Transplant Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Elevated intragraft expression of innate immunity and cell death-related markers is a risk factor for adverse graft outcome

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ARTICLE INFO

Keywords:
Toll like receptors
Complement
Apoptosis
Gene expression
Kidney transplant outcome

ABSTRACT

Background: Molecules of the innate immune response are increasingly recognized as important mediators in allograft injury during and after kidney transplantation. We therefore aimed to establish the relationship between the expression of these genes at implantation, during an acute rejection (AR) and on graft outcome.
Methods: A total of 19 genes, including Toll like receptors (*TLRs*), complement components and regulators, and apoptosis-related genes were analyzed at the mRNA level by qPCR in 123 biopsies with acute rejection and paired pre-transplantation tissue (n = 75).
Results: Before transplantation, relative mRNA expression of *BAX:BCL2* ratio (apoptosis marker) and several complement genes was significantly higher in tissue samples from deceased donors compared to living donors. During AR, TLRs and complement genes showed an increased expression compared to pre-transplant conditions, whereas complement regulators were decreased. A relatively high *TLR4* expression level and *BAX:BCL2* ratio during AR in the deceased donor group was associated with adverse graft outcome, independently of clinical risk factors.
Conclusions: Complement- and apoptosis-related gene expression is elevated in deceased donor transplants before transplantation. High *BAX:BCL2* ratio and *TLR4* expression during AR may reflect enhanced intragraft cell death and immunogenic danger signals, and pose a risk factor for adverse graft outcome.

1. Introduction

The occurrence of an acute kidney allograft rejection, associated with infiltration of recipient immune cells to the kidney, is a risk factor for adverse graft outcome [1]. The role of innate immunity including pattern recognition receptors and the complement system in rejection has been appreciated [2,3]. Toll like receptors (TLRs) are a family of transmembrane proteins that are capable of recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [4]. TLR stimulation leads to dendritic cell maturation, characterized by upregulation of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which initiate an immune response [5]. Endogenous ligands including heat-shock proteins (HSP) [6], uric acid [7], high-mobility group box 1 protein (HMGB1) [8,9], and genomic double-stranded DNA [10] may stimulate TLRs. The interaction between HMGB1 and TLR4 leads to proinflammatory responses in the graft: after kidney transplantation, recipients with a donor graft containing a genotype variant in the coding sequence of TLR4 had lower expression of proinflammatory genes MCP-1 and TNFα and higher expression of anti-inflammatory heme oxygenase 1, and they showed an increased rate of immediate graft function [11]. Association of TLR2 and TLR4 expression was found with renal ischemia reperfusion injury (IRI) and early kidney allograft outcomes [12,13]. Other TLRs have not been investigated in the context of delayed graft function (DGF) and acute rejection (AR).

The complement system plays a pivotal role in ischemia reperfusion injury and allograft rejection after transplantation [3]. The expression of complement components is significantly increased in deceased donor

Abbreviations: AR, acute rejection; ATG, antithymocyte globulins; BAX, Bcl-2-associated X protein; BCL2, B-cell lymphoma 2; CI, 95% confidence interval; DAMP, damage-associated molecular patterns; DGF, delayed graft function; FFPE, formalin-fixed and paraffin-embedded; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGB1, high-mobility group box 1 protein; HR, hazard ratio; HSP, heat-shock proteins; IRI, Ischemic renal injury; MAC, membrane attack complex; PAMP, pathogen-associated molecular patterns; TLRs, Toll-like receptors

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https://doi.org/10.1016/j.trim.2018.02.009
Received 18 January 2018; Received in revised form 13 February 2018; Accepted 14 February 2018

kidneys after cold ischemia [14,15]. Activation of the complement cascade leads to the release of anaphylatoxins (C3a and C5a) and the formation of the membrane attack complex (MAC) C5b–C9, which mediates the injury following transplantation [16,17]. C2 and C4 are essential components in the classical and lectin pathway, and C3 plays a central role in all pathways of the complement system. Complement regulators act as inhibitors of the complement cascade through various mechanisms [18,19]. For example, the decay acceleration factor (CD55) prevents the formation of C3 convertase. CD46 acts as cofactor for inactivating C3b and C4b by serum factor I. Complement receptor 1 both has decay-accelerating activity and cofactor activity. CD59 prevents the formation of MAC. Deficiency of CD55 and CD59 in experimental settings leads to increased renal ischemia reperfusion injury [20,21]. In C4d-negative biopsy specimens during allograft dysfunction local CD55 expression was related to favorable transplant outcome [22].

The role of apoptosis in IRI after kidney transplantation is increasingly being recognized [23,24]. The anti-apoptotic protein B-cell lymphoma 2 (BCL2) was significantly decreased and pro-apoptotic protein BCL2-associated X protein (BAX) was increased during normothermic ischemia [25]. The augmentation of BCL2 protects renal tubular cells from IRI through reducing renal tubular epithelial cell apoptosis [26]. High ratios of *BAX:BCL2* in pre-transplant biopsies are associated with an increased risk of DGF [27].

