Intrgraft Toll-like receptor profiling in acute renal allograft rejection

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Abstract

Background. Experimental studies have shown potential for Toll-like receptor (TLR) profiling in renal allograft in predicting renal outcome after transplantation. Our goal was to determine if profiling of TLR1–10 and TLR-related genes could be used as a prognostic value for renal function and late clinical outcome after transplantation.

Methods. TLR1–10, CD14, MD-2 and negative regulators Toll-interacting protein (TOLLIP) and single immunoglobulin domain IL-1R-related receptor (SIGIRR) and Toll-like receptor (TLR) profiling in renal allograft in association with reduced mRNA levels of TOLLIP in renal transplant recipients without rejection (NR). Analysis was performed by multiplex ligation-dependent probe amplification. TLR (-related) genes were correlated to Banff’07 classification, cellular influx, response to conventional anti-rejection therapy, renal function 12 and 24 months after rejection and graft loss.

Results. mRNA levels of most TLRs were significantly higher in acute rejection while TOLLIP mRNA level was decreased. mRNA levels of TLR1/2/4/7/8 were highly accurate in distinguishing AR from NR. TLR mRNA levels correlated to inflammatory parameters according to the Banff’07 classification and to cellular influx. Elevated mRNA level of TLR3 in acute rejection was independent from infiltrating leukocytes. TLR (-related) genes were not correlated with response to conventional anti-rejection therapy. Splice variant TLR4r3 was associated with poor renal function 24 months after transplantation, and TLR1 appeared to be associated with graft loss.

Conclusion. The elevated mRNA levels of several TLRs in association with reduced mRNA levels of TOLLIP in renal transplant biopsies of patients with acute rejection indicate a pro-inflammatory state, which may contribute to uncontrolled inflammation.

Keywords: allograft rejection; kidney; Toll-like receptors; transplantation

Introduction

Acute renal allograft rejection still occurs in 10–20% of patients after renal transplantation and causes graft loss in up to 6% in the first year after transplantation [14]. The occurrence of an acute episode of rejection is a major determinant of long-term allograft function. In rejection, the role of innate immunity has been re-appreciated [4] with special interest in Toll-like receptors (TLRs). TLRs are conserved pattern recognition receptors of which 10 are described in humans and are expressed by a variety of immune and non-immune cells and widely expressed in the kidney [16]. TLRs detect motifs of pathogens and subsequently induce innate and adaptive immunity [1,23]. In addition, they recognize endogenous molecules released during cellular injury [12]. Excessive or prolonged TLR signalling may induce profound inflammation and tissue injury. To achieve immunological homeostasis, negative feedback regulation of TLR signalling is crucial. Negative regulators of TLR signalling like single immunoglobulin domain IL-1R-related receptor (SIGIRR) and Toll-interacting protein (TOLLIP) are expressed in the kidney [16,19,25]. Despite the known roles for TLRs in the activation of innate immunity and triggering of adaptive immunity, their implication in graft rejection or acceptance remains a relatively unexplored field [3,4]. Several studies have shown potential for TLR profiling in predicting renal outcome after transplantation [7,9,13], which are mainly focused on TLR2 and TLR4. Our goal was to investigate if profiling of TLR (-related) genes including negative regulators TOLLIP and SIGIRR in renal biopsies correlates with acute rejection and could predict response to conventional anti-rejection therapy and/or late clinical outcome.

Materials and methods

Patients

Renal biopsies analysed in the present study were obtained from a cohort used in earlier studies [20,26]. Immunosuppressive medication consisted of induction therapy with basiliximab (Simulect; Novartis Pharma B.V., Arnhem, The Netherlands), prednisolone and a calcineurine inhibitor cyclosporine (Neoral; Novartis Pharma B.V.) or tacrolimus (Prograf; Astellas Pharma, The Netherlands). In addition, patients received mycophenolate mofetil (Cellcept; Roche Nederland B.V.) [20,26]. Demographic characteristics of the patients are summarized in Table 1. Written informed consent was obtained from all study patients, and the study was approved by the institutional ethical committee of the Academic Medical Center.
Clinical outcome and histopathology

Clinical outcome was determined according to the following definition: response to anti-rejection therapy was defined as a decrease in serum creatinine level to maximally 125% of the value before the clinically diagnosed episode of allograft rejection. Late clinical outcome was defined by estimation of the glomerular filtration rate 12 and 24 months after rejection episode using the Modification of Diet in Renal Disease Study equation or by graft loss (return to dialysis and/or transplantectomy). All biopsies were scored according to the Banff'07 classification [22]. Immunohistochemical staining for C4d was performed as previously described [20]. Antibody-mediated rejection was defined as C4d staining in >10% positive peritubular capillaries on paraffin-embedded material according to the Banff'07 recommendations [22].

