Molecular Phenotypes of Kidney Graft Rejection

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Key Words

Renal transplantation · Acute rejection · Gene expression · Molecular phenotype

Abstract

In the last decade, gene expression studies of kidney transplants provided an opportunity to better understand the development and regulation of kidney graft rejection. This review outlines the progress in the definition of biomarkers of rejection and, above all, concentrates on studies of the molecular phenotype of rejection. This phenotype, rather than morphological characterization, may be critical for assessing the ongoing processes in the graft and for the outcome prediction.

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Introduction

Despite advances in immunosuppression, the long-term kidney allograft survival has not improved substantially. The main cause of graft loss in the long term remains to be chronic rejection, an entity challenged in the past, yet still contained in the Banff classification [1]. In the pathogenesis of this process, both cellular and humoral immune responses have been implicated. Standard therapy of chronic rejection has not been defined to date mainly because of late diagnosis. Deterioration of kidney

graft function determined by the increase of serum creatinine frequently reflects the already developed and untreatable structural changes; however, molecular signatures of processes leading to those changes might be detected weeks before. To detect molecular fingerprints of developing injury, the identification of new noninvasive biomarkers of rejection and evaluation of intrarenal molecular events within the kidney graft in various clinical situations seem to be mandatory.

Diseases with similar morphological findings differ in their molecular patterns and this phenotype may be critical for assessing the risk of progression. Although initial knowledge comes from oncology [2], there are reports suggesting the progression of kidney diseases to be related with the intrarenal molecular phenotype [3, 4]. Recently, it has been hypothesized that molecular phenotypes of graft injury may predict the graft prognosis more accurately than the histology itself.

Animal Lessons

To understand the molecular processes of kidney graft rejection, various animal models have been studied. On the well-defined rat kidney transplantation model, Edemir et al. [5] demonstrated global changes in gene expression levels after syngeneic and allogeneic transplantation. Using DNA microarrays and qRT-PCR, they identified major immune effector-related pathways activated

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after allogeneic transplantation and related to acute rejection mechanism of tissue injury (e.g. *Ifng*, *Tnf*, *Cxcl9*, *Xcl1*, *Cxcl10*, *Ccl3*, *Ccr1*, *Ccr2*, *Ccr5*, *Cd8a*, *Fcnb*, *Gzmb*, *Gzma*, *Prf*, *Cd3d*, *Cd8*, and *Cd163* were upregulated). In parallel, they identified a number of upregulated genes linked to tissue-protective and immune counterregulation mechanisms that would balance and limit the ongoing inflammatory/immune responses (e.g. *C4bpa*, *Il10*, *Bcl2a1*, *Socs1*, *Il1rn*, *Hmox1*). These observations fit with the evolving view of immunity as a balance between effector and regulatory mechanisms. An experimental system to classify transcripts as members of pathogenesis-based sets representing biological processes in injured or diseased tissue was used by Halloran et al. [6] in their microarray studies on mouse kidney transplants. Both isografts and allografts displayed host macrophage infiltration of interstitium from day 2, followed by active injury-repair response, with inflammatory features, injury-up features and loss of epithelial features. The allografts displayed specific expression of IFNG effects and T cell transcripts. Strong 'classical' (IFNG-induced) and alternative macrophage activation features coexisted in allografts. Transcripts associated with alternative macrophage activation were prominent in acute T-cell-mediated rejection (TCMR) and were also associated with tissue remodeling [7]. The injury-repair response in TCMR required T cells but was independent of T cell cytotoxic mechanisms, B cells and antibody [8]. Some of the cytotoxic T cell transcripts, e.g. *Gzmb*, *Prf* and Fas ligand previously described as specific for rejection [9, 10], were shown not to be specific for TCMR, because they also reflect homing of effector memory T cells to injured tissues, or natural killer cells in capillaries in acute antibody-mediated rejection (AMR) [6]. Several molecules associated with graft acceptance and rejection were described using the model of rat kidney and mouse heart transplantation [11]. The long-term acceptance of graft was shown to be associated with the high levels of *Toag-1* and α -1,2-mannosidase genes both in the graft and in peripheral blood, whereas their expression was downregulated in the peripheral blood 3–5 days before acute rejection of mouse heart and rat kidney allografts.

