Inflammation in early kidney allograft surveillance biopsies with and without associated tubulointerstitial chronic damage as a predictor of fibrosis progression and development of de novo donor specific antibodies

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Abbreviations:

ABMR, antibody mediated rejection

CsA, cyclosporine

DGF, delayed graft function

dnDSA, *de novo* donor specific antibodies

DSA, donor specific antibodies

HLA A mm, HLA A mismatches

HLA B mm, HLA B mismatches

HLA DR mm, HLA DR mismatches

IFTA, interstitial fibrosis and tubular atrophy

IFTA+I, interstitial fibrosis and tubular atrophy with inflammation

OR, odds ratio

PRA, panel reactivity antibodies

TAC, tacrolimus

Abstract

Interstitial fibrosis and tubular atrophy (IFTA) associated with interstitial inflammation in non scarred areas (IFTA+i) is associated with poorer graft outcome than inflammation without IFTA or IFTA without inflammation. We evaluated if histological categories at week 6 could predict the development of interstitial fibrosis and *de novo* donor specific anti-HLA antibodies (dnDSA) at one year. Biopsies were classified according to Banff criteria as normal (i+t≤1 and ci+ct≤1), inflammation (i+t≥2 and ci+ct≤1), IFTA (i+t≤1 and ci+ct≥2) or IFTA+i (i+t≥2 and ci+ct≥2). We analyzed 598 standard immunological risk recipients. The histological diagnosis at 6 weeks was: normal (n= 206), inflammation (n=29), IFTA (n=255) and IFTA+i (n=108). Moderate/severe interstitial fibrosis (ci≥2) at 1 year was observed in 4.2% of patients with prior (6 weeks) normal histology, in 3.4% with inflammation, in 13.8 % with IFTA and in 24.5% with IFTA+i (p=0.0001). Fifty-three recipients (8.9%) had dnDSA at 1 year. Independent predictors of development of dnDSA at 1 year were: HLA-DR mismatches (OR 1.95, 95%CI 1.09-3.49), the presence of inflammation (OR 5.49, 95% CI 1.67-18.03) or IFTA+i (OR 4.09, 95%CI 1.67-10.0) in the 6 week surveillance biopsy.

Early subclinical inflammation in surveillance biopsies *with or without* tubulo-interstitial chronic lesions is associated with an increased risk of dnDSA development.

Introduction

The presence of tubulo-interstitial inflammation in early surveillance renal allograft biopsies has been associated with progression of interstitial fibrosis[1] and decreased renal allograft survival[2]. However, in these studies the presence or absence of interstitial fibrosis and tubular atrophy (IFTA) was not taken into consideration for the evaluation of subclinical inflammation. In later studies, it was observed that renal allograft survival was significantly shortened if surveillance biopsies showed IFTA with interstitial inflammation in non scarred areas (IFTA+i), while inflammation not associated with IFTA was a relatively uncommon finding and was associated with graft survival comparable to normal biopsies and biopsies with IFTA without inflammation[3-5]. The reason why IFTA+i is associated with a poor outcome has not been clarified.

The presence of inflammation in early surveillance biopsies has been associated with an enhanced donor specific memory T cell response[6] suggesting that subclinical inflammation may represent a surrogate of the alloimmune response. Recently, this notion has been reinforced by the description of a temporal association between early subclinical inflammation and an increased risk for *de novo* donor specific anti-HLA antibodies (dnDSA), as well as antibody mediated rejection[7-8]. It is not known whether inflammation in otherwise normal biopsies and in biopsies with IFTA+i imply a different risk for development of dnDSA. Additionally it is not clear whether the presence of microvascular injury in early surveillance biopsies also contributes to an increased risk of dnDSA[8].

The aim is to evaluate if the presence of IFTA+i in 6 weeks surveillance biopsies implies a differential risk for the progression of fibrosis and development of dn DSA at 1 year than the presence of inflammation not associated with IFTA.