Multiple ligation-dependent probe amplification and RT–PCR

For mRNA analysis, a second specimen of the renal biopsy was snap frozen and 10–15 10-μm sections of renal cortex were cut with a Microm HM500 cryostat (Adamas Instruments BV), collected in an Eppendorf tube containing TRIzol (Invitrogen, Breda, The Netherlands) after which RNA was directly extracted according to the manufacturer's protocol. RNA was subsequently purified by RNasey spin columns (Qiagen, Westburg, The Netherlands). TLR1–10, CD14 and MD-2 mRNA expression was analysed by multiple ligation-dependent probe amplification (MLPA) as previously described [15]. TLR6 was removed because it would give a signal on contaminating chromosomal DNA. Therefore, TLR6 was determined by RT–PCR. Thirty-one out of 36 samples of acute rejection and 14 control renal samples were usable for MLPA. Primers used for RT–PCR are described in Supplementary Material Table 1. All 36 samples of acute rejection and 14 control renal samples were usable for RT–PCR. Levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of housekeeping gene poly(A)-specific ribonuclease (PARN).

Statistical analysis

SPSS 17.0 statistical software (SPSS Benelux B.V., Gorichem, The Netherlands) for social science was used for the statistical analysis. Differential expression was determined by RT–PCR. Non-parametric Mann–Whitney rank-sum test. Spearman’s rank correlation coefficient was used to test the correlation between variables. Receiver operating characteristic (ROC) curves were used to analyse normalized mRNA levels in order to determine the cut-off point that yielded the highest combined sensitivity and specificity for distinguishing acute rejection from non-acute rejection. Area under ROC curve was determined to summarize the discriminative ability of the test. A two-tailed P < 0.05 was considered statistically significant. For MLPA data, we additionally examined Bonferroni correction to take into account the number of tests performed by adjusting the P-value. To do so, we applied a two-tailed significance level of 0.0034 (0.05/14 genes). Graphs were constructed in GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

Results

Clinical and biochemical parameters of the included patients

No statistical difference was observed between patients diagnosed with acute rejection (AR) and without acute rejection (no rejection; NR) concerning patient and donor characteristics, donor source, eGFR at 12 and 24 months, HLA mismatches, cold ischaemia time, number of biopsies from second acute rejection and anti-thymocyte globulin (ATG) treatment (Table 1).

Profiling of TLR (-related) genes in renal biopsies upon acute rejection

We first determined mRNA levels of TLR1–5/7–10, CD14 and MD-2 using MLPA. Of note, TLR6 mRNA levels were determined by RT–PCR. Except for TLR6 and TLR10, all detectable TLRs, CD14 and MD-2 were significantly increased in AR compared with NR (Figure 1A). When post hoc Bonferroni correction was applied to correct for the number of tests performed, mRNA levels of TLR1–4/7/8, CD14 and MD-2 were still significantly increased in AR compared with NR (all P < 0.0034). To verify the results from MLPA, we determined mRNA levels of the two most well-studied TLRs, TLR2 and TLR4, by quantitative RT–PCR and expressed them relatively to housekeeping gene PARN showing comparable results [NR vs AR, TLR2: 2.11 (0.83–3.59) vs 4.82 (0.0133.27), P = 0.001 and TLR4: 0.19 (0.1–1.65) vs 0.38 (0.08–5.66), P = 0.002; data are median (range)]. SIGIRR and TOLLIP mRNA levels were determined by RT–PCR. Although SIGIRR mRNA level was similar between renal biopsies from NR and AR, TOLLIP mRNA level was decreased in renal biopsies from AR compared with NR (Figure 1B).

Accuracy and specificity of mRNA diagnostic test

The ROC curve is generally accepted as a method to determine the accuracy of the test [2,18]. Results are displayed in Table 2. We analysed the ability of TLR (-related) mRNA diagnostic testing in order to distinguish between AR and NR by analysing the area under ROC curve (AUC). TLR1–4/4, TLR7–8r2, CD14 and MD-2 mRNA levels were highly accurate in distinguishing AR from NR, also when P-value was corrected for Bonferroni (AUC ~0.90 or higher [2], all P < 0.0034; Table 2). TLR6, TLR10 and SIGIRR were not significantly different from reference line (data not shown). High sensitivity (~90% or
higher) was observed with TLR2, TLR4r3, TLR8r2 and CD14 mRNA levels. Of note, TLR8r2 also displayed high specificity (93%).

