Decreased TIM-3 mRNA expression in peripheral blood mononuclear cells from nephropathy patients

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ABSTRACT. Increasing evidence shows that TIM-1 and TIM-3 influence chronic autoimmune diseases, and their expression levels in immune cells from nephritic patients are still unknown. Real-time transcription-polymerase chain reaction analysis was used to determine expression levels of TIM-1 and TIM-3 mRNA in peripheral blood mononuclear cells (PBMCs) from 36 patients with minimal change glomerulopathy (MCG), 65 patients with lupus nephritis (LN), 78 patients with IgA nephropathy (IgAN), 55 patients with membranous nephropathy (MN), 22 patients with crescentic glomerulonephritis (CGN), 26 patients with anaphylactoid purpura nephritis (APN), and 63 healthy controls. TIM-3 mRNA expression significantly decreased in PBMCs from nephritic patients (LN, $P < 0.0001$; MCG, $P < 0.0001$; MN, $P = 0.0031$; CGN, $P = 0.0464$; IgAN, $P = 0.0002$; APN, $P = 0.0392$) compared with healthy controls. In contrast, there was no significant difference in TIM-1 mRNA expression between the patients and the healthy

controls. Our results suggest that insufficient expression of TIM-3 mRNA may be involved in the pathogenesis of nephropathy.

INTRODUCTION

The T-cell immunoglobulin and mucin domain (TIM) family consists of three members (TIM-1, TIM-3, and TIM-4) on human chromosome 5q33.2, which play a critical role in regulating immune responses, including transplant tolerance, autoimmunity, and the response to viral infections (Su et al., 2008; Rodriguez-Manzanet et al., 2009; Freeman et al., 2010). TIMs have different molecular structures and expression patterns, suggesting that they have distinct functions in regulating T-cell responses. It has been reported that TIM-1 and TIM-3 are involved in organ-specific inflammatory and autoimmune diseases (McIntire et al., 2001; Sánchez-Fueyo et al., 2003; Khademi et al., 2004). TIM-4 is exclusively expressed on antigen-presenting cells, where it mediates phagocytosis of apoptotic cells and plays an important role in maintaining tolerance (Kobayashi et al., 2007).

Increasing evidence shows that TIM-1 and TIM-3 influence chronic autoimmune diseases. For example, TIM-1 mRNA is expressed in the cerebrospinal fluid mononuclear cells of patients with multiple sclerosis, and rheumatoid arthritis is associated with polymorphisms of TIM-1 (Khademi et al., 2004; Chae et al., 2004, 2005). Galectin-9, a ligand of TIM-3, induces the cell death of Th1 cells in a TIM-3-dependent manner and galectin-9-deficient mice become susceptible to collagen-induced arthritis (Zhu et al., 2005; Seki et al., 2008). *In vivo* administration of antibody to TIM-3 exacerbates the clinical and pathological severity of experimental autoimmune encephalomyelitis (Monney et al., 2002). The analysis of T-cell clones obtained from patients with multiple sclerosis revealed significantly lower expression of TIM-3 compared with normal controls (Koguchi et al., 2006).

Recently, studies have shown that TIM-1 and TIM-3 are also involved in the development of renal diseases (Nozaki et al., 2012; Yang et al., 2014). In this study, we examined the expression of TIM-1 and TIM-3 mRNA in peripheral blood mononuclear cells (PBMC) from nephritic patients and healthy controls. Our results demonstrate that the mRNA expression of TIM-3 significantly decreases in PBMC from patients with these renal diseases compared with healthy controls.

MATERIAL AND METHODS

Human subjects

The present study enrolled 36 patients with minimal change glomerulopathy (MCG), 65 patients with lupus nephritis (LN), 78 patients with IgA nephropathy (IgAN), 55 patients with membranous nephropathy (MN), 22 patients with crescentic glomerulonephritis (CGN), and 26 patients with anaphylactoid purpura nephritis (APN). The patients were all diagnosed by clinical presentation, laboratory data, and renal biopsy. We excluded patients who had a history of atopy-related disease, such as asthma or allergic rhinitis. The patients were compared with 63 healthy controls with no history of autoimmune diseases. Written informed con-

sent was obtained from all donors and the study was approved by the human ethics committee of the China Medical University.