Human Kidney Graft Biopsies

'0-Hour' Biopsies

Interestingly, evaluation of '0-hour' biopsies may detect molecular phenotypes associated with the risk of late graft dysfunction. It was shown that the transcriptome reflects

kidney quality and susceptibility to delayed graft function better than available clinical and histopathological scoring systems [12]. The expression of *TNFA*, *TGFB*, *CD25*, and *ICAM1* in '0-hour' biopsy predicted increased risk of delayed graft function and acute rejection and using these molecular markers together with clinical indicators further enhances the quality of prognosis [13]. Predictive models were built for proteinuria and graft loss 2 years after transplantation using clinical variables in combination with intragraft gene expression data [14]. Another study analyzed microarray gene expression in relation to histologic parameters of donor organs. In already injured kidney, the genes related to immunity and defense, cell communication and apoptosis were upregulated. Combination of histology with 3 from the set of differentially expressed genes (*NLRP2*, *IGJ*, and *RGS5*) increased the predictability of 1-year serum creatinine dramatically [15].

'Case Biopsies'

Earlier studies showed upregulation of Fas ligand mRNA in biopsy samples with therapy-resistant acute rejections versus samples with therapy-sensitive acute rejection [16, 17]. Besides the upregulation of granulysin, higher intrarenal mRNA expression of *NKG2D*, which is activating cytotoxicity receptor expressed by natural killer cells, was reported to be associated with the deteriorated renal function at 6 and 12 months after transplantation, mainly in grafts with acute rejection [18].

The first evidence, that early acute rejection after transplantation is not a homogeneous molecular entity, was given by Sarwal et al. [19]. On the basis of hierarchical clustering, the 3 subtypes of acute rejection were defined. The subtypes of rejection differed in the expression of genes reflecting apoptosis, infiltration and activation of lymphocytes, driven by *NFκB* and interferon- γ , transcripts from T cells, natural killer cells, and macrophages. Enhanced expression of cytotoxic T lymphocyte effector genes (*GZMA* and *RANTES*), adhesion molecules, cytokines, cytokine receptors, and growth factors, and surprisingly also markers of B cells (*CD20*, *CD74*, immunoglobulin heavy and light chains, and other molecules associated with B cell receptors) was found in the subtype of acute rejection with the most unfavorable outcome. Acute rejection samples clustered with samples from patients with chronic allograft nephropathy showed enhanced expression of genes involved in cellular proliferation and cell cycling whereas the molecular features of lymphocyte infiltration and activation were minimal in this subtype. It suggests a relatively quiescent rejection process with active tissue repair and regeneration.

Table 1. Molecular phenotypes in human kidney graft biopsies

	Biopsies n	Method used to analyze expression	Main findings
‘0-hour’ biopsies			
Mueller et al. [12], 2008	87	microarray	Transcriptome of implant biopsies reflected kidney quality and susceptibility to delayed allograft function better than clinical and histopathological scoring systems
Avihingsanon et al. [13], 2005	75	qRT-PCR	TNFA, TGFB, CD25, and ICAM1 enhanced quality of prognosis from clinical indicators
Bodonyi- Kovacs et al. [14], 2010	73	qRT-PCR	High CD3 and low PECAM were associated with worse 2-year graft function; high TNFA predicted worse 2-year graft survival
Perco et al. [15], 2009	82	microarray	NLRP2, IGJ, and RGS5 combined with histology predicted 1-year serum creatinine
‘Case biopsies’			
Nickel et al. [16], 2001	22 (19)	qRT-PCR	FASL was upregulated in therapy-resistant acute rejections
Desvaux et al. [17], 2004	34	qRT-PCR	FASL was a predictor of antirejection therapy resistance
Seiler et al. [18], 2007	109	qRT-PCR	NKG2D and granulysin upregulation was associated with restricted 6-month graft outcome
Sarwal et al. [19], 2003	67 (50)	microarray	Immune activation and cellular proliferation-related gene expression pattern distinguished subtypes of acute rejection with different outcome
Tsai et al. [20], 2006	45 (31)	immuno- histochemistry	Higher infiltration with CD20+ cells was associated with worse graft survival
Bagnasco et al. [21], 2007	74 (58)	immuno- histochemistry	B-cell-rich infiltrates were not associated with worse outcome in acute rejection
Viklicky et al. [22], 2010	64	qRT-PCR	Low expression of CD20 was associated with poor graft prognosis after early acute rejection
Sagoo et al. [23], 2010	71 ^a + 24 ^b	microarray qRT-PCR	Molecular biomarkers were able to identify tolerant patients; tolerant patients had elevated peripheral blood B cells with a decrease in the memory B cell subset
Newell et al. [24], 2010	58	microarray qRT-PCR	Tolerant patients displayed increased numbers of B cells in peripheral blood, with decreased memory pool
Bestard et al. [26], 2008	37	immuno- histochemistry	FoxP3+ Treg in subclinical rejection in the 6th month predicted better graft function 2 and 3 years after transplantation
Bunnag et al. [27], 2008	83 (77)	microarray qRT-PCR	FOXP3 was evaluated in rejection (both TCMR and AMR); no relationship between FOXP3 or FOXP3/GZMB expression and future function
Anglicheau et al. [28], 2009	33 (32)	qRT-PCR ^c	Evaluated the set of miRNAs predictive of acute rejection and allograft function
‘Protocol biopsies’			
Mengel et al. [30], 2010	107	microarray	No association of gene expressions in 6-week protocol biopsies with future rejection episodes, functional deterioration or allograft loss; early protocol biopsies reflect more injury-repair response to implantation stresses
Matl et al. [31], 2010	163	qRT-PCR	High expression of RANTES and IP-10 in subclinical rejection was associated with poor outcome