Material and methods

a) Patients

All patients with a negative cytotoxic T and B cell cross-match, panel reactive antibody (PRA) ≤ 20% and donor specific antibodies (DSA) negative before transplantation, receiving a single kidney transplant at Oslo University Hospital Rikshospitalet between January 2009 and December 2012 were considered and patients were followed during the first year after transplantation. At our tissue-typing laboratory a PRA of 20% or less is a borderline value and patients with a PRA ≤ 20% are considered as normal immunological risk. Only patients receiving a calcineurin-based immunosuppressive regimen with a surveillance biopsy at 6 ±2 weeks and 1 year (±2 month) and an anti-HLA antibody determination at the time of both biopsies were included in the present analysis. Patients receiving a kidney from a HLA identical donor, ABO incompatible graft, were excluded. The study was approved by the South-Eastern Regional Committee for Medical and Health Research Ethics in Norway and was performed in accordance with the Declaration of Helsinki and is consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. All patients gave their written informed consent.

b) Biopsies

Two cores were obtained with ultrasound guidance using an 18 gauge spring-loaded biopsy gun, one for histology (hematoxylin eosin and saffron, periodic acid-Schiff and Masson's trichrome) and one for C4d. Both biopsies contained at least one glomerular and one arterial profile and sufficient tubulo-interstitial tissue to grade interstitial inflammation (i), tubulitis (t), interstitial fibrosis (ci) and tubular atrophy (ct). C4d was stained with indirect immunofluorescence on frozen sections (monoclonal antibody; Quidel, San Diego, CA, USA). Grade 3 diffuse C4d staining in >50% of peritubular capillaries was classified as positive.

c) Histological classification

Renal lesions were graded according to the Banff criteria[9-10]. Biopsies were classified into 4 groups; a) normal histology (i+t≤1 and ci+ct≤1), b) inflammation (i+t≥2 and ci+ct≤1), c)

interstitial fibrosis and tubular atrophy (IFTA) (i+t≤1 and ci+ct≥2) and d) IFTA associated with interstitial inflammation in otherwise normal areas (IFTA+i) (i+t≥2 and ci+ct≥2). Areas with fibrotic scars were excluded from evaluation.

d) Anti-HLA antibodies

Immunomagnetic cytotoxic T and B cell cross-matches were done at transplantation in all recipients. Additional testing on the Luminex platform LX200, using the LSM12-screening kit (One Lambda) for identification of HLA class I and class II was performed. IgG antibody specificities was examined using single antigen coated flow beads provided by One Lambda at transplantation and at the time of the 6 weeks and 1 year surveillance biopsies. We used a mean fluorescence intensity of 1000 as a cutoff value. As a negative control we used serum (LS-NC) delivered by the kit producer (One Lambda). No patient had HLA DSA or PRA >20% prior to or at transplantation.

e) Clinical variables

All patients received induction therapy with basiliximab followed by a calcineurin inhibitor based regimen (either cyclosporine or tacrolimus) in combination with mycophenolate and prednisone as previously described[11]. Cyclosporine based immunosuppression was used in recipients older than 50 years and in younger patients with impaired glucose tolerance test prior to transplantation as a strategy to limit the impact on post transplant diabetes mellitus. Acute cellular rejections were treated with intravenous methylprednisolone, total dosage of 1375 mg, divided into 5 infusions. The oral prednisolone dose was increased to 30 mg/day and tapered 5 mg every other week. In case of steroid resistant rejection, rabbit anti-thymocyte globulin was administered. Acute antibody mediated rejection (ABMR) was additionally treated with plasmapheresis and/or intravenous immunoglobulin. Routinely, 5 plasmapheresis sessions were performed with replacement preferentially 4% albumin in Ringer (50 ml/kg bodyweight). In therapy resistant cases rituximab or rabbit anti-thymocyte globulin was administered. Delayed graft function (DGF)

was defined as the need of at least one dialysis session during the first 7 days after transplantation.

f) Statistical analysis

Results are expressed as frequencies for categorical variables or as the mean ± standard deviation for continuous variables. Chi squared, Kruskal-Wallis and ANOVA were used to compare categorical, ordinal or non-normally distributed continuous variables, and continuous normally distributed variables, respectively. All p-values were two-tailed and a p-value less than 0.05 were considered significant. Binomial and multinomial logistic regression analyses were carried out to study independent predictors of outcome variables. Variables with a p value less than 0.05 in the univariate analysis were included in the multivariate analysis.