**Correlation between TLR (-related) genes and Banff’07 classification**

Correlation between TLR (-related) genes and histological grading according to Banff’07 classification (tubulitis, interstitial infiltrate, glomerulitis, arteritis, interstitial fibrosis, tubular atrophy, vascular thickening and mesangial increase) and C4d positivity were determined. A summary with significant correlations is displayed in Table 3. TLR1, TLR4, TLR7, TLR8 and MD-2 mRNA levels correlated with tubulitis and interstitial inflammation. TLR1–4, TLR8, CD14 and MD-2 mRNA levels correlated with arteritis. TLR3 and TLR10 mRNA levels were correlated with mesangial increase and interstitial fibrosis, respectively. When post hoc Bonferroni P-value correction was applied to correct for the number of tests performed, TLR2, TLR4r1/3 and TLR8r2 were especially correlated to arteritis and TLR4r1/3 also for interstitial infiltrate (all P < 0.0034). TLR6, TOLLIP and SIGIRR mRNA levels did not correlate to any histological grading (data not shown). Positive C4d immunostaining in peritubular capillaries is a surrogate marker for antibody-mediated rejection [6]. Five out of 36 biopsies had a cellular and antibody-mediated rejection while other biopsies displayed a cellular-mediated rejection only. No difference in mRNA levels of TLR (-related) genes was observed between C4d-negative and C4d-positive biopsies (data not shown).

**Correlation between TLR (-related) genes and cellular influx**

The hallmark of acute allograft rejection is the infiltration of numerous inflammatory cells into the graft [22]. Therefore, the mRNA levels of TLR-related genes observed in renal biopsies upon AR might be related to the inflammatory infiltrate. As expected, biopsies from AR showed significantly higher mRNA levels of CD3ε, CD20 and CD68 (representing T cells, B cells and macrophages, respectively) compared with NR [NR vs AR—CD3ε: 0.96 (0.23–12.52) vs 5.02 (0.48–26.14), P < 0.0005; CD20: 0.64 (0.01–14.03) vs 5.01 (0.11–109.10), P < 0.001;...
CD68: 13.60 (4.42–70.40) vs 38.70 (5.24–271.00), P < 0.005; data are median (range)]. Correlations between mRNA levels of TLR (-related) genes and CD3ε, CD20 and CD68 mRNA levels are summarized in Table 4. Except for TLR3, TLR (-related) genes correlated with one or more markers of leukocytes.

**Association between TLR (-related) genes and response to therapy**

To determine if TLR (-related) genes have a prognostic value for response to conventional anti-rejection therapy, we compared mRNA levels of these genes within the group of acute rejection. Measurable mRNA levels of TLR1–10, CD14, MD-2, TOLLIP and SIGIRR mRNA levels were not associated with response to anti-rejection therapy (Figure 2).

**Association between TLR (-related) genes and late renal outcome**

As expected, eGFR at 12 and 24 months after transplantation was significantly improved in patients responding to conventional anti-rejection therapy [non-responders vs responders—12 months: 52 (23–98) vs 45 (18–72), P < 0.05; 24 months: 56 (25–85) vs 41 (11–54), P < 0.01; data are median (range)]. We determined if TLR (-related) genes could predict late renal outcome. Except for splice variant TLR4r3 mRNA, which correlated with poor renal function defined by eGFR at 24 months, no correlation could be found between other TLR (-related) genes at time of acute rejection and late renal function (data not shown). Within the acute rejection group of 35 patients, five patients returned to dialysis. TLR1 mRNA level was associated with return to dialysis (Figure 3). We analysed the ability of TLR1 gene expression to distinguish the two groups using ROC curve showing moderate accuracy of this diagnostic test (AUC 0.831 ± 0.085, P = 0.021, sensitivity 80% and specificity 81%). TLR4r1, TLR4r4 and MD-2 mRNA levels showed a trend associated with return to dialysis (Figure 3; TLR4r1, P = 0.096; TLR4r4, P = 0.068; MD-2, P = 0.060). Other determined genes were not associated with return to dialysis.