Table 1 (continued)

	Biopsies n	Method used to analyze expression	Main findings
Scherer et al. [32], 2003	17	microarray qRT-PCR	Ten genes were differentially expressed in 6-month protocol biopsies in patients developing chronic rejection within the following 6 months compared with patients who remained stable
Park et al. [33], 2010	151	microarray qRT-PCR	The combination of fibrosis and inflammation (IFNG, UBD, CXCL9, CXCL10, CD14, FOXP3 upregulation) in 1-year protocol biopsies was associated with reduced graft function and survival
'Late biopsies' Pribylova- Hribova et al. [34], 2006	174	qRT-PCR	TGFB1 mRNA upregulation in chronic allograft nephropathy predicted poor renal graft function within the 18-month follow-up
Hribova et al. [35], 2007	74	qRT-PCR	Higher expression of TGFB1 and MCP1 in chronic allograft nephropathy was associated with higher risk of graft loss
Einecke et al. [36], 2010	105	microarray	Transcripts reflecting tissue injury (e.g. ITGB6, HAVCR1, LTF, ADAMTS1, SERPINA3, MMP7, C3, COL1A2), dedifferentiation and epithelial-to-mesenchymal transition (NNMT, VCAN), and tissue remodeling, but not those reflecting inflammation (IFNG effects and T cell or macrophage infiltration), predicted graft failure
Famulski et al. [37], 2010	234 (173)	microarray qRT-PCR	The canonical form of TCMR in late biopsies was defined on the basis of molecular phenotype and independent of histopathology with AMATs and GRITs as the main feature and with the best prognosis despite severe inflammation
Sis et al. [40], 2009	212/169	microarray	High endothelial-associated transcript expression with antibody predicts graft loss in AMR patients better than C4d
Figures in parentheses indicate number of patients. ^a Training set. ^b Test set. ^c Taqman low-density array.			

The role of intra-graft B cells in renal transplantation is the subject of many studies with controversial results. The B cell infiltrate during acute rejection was shown to be associated with poor prognosis in some [19, 20] but not all reports [21]. In early acute rejection, we showed several genes to be expressed differentially between AMR and TCMR and distinct molecular phenotypes were associated with poor kidney graft prognosis. Expression profiles suggesting lack of regulation defined as lower expression of FOXP3, TGFB1, RANTES and especially CD20, a marker of B cells, were observed within the graft that failed as a consequence of AMR [22]. What is the explanation of such differences in the published literature? The role of B cells turns out to be very complex and varied. Activated B cells are known to have the potential to serve as antigen-presenting cells, sustain T cell response in the graft and they may be related to more severe rejection outcome. B cells, however, may also play a role in

transplantation tolerance, as indicated in recent reports from the ITN and RISE consortia [23]. Drug-free tolerant patients displayed increased numbers of B cells in peripheral blood, with a decreased memory pool and an increase in transitional and naïve B cell subsets. Enhanced expression of B cell differentiation and activation genes in tolerant patients was also reported [24]. The current view on the role of B cells in kidney graft rejection as well as in graft tolerance was reviewed by Zarkhin et al. [25].