Results

During the study period 1156 kidney transplants were performed. A total of 598 patients with a 6 week and 1 year biopsy with sufficient tissue for evaluation and with evaluation of dnDSA at the time of early and late biopsy were included. Reasons for exclusion are summarized in Figure 1. Of note, 242 patients were excluded because 6 weeks biopsy or 1 year biopsy was not performed. The reason for this was graft loss before 6 weeks in 9 patients, graft loss between 6 weeks and 1 year in 6 patients, patient death before 6 weeks in 1 patient and patient death between 6 weeks and 1 year in 25 patients. Reasons for graft failure during the first year were 6 cases of primary vascular problems, 4 acute rejection episodes, 2 non-viable kidneys, one recurrence of primary kidney disease and 2 not further determined and causes of death during the first year were 8 cases of septicemia, 6 malignancies, 2 myocardial infarctions, 2 cerebrovascular accidents, 2 pulmonary infections and one of each of mesenteric infarction, cardiac arrest, cystic liver disease, accident unrelated to ESRD, suicide and one of uncertain cause. In 201 patients one or both biopsies were not performed due to medical contraindication (n=24), declined consent (n=16), early

transfer to the local hospital responsible for long-term follow-up (n=48), capacity problems at the laboratory (n=57), or were alive but did not meet for the 1-year investigation at the transplant center (n=56).

Histological diagnosis at 6 weeks

A total of 598 surveillance biopsies obtained 6 weeks after transplantation were evaluated. Of those, 567 were representative according to Banff criteria showing at least 7 glomeruli and 1 artery and 31 biopsies were under the threshold of adequacy showing between 1 and 6 glomeruli. Histological diagnoses were: normal (206), inflammation (29), IFTA (255) and IFTA+i (108). Thus, 12.3% (29 out of 235) of patients had inflammation in biopsies without IFTA while 29.8 % (108 out of 363) of patients had inflammation in biopsies already displaying IFTA ($p<0.0001$).

Clinical variables associated with histological diagnosis at 6 weeks

Characteristics of patients according to histological diagnosis at 6 weeks are summarized in table 1. For multinomial logistic regression the reference category was normal histology. The number of HLA-DR mm (Odds Ratio –OR- 2.59 and 95% confidence interval --95%CI- 1.28-5.25) and the use of cyclosporine in comparison to tacrolimus (OR 2.32 and 95%CI 1.01-5.26) were independent predictors of inflammation at 6 weeks. Independent predictors of IFTA were donor age (OR 1.06, 95% CI 1.04-1.08), male donor gender (OR 1.96, 95%CI 1.29-2.99) and HLA-B mm (OR 1.44, 95%CI 1.05-1.98). Finally, independent predictors of IFTA+i were HLA-DR mm (OR 2.16, 95% CI 1.39-3.34), use of cyclosporine in comparison to tacrolimus (OR 1.96, 95%CI 1.17-3.33), HLA-B mm (OR 1.79, 95% CI 1.19-2.69), donor age (OR 1.035, 95% CI 1.02-1.05) and male donor gender (OR 1.76, 95% CI 1.05-2.96).

Serum creatinine at 6 weeks and at 1year according to histological diagnosis at the time of the early surveillance biopsy is summarized in figure 2.

Evolution of histological lesions between 6 weeks and 1 year

From 6 weeks to one year after transplantation, Banff scores for interstitial inflammation (0.34±0.70 vs 0.32±0.69) and tubulitis (0.48±0.78 vs 0.46±0.75) were not significantly changed. Banff scores for interstitial fibrosis showed however a significant increase from 0.67±0.59 to 0.85±0.72 ($p<0.0001$) and tubular atrophy from 0.80±0.56 to 0.99±0.66 ($p<0.0001$).