**Discussion**

In our study, mRNA levels of different TLRs and TLR regulator TOLLIP were respectively elevated and decreased in acute rejection biopsies compared with non-acute rejection biopsies and were correlated to inflammatory parameters according to the Banff'07 classification. Interestingly, TLR3 mRNA level was elevated in biopsies displaying acute rejection but, unlike most TLRs, was not correlated to cellular infiltrate, suggesting that this molecule in particular is up-regulated in renal parenchymal cells.

Several experimental studies have shown the contribution of TLRs in renal diseases and rejection [10,21]. However, knowledge about expression and contribution of TLRs in human allograft rejection is limited and studies are focused on TLR2 and TLR4 [7,9,13]. We are the first to determine all TLRs in biopsies from patients with AR. Earlier studies showed that in renal disease like acute or chronic rejection,
TLR4 mRNA levels are elevated compared with the control group or patients with operational tolerance [7,13]. TLR4 mRNA level was also significantly higher in peripheral blood mononuclear cells (PBMCs) of patients with chronic rejection compared with PBMCs of patients with stable graft function and displayed a higher percentage of TLR4-expressing monocytes [7]. These data implicate that TLR4 plays a potential pathophysiological role in the immune response during rejection. TLR4 mRNA transcripts are alternatively spliced into four mRNA molecules. In our study, the four different splice variants of TLR4 were measured using MLPA. Splice variant TLR4r3 showed a trend associated with poor renal function at 12 months and was significantly associated with poor renal outcome at 24 months. Variant TLR4r3 lacks the possibility for translocation of the TLR4 protein to the cell surface [17], suggesting that TLR4r3 might be a soluble factor with inhibitory properties [11]. Further studies are needed to determine if this splice variant is of significance in acute rejection.

TOLLIP associates directly with TLR2 and TLR4 and plays an inhibitory role in TLR-mediated cell activation. Negative regulation of the TLR signalling by TOLLIP, therefore, serves to limit the production of pro-inflammatory mediators during inflammation. To our knowledge, we are the first to describe TOLLIP mRNA in renal biopsies during acute rejection. The relative down-regulation of TOLLIP mRNA levels which were observed might well lead to an imbalance between pro- and anti-inflammatory mechanisms and to an uncontrolled inflammation upon acute renal rejection. In contrast to TOLLIP, another negative regulator of TLR signalling SIGIRR was not down-regulated upon acute rejection.

TLR3 is known to detect double-stranded viral RNA [5], and recently Cavassani et al. showed that TLR3 recognizes endogenous RNA released by necrotic cells [8]. TLR3 mRNA level was elevated in acute rejection and, unlike most other TLRs, was not correlated with the influx of inflammatory cells, suggesting that elevated TLR3 levels in acute rejection originate from renal parenchymal cells. TLR3 is constitutively expressed at low levels by tubular epithelial cells and is up-regulated upon stimulation in vitro [24]. Hypothetically, elevated TLR3 expression on tubular epithelial cells could contribute to the regulation of inflammation during acute rejection.

**Fig. 2.** Gene profiling in non-responders and responders to conventional anti-rejection therapy. mRNA levels of TLR1–4, 6–8, 10, CD14 and MD-2 (A), TOLLIP and SIGIRR (B) in renal biopsies from patients defined as non-responders (No) and responders (Yes). Data are expressed relatively to housekeeping gene PARN. Results are reflected in box-and-whisker graphs showing range, lower quartile, median and upper quartile. TLR r1/3/4: different probe sets were generated corresponding with different splice variants.

**Fig. 3.** Gene profiling and graft loss. mRNA Levels of TLR1–4, 6–8, 10, CD14 and MD-2 (A), TOLLIP and SIGIRR (B) in biopsies from patients that do not return to dialysis (No) and patients that do return to dialysis (Yes). Data are expressed relatively to housekeeping gene PARN. Results are reflected in box-and-whisker graphs showing range, lower quartile, median and upper quartile. TLR r1/3/4: different probe sets were generated corresponding with different splice variants. *P < 0.05 vs No (not corrected for Bonferroni).
In conclusion, mRNA levels of most TLRs were significantly higher in acute rejection and correlated to cellular influx and inflammatory parameters according to the Banff’07 classification. However, the elevated mRNA level of TLR3 in acute rejection was independent from infiltrating leukocytes and may be related to activated renal parenchymal cells. The elevated mRNA levels of TLR2 and TLR4 in association with reduced mRNA levels of TOLLIP indicate a pro-inflammatory state in renal tissue during acute rejection, which may contribute to uncontrolled inflammation.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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