In the present study, we examined innate-immune-related and apoptosis-related markers in kidney biopsies of 125 patients before transplantation and during an acute rejection episode, and investigated their relation to clinical outcome.

2. Methods

2.1. Patient characteristics

Patients who had received a kidney allograft at the Leiden University Medical Center (LUMC) during 1995–2005 were included. A total of 123 for-cause biopsy samples in case of clinical suspicion of AR were obtained within 6 months after transplantation, and 77 pre-transplantation biopsies (75 biopsies paired to the subsequent AR biopsy) were taken at time of transplantation before reperfusion. Patient characteristics are shown in Table 1. Delayed graft function was defined as dialysis-dependency in the first week after transplantation.

2.2. Ethics

Written informed consent was obtained from donors for use of part of the human material for scientific purposes. The study were performed in accordance with the Declaration of Helsinki Good Clinical Guidelines and approved by the local medical ethics committee.

2.3. Gene selection

The innate immune related genes (*TLR1-TLR10*), potentially acting as initiators of inflammation, were studied. The key complement component (*C2*, *C3*, *C4*) and complement regulators (*CR1*, *CD46*, *CD55*, *CD59*), which inhibit complement activation, were included. The apoptosis related genes *BAX* and *BCL2*, which may be associated with IRI and DGF, were also tested.

2.4. RNA extraction and cDNA synthesis

RNA isolation and quality check, and cDNA synthesis were performed as described previously [28].

2.5. Real time quantitative PCR analysis

Optimal primers pairs were selected using Primer 3 version 4.0.0.

Table 1
Demographics of patient cohort.

Variable	Number (%)
Recipient age (≥ 50 years)	53 (43.1%)
Recipient gender (Female)	40 (32.5%)
Donor age (≥ 50 years)	52 (42.6%)
Donor gender (Female)	74 (60.7%)
Donor type (Living)	24 (19.5%)
Time from transplant to rejection (days, IQR)	14 (9–37)
First transplantation (Yes)	103 (84.4%)
HLA–A/B matching (Yes)	20 (16.4%)
HLA-DR matching (Yes)	43 (35.2%)
Virtual PRA (0–5%)	81 (66.4%)
DGF (Yes)	33 (28.7%)
Steroid responsiveness	68 (56.2%)
Cold ischemia time (≤ 18 h)	31 (29.8%)
Banff score	
Glomerulitis (g = 0/1/2/3)	74/25/7/3
Interstitial inflammation (i = 0/1/2/3)	5/44/36/24
Tubulitis (t = 0/1/2/3)	11/39/38/21
Intimal arteritis (v = 0/1/2/3)	62/24/7/7
Interstitial fibrosis (ci = 0/1/2)	61/41/7
Tubular atrophy (ct = 0/1/2)	60/44/5
C4d diffuse positive	14 (11.4%)
Rejection characteristics	
No rejection	7 (5.7%)
Borderline rejection	33 (27.0%)
Interstitial rejection	42 (34.4%)
Vascular rejection	40 (32.8%)
Graft survival (Death censored)	
> 1 year	106 (92.2%)
> 6 year	101 (87.8%)

HLA, human leukocyte antigen; PRA, panel reactive antibodies; DGF, delayed graft function.

To prevent amplification of genomic DNA, reverse and forward primers were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. All primer sets were tested on control cDNA, and PCR efficiencies were between 90% and 110%. The 15-μL qPCR reaction contained 3 μL of 25-times-diluted cDNA, 15 pmol forward and reverse primers, 7.5 μL of PCR Mix (Applied Biosystems by Life Technologies, Austin, Texas, USA), and nuclease-free water [29]. Relative gene expression levels were normalized to the geometric mean of the reference genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.6. Immunohistochemistry

Immunohistochemical studies were performed on an independent set of 34 formalin-fixed and paraffin-embedded (FFPE) kidney biopsy samples: 25 from patients with AR and 9 protocol biopsies from patients with stable graft function. Patients included in this group were transplanted between 2006 and 2015. Monoclonal anti-human antibodies against BAX (ab32503, Abcam, 1:1400 dilution), BCL2 (Sp66, Ventana), TLR4 (ab22048, Abcam, 1:800 dilution), and TLR9 (clone 26C593.2, Novus, 1:800 dilution) were used for immunohistochemistry on sequential 4-μm sections. Staining procedures have been described in a previous publication [30]. Semi quantitative scoring of the number of BCL2-, TLR4-, and TLR9 positive tubular epithelial cells was performed blindly by two observers using a scale from 0 to 5 (0 = 0%, 1 ≤ 10%, 2 = 10–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100%).

2.7. Statistical analyses

Gene expression differences in paired (PreTx, AR) tissue samples were analyzed using Wilcoxon signed ranks test. Differences in gene expression between deceased and living donors and the occurrence of DGF were assessed by Mann-Whitney *U* tests (two-sided). Correlations between innate immunity mRNA expression levels and mRNA