A total of 72 out of 598 patients (12.04%) had a ci score ≥ 2 at one year. In figure 3 the relationship between histological diagnosis at 6 weeks and ci score ≥ 2 at 1 year is shown. In table 2, clinical data and histological diagnosis at 6 weeks according to the presence of a ci score ≥ 2 at 1 year are summarized. Logistic regression analysis showed that donor age (OR 1.04 and 95% CI 1.01-1.07), the presence of an episode of acute rejection before the 6 week biopsy (OR 2.48 and 95% CI 1.18-5.20), the presence of an episode of acute rejection between 6 week and one year biopsy (OR 2.38 95 % CI 1.08-5.30) and the presence of IFTA+i in the 6 week biopsy (OR 4.21 and 95% CI 4.2 and 95% CI 1.80-9.90) were independent predictors of a ci score ≥ 2 at one year.

Predictors of dnDSA at 1 year

A total of 53 out of 598 patients (8.9%) had dnDSA at one year. In table 3, clinical data and histological diagnosis at 6 weeks according to the presence of dnDSA at one year are summarized. Logistic regression analysis including clinical and histologic data at 6 weeks showed that the number of HLA-DR mm (OR 1.95 and 95% CI 1.09-3.49), inflammation in 6 week biopsy (OR 5.49 and 95% CI 1.67-18.03) and IFTA+i at 6 weeks (OR 4.09 and 95% CI 1.67-10.05) were independent predictors of dnDSA at 1 year. The analysis was repeated excluding the 12 patients with dnDSA at 6 weeks and the results not modified by excluding these patients (data not shown)

Microcirculatory injury, C4d deposition and dnDSA

There was no association between microcirculatory injury at 6 weeks and dnDSA at 1 year but peritubular capillaritis and C4d deposition at 1 year were more common in patients displaying dnDSA (Table 4).

Discussion

The main finding of this surveillance biopsy analysis was that tubulo-interstitial inflammation in biopsies without IFTA and in biopsies displaying IFTA+i at 6 weeks was associated with an increased prevalence of dnDSA at one year. Of note, the odds ratio for dnDSA was similar in patients with inflammation and IFTA+i. On the contrary, microcirculatory injury at 6 weeks was not associated with an increased prevalence of dnDSA at 1 year, while the presence of dnDSA at 1 year was associated with microcirculatory injury at the same time.

Currently antibody mediated rejection (ABMR) is regarded as a main cause of renal allograft failure[12] and the presence of DSA as an underlying necessity for its appearance[13]. Our understanding of ABMR has evolved in the last decade. Acute ABMR was initially included in the 2003 Banff classification[14], chronic AMBR in Banff 2005[15], and subclinical ABMR[10, 16] was described few years later. ABMR in patients with preformed and dnDSA constitutes an evolving process that is manifested by different clinical phenotypes[17]. In the present study, we confirm that there is a temporal association between early subclinical tubulo-interstitial inflammation and an increased risk for development of dnDSA[7-8, 18-19]. Altogether, these observations suggest that tubulo-interstitial inflammation may constitute a trigger of the complex process leading to dnDSA synthesis. In our study, microcirculatory injury at 6 weeks was not associated with presence of dnDSA one year after transplantation, while one-year dnDSA were associated with the presence of microcirculatory injury in one-year biopsies. Consequently, our data may indicate that microcirculatory injury is not a trigger but a consequence of dnDSA.

The risk of dnDSA at one year was similar in patients with inflammation and IFTA+i, suggesting that inflammation constitutes a risk factor for dnDSA regardless of the presence or absence of fibrosis. This observation, as far as we know, has not been made before. In previous studies, it has been described that IFTA+i, but not inflammation in the absence of IFTA is associated with decreased graft survival. On the other hand, studies evaluating the relationship between early inflammation and the risk of dnDSA did not clarify whether the presence or absence of fibrosis in patients with inflammation modulates the risk of dnDSA. Thus even mild inflammation in biopsies without fibrosis cannot be considered a non significant lesion.