Preparation of PBMC and extraction of RNA

PBMCs were separated by density gradient centrifugation from peripheral blood anticoagulated with sodium citrate. Cells from the interphase were collected and washed twice with phosphate-buffered saline. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer protocol and quantified by photometrical measurement.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

One microgram of total RNA was reverse transcribed to cDNA in a 20 μL total volume system using a reverse transcription reaction kit (Promega). Real-time PCR was performed using the Express SYBR GreenER qPCR Supermix Universal Kit (Invitrogen) on a Rotor-gene 6000 system (Qiagen). The 25-μL PCR mixture contained 2 μL reverse-transcribed product, 12.5 μL SYBR GreenER Supermix, 8.5 μL RNase-free water, 1 μL forward, and 1 μL reverse primers. The reaction was performed on a 72-well optical plate on triplicate. The first step of PCR protocol was 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, and the second step was 60°C for 30 s. A melting-curve analysis was performed to ensure specificity of the PCR products, all of which were subjected to electrophoresis on an agarose gel for confinement to a single band of the expected size. The expression of TIM-1 and TIM-3 was normalized to β-actin and determined using the comparative ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Primers were as follows: TIM-1 forward: 5'-GCTTTGCAAAATGCAGT TGA-3', and reverse: 5'-TGTT-GAATGCCAGATGAAA-3'; TIM-3 forward: 5'-GACTTCAC TGCAGCCTTTCC-3', and reverse: 5'-GATCCCTGCTCCGATGTAGA-3'; β-actin forward: 5'-TACAGCTTCACCACCACAGC-3', and reverse: 5'-AAGGAAGGCTGGAAAAGAGC-3'.

Statistical analysis

Data were managed and stored using the SPSS 16.0 statistical software. The differences of relative mRNA levels between patient and control groups were tested by the Mann-Whitney *U*-test. The tests were two-tailed, and P values < 0.05 were considered to be statistically significant.

RESULTS

A total of 282 blood samples were obtained from patients with the following biopsy-confirmed diagnoses: MCG, LN, IgAN, MN, CGN, and APN. Sixty-three healthy blood donors with a mean age of 42 ± 16 years (median age 38, range 19-80 years), 18 men and 45 women, served as healthy controls. The general characteristics of the patients and healthy controls are listed in Table 1.

TIM-1 and TIM-3 mRNA expression levels in PBMCs from healthy controls and patients with different renal diseases were assessed by real-time RT-PCR. The results revealed that the mRNA expression of TIM-3 significantly decreased in PBMC from patients with the renal diseases (LN, 0.79 ± 0.72, P < 0.0001; MCG, 0.46 ± 0.39, P < 0.0001; MN, 1.14 ± 1.25, P

= 0.0031; CGN, 0.87 ± 0.64, P = 0.0464; IgAN, 0.83 ± 0.68, P = 0.0002; APN, 1.77 ± 3.01, P = 0.0392) compared with healthy controls (HC, 1.26 ± 0.84) (Table 1 and Figure 1). In contrast, there was no significant difference in TIM-1 mRNA expression between the patients and the healthy controls (Data not shown).

Table 1. Clinical characteristics and TIM-3 mRNA expression in peripheral blood mononuclear cells (PBMCs) from the healthy controls and patients with different glomerular diseases.

Diagnosis	HC (N = 63)	LN (N = 65)	MCG (N = 36)	MN (N = 55)	CGN (N = 22)	IgAN (N = 78)	APN (N = 26)
Male/female	18/45	15/50	12/24	18/37	8/14	19/59	7/19
Age (years)	42 ± 16	51 ± 19	38 ± 11	46 ± 18	55 ± 14	45 ± 16	39 ± 13
	38 (19-80)	50 (18-80)	38 (20-62)	44 (19-74)	56 (23-61)	46 (18-76)	41 (20-67)
BMI (kg/m²)	26 ± 7	23 ± 8	27 ± 5	24 ± 9	29 ± 10	32 ± 12	25 ± 6
	25 (21-39)	22 (18-40)	25 (22-45)	26 (20-45)	31 (17-55)	32 (21-60)	23 (20-56)
TIM-3 mRNA	1.26 ± 0.84	0.76 ± 0.58***	0.49 ± 0.39***	0.93 ± 0.84**	0.90 ± 0.64*	0.82 ± 0.68***	0.87 ± 0.61*
	1.10 (0.09-3.97)	0.61 (0.005-3.10)	0.46 (0.004-1.77)	0.59 (0.08-3.76)	0.73 (0.12-2.87)	0.68 (0.05-4.17)	0.69 (0.13-2.30)

HC, healthy control; LN, lupus nephritis; MCG, minimal change glomerulopathy; MN, membranous nephropathy; CGN, crescentic glomerulonephritis; IgAN, IgA nephropathy; APN, anaphylactoid purpura nephritis; BMI, body mass index. All values are reported as means ± SD in the upper rows and median (minimum-maximum) in the lower rows. *P < 0.05, **P < 0.005, ***P < 0.0001 compared with healthy controls.
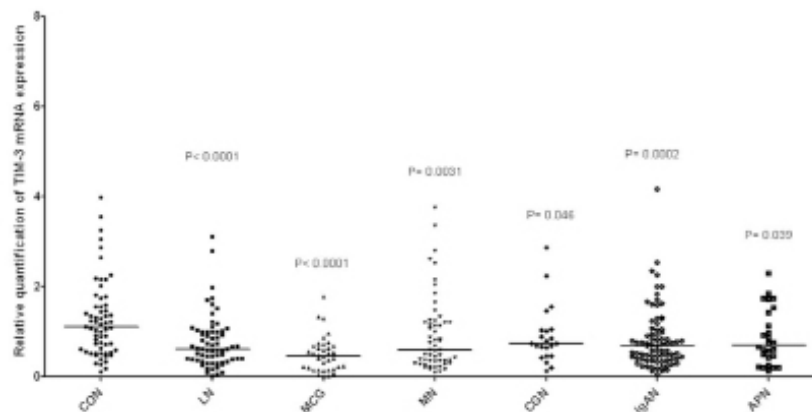
Figure1. Relative expression levels of TIM-3 mRNA in peripheral blood mononuclear cells (PBMCs) from healthy controls (HC, N = 63) and patients with different glomerular diseases [lupus nephritis (LN), N = 65; minimal change glomerulopathy (MCG), N = 36; membranous nephropathy(MN), N = 55; crescentic glomerulonephritis (CGN), N = 22; IgA nephropathy (IgAN), N = 78; anaphylactoid purpura nephritis (APN), N = 26]. P values of differences between the healthy controls and patients are defined by Mann-Whitney statistics.

