Biochemical and Biophysical Research Communications 390 (2009) 307–312

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

An emerging role of deubiquitinating enzyme cylindromatosis (CYLD) in the tubulointerstitial inflammation of IgA nephropathy

Tai-Gen Cui [a,1], Tomonaga Ichikawa [b,1], Ming Yang [a], Xiaoyu Dong [b], Jinqing Li [b], Taixing Cui [b,*]

[a] Department of Nephrology, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China
[b] Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29208, USA

ARTICLE INFO

Article history:
Received 21 September 2009
Available online 1 October 2009

Keywords:
IgA nephropathy
CYLD
Tubular epithelial cells
Proteinuria
Tubulointerstitial inflammation

ABSTRACT

Immunoglobulin A (IgA) nephropathy is an important cause of end-stage kidney disease (ESKD). Tubulointerstitial inflammation and subsequent fibrosis appear to be a major contributor of the disease progression to ESKD; however, the underlying mechanism is poorly understood. Herein, we report that a unique feature of CYLD expression in kidneys of patients with IgA nephropathy and a CYLD-mediated negative regulation of inflammatory responses in human tubular epithelial cells. Immunochemical staining revealed that CYLD was predominantly expressed in renal tubular epithelial cells in 81% of the patients (37 cases) with proteinuric IgA nephropathy. Patients with positive CYLD had significantly less tubulointerstitial lesions and higher estimated glomerular filtration rate (eGFR) levels when compared with those negative. Logistic regression analysis indicated that eGFR was a predictor for the CYLD expression. In cultured human tubular epithelial HK-2 cells, tumor necrosis factor-alpha (TNFα) up-regulated CYLD expression. Adenoviral knockdown of CYLD did not affect albumin-, hydrogen peroxide (H_2O_2)-, tunicamycin- or thapsigargin-induced cell death; however, it enhanced TNFα-induced expression of intracellular adhesion molecule (ICAM)-1 as well as activation of c-Jun N-terminal kinase (JNK). Moreover, monocyte adhesion to the TNFα-inflamed HK-2 cells was significantly increased by the CYLD shRNA approach. Taken together, our results suggest that CYLD negatively regulates tubulointertitial inflammatory responses via suppressing activation of JNK in tubular epithelial cells, putatively attenuating the progressive tubulointerstitial lesions in IgA nephropathy.

Introduction

IgA nephropathy is the most common primary glomerulonephritis world wide. A complete remission occurs in less than 10% of all patients, whereas others more commonly are associated with slowly progressive chronic renal impairment with 25–40% of any cohort developing end-stage renal failure within 30 years of diagnosis [1,2]. A hallmark of IgA nephropathy is the deposition of immunoglobulin A and complement components in the glomerular mesangium. While molecular basis of IgA nephropathy is not fully understood, it has been documented that the deposition of polymeric IgA triggers glomerular immuno-inflammatory injury and subsequent proteinuria may further induce tubulointerstitial lesions, contributing to the progression of IgA nephropathy [1,3,4]. Intriguingly, recent studies have highlighted a pathological significance of the ubiquitin–proteasome system in human kidney diseases including IgA nephropathy [5–7]. Nevertheless, critical mediators of the tubulointerstitial injury in IgA nephropathy remain to be determined.

Emerging evidence has revealed that deubiquitinating enzymes (DUBs) act as crucial regulators in diverse biological processes [8]. Approximately 100 DUBs have been identified and CYLD is one of the best studied [9,10]. CYLD was originally identified as a tumor suppressor gene of human familial cylindromatosis. Subsequent studies have demonstrated that CYLD negatively regulates nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) pathways, and plays a critical role in immunity, lipid metabolism, spermatogenesis, osteoclastogenesis antimicrobial defense, and inflammation. CYLD is expressed in multiple tissues including kidney [11]; however, its physiological or pathological relevance in kidney remains unknown.