Risk factors associated with inflammation in biopsies without IFTA were the number of HLA-DR mm and the use of cyclosporine in comparison to tacrolimus, which can be considered surrogates of the alloimmune response. Donor age and male gender, representing surrogates of the quality of the kidney, but also HLA-B mm, were associated with IFTA. Finally, IFTA+i was associated with both: surrogates of the alloimmune response, i.e. use of cyclosporine, HLA-DR and HLA-B mm, as well as surrogates of the quality of the kidney such as donor age and male gender. Of note, the number of HLA-B mm can be considered a surrogate of the alloimmune response and was associated with IFTA. This association suggests that apart from preexisting donor related chronic damage, alloimmune response might contribute to interstitial fibrosis in the absence of significant inflammation. This idea is in agreement with the observation that inflammation related genes are upregulated in patients with chronic injury[20-21].

The prevalence of subclinical inflammation ranges from 5 to more than 50% in different studies[22-23]. Discrepancies between studies, can be explained by timing of surveillance biopsy[24], interobserver variability[25] and especially by the type of immunosuppression[26]The prevalence of subclinical inflammation ranges from 5 to more than 50%. Discrepancies between studies can be explained by timing of surveillance biopsy, interobserver variability and especially by the type of immunosuppression. The prevalence and intensity of

inflammation is lower in tacrolimus than cyclosporine treated patients, a result that was confirmed in the present study. However, it is not known whether the use of immunosuppressive regimens associated with a low prevalence of subclinical inflammation are also associated with a decreased risk of dnDSA. In the present study, the probability to develop dnDSA was similar in patients treated with cyclosporine and tacrolimus. Nevertheless, in some clinical trials it has been described that the risk of dnDSA is associated with the immunosuppressive schedule. Unfortunately, in these trials surveillance biopsies were not done. Thus, the question whether prevention of early inflammation constitutes a strategy to prevent dnDSA deserves further study.

Despite the association between inflammation and dnDSA, it is not clear whether the use of immunosuppressive regimens associated with a low prevalence of subclinical inflammation are associated with a lower risk of developing anti HLA antibodies. This possibility has been suggested by the observation that conversion from a cyclosporine to an everolimus based regimenconversion from a tacrolimus to a sirolimus based regimen is associated with an increased probability to develop anti-HLA antibodies[31-32]. In our study the risk of developing dnDSA by one year after transplantaiton was not different in cyclosporine and tacrolimus treated patients.

The presence of IFTA+i, but not inflammation without IFTA at 6 weeks was an independent predictor of the presence of moderate to severe interstitial fibrosis (ci score ≥ 2) at one year. Other independent predictors of the presence of ci score ≥ 2 at one year were donor age, , the presence of a clinical episode of acute rejection before the 6 weeks surveillance biopsy and the presence of an episode of acute rejection between the two surveillance biopsies. Altogether, these data not only suggest that clinical episodes of acute rejection constitute a driving force for the progression of fibrosis but also point out that in patients with fibrosis, superimposed inflammation accelerates the progression of fibrosis during the first year.

Our study has limitations. The most important one is the short follow up of patients not allowing us to evaluate the relationship between inflammation, progression of interstitial fibrosis, dnDSA and graft survival. Moreover, the relationship between type of treatment and inflammation or dnDSA cannot be properly analyzed since cyclosporine was subscribed to patients at increased risk for post-transplant diabetes mellitus.

In summary, early subclinical inflammation in biopsies with or without tubulo-interstitial chronic lesions, together with HLA DR mm, are associated with an increased risk of development of dnDSA one year after transplantation. In patients with IFTA+i in early biopsies there is an increased risk for the progression of fibrosis at one year. Since type of immunosuppressive treatment modulates the severity of subclinical inflammation, it will be worth to explore whether treatment regimens associated with low prevalence of subclinical inflammation may in turn prevent the appearance of dnDSA.

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