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Renal expression of Toll-like receptor 2 and 4: Dynamics in human allograft injury and comparison to rodents

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ABSTRACT

Activation of the innate immunity through Toll-like receptors (TLRs) has been postulated to play an important role in the pathophysiology of renal allograft dysfunction. TLR2 and TLR4 dynamics in different human post-transplant pathological entities has never been studied. Therefore, we evaluated pre- and post-transplantation protein expression of TLR2 and TLR4 in human kidney biopsies.

Human kidney biopsies obtained from living kidney donors and patients with acute tubular necrosis, acute cellular and vascular rejection and interstitial fibrosis/tubular atrophy (IF/TA) were used. Translating results from animal studies to the clinical situation is highly important considering the upcoming clinical studies with TLR inhibitors in human renal transplantation. Hence, the TLR2 and TLR4 expression in healthy mouse and rat kidneys was analyzed and compared with human kidneys. In healthy human kidneys, TLR2 is expressed on the endothelium and Bowman's capsule, while TLR4 is expressed on the endothelium only. No tubular staining was found for both receptors in human kidneys. In contrast to human biopsies, TLR2 and TLR4 expression in rodents was observed on tubular epithelial cells. In all acute rejection human biopsies, increased infiltration of $TLR4^{+}$ leukocytes was observed. In conclusion, a discrepancy exists between human and rodent renal TLR expression, which suggests careful attention when translating results from rodent studies to the human situation. Additionally, this study revealed human TLR2 and TLR4 expression dynamics in human biopsies pre- and post-transplantation.

1. Introduction

Renal transplantation currently represents the preferred treatment for the majority of patients with end-stage renal disease (ESRD). The number of patients with ESRD has doubled during the last decade in Europe and the United States (Grassmann et al., 2005). Advancements in renal transplantation immunology has led to a success rate of >90% for the first year, a result of the decreased incidence of acute rejection (Kasiske et al., 2005). Unfortunately, long-term renal allograft survival has not improved significantly over the past decade (Meier-Kriesche et al., 2004). Mechanisms responsible for long-term renal allograft loss are both immune and non-immune mediated (Nankivell and Kuypers, 2011). Early pre- and post-transplantation events such as donor brain death, tubular injury caused by ischemia-reperfusion injury (IRI) and episodes of acute rejection have an important impact on late chronic kidney injury and subsequent allograft dysfunction (Nankivell and Chapman, 2006). Recently, innate immune activation through Toll-like receptors (TLRs) has been demonstrated to be an important driver in the pathogenesis of renal IRI and acute rejection (Dessing et al., 2010; Kruger et al., 2009; McDaniel et al., 2013; Naesens et al., 2009). At this moment, 10 human and 12 murine TLRs have been identified and each receptor recognizes a multitude of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Kawai and Akira, 2010; Sloane et al., 2010). Upon activation, TLR signaling causes release of cytokines and chemokines resulting in cell apoptosis,

Abbreviations: ATN, acute tubular necrosis; DAMPs, damage-associated molecular pattern molecules; ESRD, end-stage renal disease; IF/TA, interstitial fibrosis and tubular atrophy; IRI, ischemia-reperfusion injury; PAMPs, pathogen-associated molecular pattern molecules; TLRs, Toll-like receptors.

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bacterial death, activation of adaptive immunity and inflammation (Yamamoto and Takeda, 2010).

TLR2 and TLR4 have found to be expressed on renal tubular epithelial cells on RNA level (Tsuboi et al., 2002) and after IRI in mice their expression is increased (Tsuboi et al., 2002; Wolfs et al., 2002). Importantly, it was demonstrated that TLR2$^{-/-}$ or TLR4$^{-/-}$ mice are protected from renal IRI, supporting the hypothesis of a key role for both receptors in mediating IRI (Leemans et al., 2009; Wu et al., 2007). No such data is available for human IRI. It has not been fully established whether TLR expression in renal cells or TLR expression on infiltrating cells influences renal allograft injury during acute rejection. The expression pattern of TLR2 and TLR4 in normal human kidneys compared to IRI and acute rejection induced renal changes is unknown. With regards to the introduction of TLR inhibitors in human transplantation clinical studies to prevent IRI (Reilly et al., 2013), the abovementioned issues need to be addressed. Therefore, it is important to understand the expression pattern of TLR2 and TLR4 in healthy human and diseased conditions in humans as well as in rodents.

