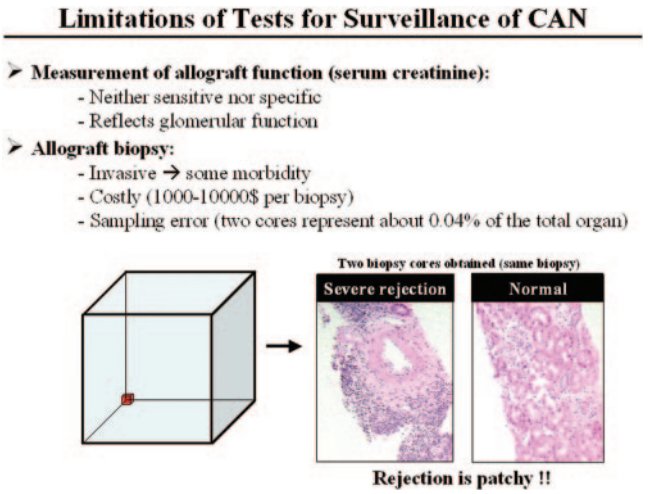

Figure 2. The limitations of current noninvasive (serum creatinine) and invasive (biopsy) test for surveillance of CAN are shown. Morbidity, cost, and the possibility of sampling error are limitations of the protocol biopsy.

functional assays of kidney dysfunction, with the notable exception of proteinuria that should probably warrant early investigation (45).

The evidence in both adult and pediatric renal transplantation indicates that clinically overt rejection is only a portion of the burden of alloimmune injury sustained by the graft during its lifetime. Subclinical rejection may represent a substantial proportion of that burden, the pathogenicity of which is now recognized. The optimal use of immunosuppressive agents requires that the inflammatory status of the graft be known. The tests that are needed for this assessment ideally would be noninvasive, allowing for frequent sampling, and be capable of detecting subclinical inflammation with satisfactory sensitivity and specificity. The urine may be the ideal medium to look for candidate tests that may include the products of alloactivated or cytotoxic cells (46), chemokines and cytokines (47,48), the patterns of proteins detected by proteomic techniques (49), or those of low molecular weight metabolites detected by magnetic resonance (50). Detection of subclinical inflammation by these means may allow for the tailoring of the intensity of immunosuppression to the inflammatory status of the graft and result in the reduction of both the incidence of late allograft losses and the unwanted side effects of immunosuppressive therapy.

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