In the present study, we explored the pathological relevance of CYLD by examining its expression profile in kidney biopsy tissues of the patients with IgA nephropathy. In addition, we examined a role of CYLD in regulating inflammatory responses in human tubular epithelial cells. Our results indicate that CYLD might act as a negative regulator of tubulointerstitial inflammatory responses, ameliorating the tubulointerstitial injury in IgA nephropathy.

* Corresponding author.
E-mail address: taixing.cui@uscmed.sc.edu (T. Cui).
[1] These authors contributed equally to the study.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.09.119

Material and methods

Kidney biopsy specimens and patients' clinical data. Paraffin-embedded kidney biopsy tissues had been selected from a kidney biopsy bank of the patients who were diagnosed as IgA nephropathy with proteinuria at the Beijing Chaoyang Hospital, Capital Medical University, China. Kidney biopsy specimens of the patients who received steroid and/or immunosuppressive agents were excluded to minimize therapeutic influences on CYLD expression. As a result, 37 cases of patients with proteinuric IgA nephropathy were subjected to the present study. Medical records of biochemical examination and kidney pathology of these patients were retrieved from the hospital database. Serum creatinine was measured by Jaffe's method (Synchron LX 20, Beckman Coulter). Estimated glomerular filtration rate (eGFR) was calculated using simplified MDRD equation [12]. Dipstick urinalysis and microscopic examination were carried out using fresh first morning urine sample. Proteinuria was defined as urinary protein excretion exceeding 0.15 g/day. Hematuria was defined as urinary red blood cells $\geqslant$3/HP by microscopic examination. The slides of kidney biopsy samples were re-examined under light microscopy by one single pathologist, and tubulointerstitial lesions were semi-quantitatively scored as previously described [13]. Briefly, lesions of interstitial cell infiltration, fibrosis and tubular atrophy were graded, respectively, from 0 to 3 according to the percentage of occupying area, and then added up together to obtain a final score. A minimum of ten glomeruli was required in the kidney biopsy specimens for light microscopy to ensure a definitive diagnosis. All patients had signed consent forms before performing kidney biopsy and agreed with use of the kidney samples for research purpose. The study was approved by the hospital ethics committee.

Immunohistochemistry. CYLD expression was examined on tissue sections of the paraffin-embedded kidney biopsy specimens with a rabbit anti-CYLD polyclonal antibody (H-419, sc-25779, Santa Cruz Biotechnology) as described previously [14]. Tissue sections were finally counterstained with hematoxylin.

Cell culture, adenoviral infection, and cell viability assay. HK-2 cells (American Type Culture Collection), an immortalized proximal tubule epithelial cell line from normal human kidney [15], were cultured in low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum. HK-2 cells at 90% confluence were infected with adenovirus of control scramble shRNA and CYLD shRNA targeting human as well as mouse and rat CYLD mRNAs (Welgen, Inc.) in serum free DMEM for 48 h. Efficacy of the adenovirus of CYLD shRNA was validated by quantitative real time-polymerase chain reaction and Western blot (Supplementary Fig. 1). Cell viability was measured by CyQUANT reagents (Invitrogen).