We hypothesize that TLRs play a role in renal allograft injury. To assess this, renal protein expression of TLR2 and TLR4 will be assessed in different patterns of renal injury after transplantation: acute tubular necrosis (ATN), acute allograft rejection (Banff grade Ia, Ib, IIa, IIb) and interstitial fibrosis/tubular atrophy (IF/TA). Renal biopsies taken from healthy human kidneys were used as basal expression of TLR2 and TLR4. Furthermore, mouse and rat TLR expression will be analyzed and compared to the human TLR expression in order to determine whether renal allograft injury in animal models can be translated to the clinical situation.

2. Materials and methods

2.1. Immunohistochemistry

For the detection of TLR2 and TLR4, frozen kidney sections (4 μm, with the exception of 2 μm for human biopsies) were first dried by airflow, fixed in cold acetone and blocked with 0.09% H_2O_2 in PBS. The sections were stained with a specific antibody to TLR2 or TLR4 for human, mouse and rat (Table 1). A number of primary antibodies were kindly provided by Hycult (Uden, The Netherlands). Horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Glostrup, Denmark) were used. As negative control, the primary antibody was replaced by isotype controls. All antibodies were diluted in 1% BSA/PBS and if needed, 1% normal human serum was added (Sigma–Aldrich, St. Louis, MO, USA) or rat/mouse normal serum. The antibodies were finally stained using 3-amino-9-ethylcarbazole (AEC) with 0.03% H_2O_2. The sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and analyzed by light microscopy. For the anti-TLR4 antibody sc-12511, the slides were blocked with normal donkey serum to eliminate background staining.

For the staining with anti-mouse TLR2 monoclonal antibodies clone 2.5 and 2.7, biotin labeling was used. The sections were prepared as previously described and blocked with a Biotin Blocking system (X0590, Dako) before applying the primary antibody. The biopsies were then stained with a specific primary antibody to TLR2 for mouse and rat sections. As secondary antibody, Streptavidin horseradish peroxidase (P0397, Dako) was used and then exposed to AEC.

All antibodies were also tested with the same concentration on paraffin sections (human, mouse and rat healthy kidney sections were used). After deparaffinisation, all different antigen retrieval methods were tested (glycine, Tris/EDTA, citrate, pepsin and protease). After these steps, the same protocol as frozen sections staining was used.

2.2. Kidney biopsies

In order to compare the expression of TLR2 and TLR4 in different types of human kidney injury after transplantation, human kidney biopsies were stained. The analyzed renal injury patterns (minimum $n = 6$ per group) were acute tubular necrosis (ATN), acute rejection (Banff Ia, Ib, IIa, IIb) and interstitial fibrosis and tubular atrophy (IF/TA, at least 30%). Living donor renal biopsies were used as healthy controls; the biopsy was taken before kidney retrieval and before renal blood vessels were clamped. A 16-gauge needle (Acecut®, TSK Laboratory, Japan) was used to obtain kidney transplant biopsies. Frozen sections were incubated with anti-TLR2 monoclonal antibody (clone 2.3) and anti-TLR4 monoclonal antibody (76B357.1).

2.3. Scoring of the kidney biopsies

The biopsies were scored by two independent observers in a blinded fashion. For every section, the presence of TLR2$^+$ and TLR4$^+$ inflammatory cells, glomeruli, interstitial space, endothelium and tubuli was scored independently. They were given a score ranging from 0 to 4. Score zero meaning there was no TLR2 or TLR4 positive staining and the scores ranging from 1 to 4 showing the intensity and quantity of TLR positive cells.

2.4. Tissue specimen

For TLR expression analysis in mice and rat, kidneys from healthy animals were used ($n = 5$).

2.5. Western blot

Aliquots (20 and 40 μg) of peripheral blood mononuclear cells with 5× sample buffer were heated up to 95 °C. The samples were then immediately cooled on ice. Gel electrophoresis was performed at 110 V. The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS. Subsequently, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 350 mA. The blot buffer contained 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were blocked in 5% skim milk in TBS-0.1%Tween and then incubated with the primary antibody overnight at 4 °C in 5% skim milk in TBS-0.1%Tween. As control, the primary antibody was omitted. Subsequently, the membranes were incubated with the appropriate HRP-labeled secondary antibody (refer to Table 1). Chemiluminescence technology (Supersignal, Pierce, Rockford, IL) was used for detection.

2.6. Statistical analysis

Data analysis was performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the mean ± SEM. A student's t-test was used to compare TLR2 and TLR4 expression within one biopsy type. Statistical differences between the different groups were analyzed by using one-way ANOVA followed by post hoc Tukey's Multiple comparison test. For the demographic characteristics, the Kruskall–Wallis test was used for continuous variables and the Chi-square test for categorical variables. All p-values ≤ 0.05 were considered significant.

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