DISCUSSION

TIM-1 was the first member of the TIM gene family to be identified and is expressed on T-cells. TIM-3 is expressed on terminally differentiated murine Th1 cells and functions to inhibit aggressive Th1-mediated auto- and allo-immune responses (Sánchez-Fueyo et al., 2003). In this study, we explored the mRNA expression of TIM-1 and TIM-3 in PBMCs from patients with different renal diseases and healthy controls using real-time RT-PCR.

The immune system is involved in many types of renal diseases, but there is no universally accepted definition of the term "autoimmune kidney disease". Whether all kinds of

glomerulonephritis should be considered as autoimmune disease is debatable, but immune mechanisms are important in all of them (Segelmark and Hellmark, 2010).

LN is a common and serious complication of systemic lupus erythematosus patients, and is considered to be the crucial factor in the prognosis of the disease (Liu and Zhou, 2012). Immune cells, cytokines, and epigenetic factors have all been recently implicated in LN pathogenesis (Schwartz et al., 2014). Primary IgAN chemical investigations have revealed that IgA1 molecules from patients differ in their glycosylation pattern compared with IgA1 from healthy subjects. It is possible that the formation of IgG anti-IgA antibodies is necessary to induce sufficient inflammation for the disease process (Tumlin et al., 2007). A strong correlation between LN and IgAN, and chronic glomerular injury with poor outcome has been revealed. MCG is the most frequent form of nephritic syndrome in children but can also occur in adults. Clinical and experimental studies have suggested that MCG is an immune-mediated disease (Weening et al., 2013). The molecular basis for MCG is still uncertain and is one of the last enigmas in renal pathology. MN is a form of immune-mediated glomerular injury in which immune complexes of immunoglobulins accumulate in a granular pattern (Segelmark and Hellmark, 2010). Our findings showed that the mRNA expression of TIM-3 significantly decreased in PBMCs from these nephritic patients. However, our results showed that the difference in the expression of TIM-3 mRNA was only marginal between patients with CGN or APN and healthy controls. In contrast, the mRNA expression of TIM-1 in PBMCs from nephritic patients did not reach statistical significance when compared with healthy controls.

It has been demonstrated that TIM-3 negatively regulates auto-immunity or inflammatory diseases including multiple sclerosis and neuritis, as well as nephritis (Koguchi et al., 2006; Schroll et al., 2010; Zhang et al., 2011). TIM-3 is preferentially expressed on Th1 cells and negatively regulates Th1 T-cell responses. It can also affect Th2-driven diseases by modulating the balance between Th1 and Th2 type responses indirectly. Breaking the Th1/Th2 balance by TIM-3 blockade may result in renal damage (Lee et al., 2011). Moreover, the TIM-3 ligand can improve proteinuria and inhibit anti-dsDNA antibody production by adjusting T-cell function, although TIM-3 may not be directly involved in the process (Moritoki et al., 2013). The fact that the TIM-3 ligand reduces TIM-3^{+}CD8^{+} T-cells in MRL/lpr mice suggests that TIM-3^{+}CD8^{+} T-cells are associated with lupus pathogenesis, since infiltrating CD8^{+} T-cells into a lupus kidney indicates that they have the potential to mediate kidney injury (Winchester et al., 2012). It has been reported that TIM-3 negatively regulates the activation of macrophages in the kidney during nephrotoxic serum nephritis, and TIM-3 blockade aggravates nephritis by increasing the number of kidney-infiltrating inflammatory cells, which provides the evidence that TIM-3 exerts protective roles in the course of nephritis (Schroll et al., 2010). Therefore, it seems that stimulation of TIM-3 expression may be a promising target for therapeutic intervention in autoimmune nephropathy.

In conclusion, we have demonstrated a decreased mRNA expression of TIM-3 in different nephritic patients, indicating the functional involvement of TIM-3 in the pathogenesis of renal diseases and highlighting a promising target for therapeutic intervention.

ACKNOWLEDGMENTS

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