A role of regulatory T cells in acute rejection is widely discussed as well. We showed a low FOXP3 intrarenal transcript to be associated with poor outcome of early antibody-mediated rejection [22]. Similarly, it was shown that the presence of FoxP3+ Treg in subclinical rejection in the 6th month predicts better graft function 2 and 3 years after transplantation [26]. Although the expression of FOXP3 in transplant biopsies is studied due to its role in a population of regulatory T cells, Bunnag et al. [27]

showed evaluated expression of FOXP3 in rejection (both TCMR and AMR). They observed no relationship between the levels of FOXP3 or FOXP3/GZMB expression and future function.

Another enhanced step in studying gene expression is the focus on microRNAs (miRNAs). miRNAs are small, highly conserved RNA molecules acting as posttranscriptional regulators, and so controlling gene expression. Anglicheau et al. [28] reported that a number of tubular epithelial cell miRNAs are underexpressed during acute cellular rejection, while others are overexpressed, probably reflecting the graft infiltration by immune cells. They evaluated the set of miRNAs predictive of acute rejection and allograft function. miRNAs have also been reported to play a role in the regulation of the fibrosis process including epithelial-to-mesenchymal transition, so they also influence later states of renal allografts [29].

'Protocol Biopsies'

The question is, in general, whether a distinct molecular phenotype defined as upregulation of specific mRNA clusters may predict graft prognosis better than routine histology itself. Mengel et al. [30], in a larger study of 6 weeks' protocol biopsies early after transplantation, did not find any association of gene expressions with future rejection episodes, functional deterioration or allograft loss. The authors concluded that early protocol biopsies reflect more injury-repair response to implantation stresses and have little relationship to graft outcome. Recently, by analyzing the intrarenal expression of 9 genes (cytokines, chemokines, markers of T/B/plasma cell infiltration) in 163 protocol biopsies at 12 weeks, we showed the high expression of chemokines RANTES and IP-10 in subclinical rejection including borderline changes to be associated with the higher risk of premature graft loss [31]. Clearly, this study was small and thus should be considered as preliminary and validation studies in larger cohorts are necessary. Scherer et al. [32] analyzed microarray expression profiles in 6-month protocol biopsies, in order to determine genes predictive of chronic rejection development 12 months after kidney transplantation. Up to now, they have identified 10 genes differentially expressed in patients developing chronic rejection within the following 6 months compared to patients who remained stable during follow-up. Such data support the hypothesis that changes in gene expression patterns in the transplanted kidney graft may be detected early prior to the apparent clinical and histological manifestation of chronic rejection. Moreover, kidney graft recipients with normal morphology or only interstitial fibrosis and tubu-

lar atrophy and without inflammation in 12-month protocol biopsies showed significantly better survival than patients with interstitial fibrosis and tubular atrophy together with subclinical inflammation. The combination of fibrosis and inflammation was associated with increased activity of innate immune pathways including IFNG and TLR responses as well as increased T cell immunity (IFNG, UBD, CXCL9, CXCL10, CD14, FOXP3) and downregulation of protective gene products (TOLLIP, VEGF, BCL2) [33].

'Late Biopsies'

TGFB1 was shown to be upregulated in both acute and chronic rejection of kidney allograft. The expression of TGFB1 during acute and chronic rejection and its relation to transplant outcome was reported [34] by our group. We demonstrated no association of TGFB1 mRNA levels with the short-term acute rejection outcome, but TGFB1 mRNA upregulation in chronic allograft nephropathy predicted poor renal graft function within the 18-month follow-up. This might indicate the importance of the fibrosis phenotype in late-stage kidney grafts. Similarly, higher expression of intrarenal proinflammatory genes in biopsies with chronic allograft nephropathy was shown to be able to discriminate patients at a higher risk for earlier allograft failure. Kaplan-Meier survival analysis revealed that proteinuria and higher expression of TGFB1 and MCP1 in transplant patients with chronic allograft nephropathy were a prognostic of poor graft outcome. This suggests that unfavorable outcome depends on tissue remodeling and fibrosis together with active immune processes in late biopsy samples [35].