Monocyte adhesion assay. Adhesion of monocyte to HK-2 cells was assessed utilizing fluorescein labeled THP-1 cells. Briefly, HK-2 cells were treated with TNFα (5 ng/ml) in serum free DMEM for 16 h in 24-well plates. THP-1 cells were labeled with CellTracker CMFDA (Invitrogen C2925) according to the manufacturer's instructions. Fluorescein labeled THP-1 cells (5×10^5 cells/well) were incubated with for 15 min at 37 °C, and then each well was washed with phosphate-buffered saline three times to separate nonadherent monocytes. Relative amount of adherent cells were quantified by measuring the fluorescence at excitation 485/20 nm and emission 528/20 nm using a fluorescence plate reader (Bio Tek Synergy 2).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time-PCR (Q-PCR). Total RNA purification, RT reaction, and Q-PCR were performed as described previously [14]. Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers that were used for Q-PCR are as follows: forward primer (5′-TGCCTTCCAACTCTCGTCTTG-3′) and reverse primer (5′-AATCCGCTCTTCCCAGTAGG-3′) were used for PCR amplification of human CYLD (NM_015247) to yield a 228-bp product. Forward primer (5′-CCGGAAGGTGTATGAACTG-3′) and reverse primer (5′-TCCATGGTGATCTCTCCTC-3′) were used for PCR amplification of human ICAM-1 (NM_000201) to yield a 318-bp product. Forward primer (5′-ACCACAGTCCATGCCATCAC-3′) and reverse primer (5′-TCCACCACCCTGTTGCTGTA-3′) were used for PCR amplification of human GAPDH (NM_002046) to yield a 451-bp product.

Western blot. Cell lysates were subjected to immunoblot analysis using antibodies of anti-phospho-ERK (cat# 9101), anti-phospho-p38 (cat# 9211) and anti-phospho-JNK (cat# 9251) (Cell Signaling Technology), as well as anti-ICAM-1 (M-19, sc-1511), anti-ERK (K-23, sc-94), anti-JNK (FL, sc-571), anti-p38 (A-12, sc-7972) and anti-GAPDH (FL-335, sc-25778) (Santa Cruz Biotechnology).

Statistical analysis. Data are expressed as mean ± s.d. Comparison between groups was done by two-sample *t*-test. The association between categorical variables was examined using χ^2 test. Binary logistic regression analysis was performed to evaluate the effect of various exposure variables including sex, disease course, eGFR, tubulointerstitial lesion score on CYLD expression. A p value of <0.05 was considered significant. Statistical analysis was performed with Statistical Package for Social Sciences version 13.0 (SPSS, Inc., Chicago, IL).

Results

CYLD expression is positively associated with kidney function in IgA nephropathy

Patients of IgA nephropathy with proteinuria have high risk of developing renal insufficiency whereas the patients with isolated hematuria hardly develop renal dysfunction [16]. Therefore, we focused on expression pattern of CYLD in kidneys of the patients with proteinuric IgA nephropathy to explore a potential role of CYLD in the pathogenesis of IgA nephropathy. Immunochemical staining revealed that CYLD expression was detected in 30 out of the 37 kidney biopsy samples (Fig. 1). CYLD was expressed predominantly in the tubular epithelial cells but not detected in the glomeruli (Fig. 1A). Of interest, patients with positive CYLD staining had higher eGFR levels (86.68 ± 3 2.12 vs 37.42 ± 29.14 ml/min/1.73 m^2, $p = 0.001$) and less tubulointerstitial lesions (4.20 ± 3.01 vs 7.29 ± 2.93, $p = 0.019$) when compared to those with negative CYLD (Fig. 1B). These results demonstrate that tubular expression of CYLD is associated negatively with tubulointerstitial lesion formation, whereas positively with renal function in patients of proteinuric IgA nephropathy.

CYLD negatively regulates TNFα-mediated pro-inflammatory signaling in tubular epithelial cells

Because tubulointerstitial injury plays a crucial role in the progression of IgA nephropathy [1,3,4], we hypothesized that CYLD deficiency in tubular epithelial cells could exaggerate tubulointerstitial lesions, thereby leading to progressive renal dysfunction in proteinuric IgA nephropathy. To test this hypothesis, we applied CYLD RNA interference (RNAi) approach to determine a pathophysiological significance of CYLD deficiency in the regulation of tubulointerstitial homeostasis *in vitro* utilizing cultured HK-2 cells, a human adult tubular epithelial cell line. Firstly, we generated adenovirus of CYLD shRNA to knock down CYLD expression. Adenoviral over-expression of CYLD shRNA dose-dependently inhibited CYLD mRNA expression with an efficacy of >90% knockdown of