This hypothesis was confirmed by a large study of Eicken et al. [36]. They analyzed gene expression in 105 for-cause biopsies using microarrays to identify genes associated with graft loss and developed the molecular risk scores strongly associated with graft loss. They found that the transcripts predicting graft failure were those reflecting tissue injury (e.g. ITGB6, HAVCR1, LTF, ADAMTS1, SERPINA3, MMP7, C3, COL1A2), dedifferentiation and epithelial-to-mesenchymal transition (NNMT, VCAN), and tissue remodeling, but not those reflecting inflammation (IFNG effects and T cell or macrophage infiltration). Risk predictions were accurate when applied to the late biopsy population. It indicates that the active ongoing tissue response to injury is the final common pathway linking mechanisms of inflammation and noninflammatory disease states to parenchymal loss, dysfunction and kidney failure. Similarly, in progressive but not in stable proteinuric disease, proximal

tubular epithelial cells showed elevated expression of genes of intracellular hypoxia response and VEGF signaling pathways (e.g. CREBBP, PTK2B, NOS1, MAPK1, HIF1A) [3]. Famulski et al. [37] defined the canonical form of TCMR (cTCMR) in late biopsies on the basis of molecular phenotype and independent of histopathology. The molecular features of cTCMR were expression of alternative macrophage activation transcripts (AMAT1) and high levels of IFNG-induced transcripts (GRIT1). The cTCMR biopsies were the most severely inflamed, but surprisingly had the best prognosis. The importance of molecular phenotype can be further illustrated on chronic AMR. Although the diagnosis of AMR is based on the presence of circulating donor-specific antibodies, histopathologic evidence of tissue injury and C4d-positive staining [1], there is recent evidence that some type of chronic AMR can occur without C4d+ [38, 39]. AMR is dominated by endothelial damage in microcirculation. Endothelial-associated transcripts identified from the literature were reported to be upregulated in both, C4d-negative and C4d-positive forms of AMR. Moreover, high endothelial-associated transcript expression with alloantibody predicted poor kidney graft outcome and graft loss better than C4d [40].

Noninvasive Markers of Graft Prognosis

When searching for noninvasive methods to find biomarkers in renal transplantation, urine seems to be the optimal fluid for investigation of different intra-graft processes. Earlier studies showed urine levels of mRNAs for PRF and GZMB to be associated with and even predictive of acute rejection. Muthukumar et al. [41] analyzed transcripts from urine sediment and found FOXP3, CD25, CD3E, and PRF mRNA in 36 renal allograft recipients with acute rejection, 18 subjects with chronic allograft nephropathy and 29 subject with normal biopsy results. They found expression levels of all investigated genes to be upregulated in acute rejection as compared with chronic allograft nephropathy or normal biopsies, but only FOXP3 mRNA urinary levels were predictive of the acute rejection outcome or graft failure. Matz et al. [42] showed IP-10 expression in urine to identify patients with ongoing acute rejection episodes several days before serum creatinine elevation and biopsy indication. Elevated levels of IP-10 protein within the first 4 posttransplant weeks were predictive of restricted graft function at 6 months even in the absence of acute rejection. Finally, Afaneh et al. [43] reported that acute rejection could be

predicted by the urinary levels of mRNA for OX40, OX40L, PD-1, and FOXP3. The acute rejection reversibility was predicted by the levels of mRNA for OX40, OX40L, and FOXP3, whereas only OX40 mRNA levels predicted graft loss after acute rejection.

Besides the studies of mRNA expression in urinary cells, urine proteomics becomes an important field in the study of markers of renal graft dysfunction. Although several studies identified a 'rejection pattern' that is able to discriminate samples with acute rejection from samples with stable graft function [44, 45], the influence of immunosuppressive drugs and possible infections on the urine proteome remains to be discussed [46]. Also differences in urinary polypeptide patterns related to chronic allograft dysfunction have been identified recently. O'Riordan et al. [47] found several urinary proteins that are associated with chronic allograft nephropathy. Following the latest Banff classification, Quintana et al. [48] showed that the composition of the proteome differs between interstitial fibrosis and tubular atrophy and chronic active AMR.

Conclusion

Molecular biomarkers are much more specific than routine laboratory tests used for graft monitoring and can discriminate between different mechanisms of injury. Analyzing the role of distinct molecular phenotypes of kidney graft diseases using molecular pathology methods may help to better define patients at risk that need early or different intervention. On the other hand, such techniques may identify patients in whom the alloimmune response has been silenced and who do not need more than minimal immunosuppression.

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