Biochemical and Biophysical Research Communications 461 (2015) 575–581

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Necroptosis mediated by *receptor* interaction protein kinase 1 and 3 aggravates chronic kidney injury of subtotal nephrectomised rats

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ARTICLE INFO

Article history:
Received 25 March 2015
Available online 20 April 2015

Keywords:
Necroptosis
RIP1
RIP3
Chronic kidney disease

ABSTRACT

Necroptosis, an alternative mode of programmed cell death, has crucial pathophysiological roles in many diseases, but its effect on chronic kidney disease (CKD) is poorly understood. Therefore, we assessed necroptosis and its pathophysiological effects in a widely used remnant-kidney rat model. *We* found that necroptotic cell death and the highest level of receptor interaction protein kinase 1 (RIP1) and receptor interaction protein kinase 3 (RIP3), critical signalling molecules for necroptosis, appeared 8 weeks after subtotal nephrectomy (SNX) surgery. After treatment with Necrostatin-1 (Nec-1), renal function and renal pathologic changes were significantly improved; the overexpression of RIP1, RIP3, mixed lineage kinase domain-like (MLKL) and dynamin-related protein 1 (Drp1) was reduced; and necroptosis was inhibited. These results indicated that necroptosis mediated by RIP1 and RIP3 participates in the loss of renal cells of subtotal nephrectomised rats and might be one of main causes of the excessive loss of renal cells during the sustained progression of renal fibrosis.

1. Introduction

The pathological changes of chronic kidney disease (CKD) begin with the excessive loss of critical nephrons, which is followed by progressive glomerular sclerosis, the collapse of peritubular capillaries and tubular interstitial fibrosis. *Whatever the nature of the* insult, even if the initial lesion has healed or improved, the remaining nephrons continue to be lost and renal fibrosis lesions progress until end-stage renal failure has occurred. Therefore, exploring the mechanism of the progressive loss of nephrons and blocking this process are widely believed to be effective ways to prevent the progression of CKD.

Necrosis is believed to occur as an accident or unregulated event. However, recent studies showed that some types of cell necrosis share similar features with programmed cell death, which can be regulated [1,2]. *This type of* cell death mode is named as necroptosis [3].

Necroptosis is a caspase-independent programmed cell death that is mediated by death receptors and regulated by accurate cell signal pathways [3]. In the necroptosis pathways, receptor interaction protein kinase 1 and 3 (RIP1/3) play a vital role [4–6]. In response to upstream signal molecules, RIP1 undergoes a series of ubiquitination, deubiquitination and phosphorylation events. When caspase-8 is inhibited, the activated RIP1 triggers the phosphorylation of RIP3 kinases and forms the necrosome, which can drive necroptosis [7]. Necrostatin-1 (Nec-1, a specific inhibitor of RIP1) can inhibit RIP1 kinase activity and stop necroptosis [8].

Recent studies found that necroptosis is the main form of tubular cell loss in acute ischemia/reperfusion renal injury [9]. However, the role of necroptosis in the progression of CKD is still unknown. Therefore, we sought to *test* the hypothesis that necroptosis is an important form of renal cell death during the

Abbreviations: CKD, chronic kidney disease; RIP1, receptor interaction protein kinase 1; RIP3, receptor interaction protein kinase 3; SNX, subtotal nephrectomy; Nec-1, Necrostatin-1; MLKL, mixed lineage kinase domain-like; PGAM5, phosphoglycerate mutase family member 5; Drp1, dynamin-related protein 1; ROS, reactive oxygen species; GSI, glomerular sclerosis index; TIS, tubulointerstitial damage scores; DAPI, 4′, 6-diamidino-2-phenylindole; RHIM, RIP homotypic interaction motif; TNFR1, tumor necrosis factor receptor1; qPCR, *quantitative* real-time PCR.

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http://dx.doi.org/10.1016/j.bbrc.2015.03.164
0006-291X/

progression of CKD and *is* mediated by RIP1 and RIP3 signalling pathways.

2. Materials and methods

2.1. Animals

Eight-week-old healthy male Sprague–Dawley (250 g–300 g in body weight) rats used in this study were supplied by the *Animal Laboratory Centre* of Chongqing Medical University. All rats were housed under standard conditions with a 12-h light–dark cycle, given free access to water and fed a standard rodent diet. All animal experimental protocols were performed in strict accordance with standards stated in the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the biomedical research ethics committee of Chongqing Medical University.

2.2. Establishment of the subtotal nephrectomised rat model

After a 7-day adaptation period, Sprague–Dawley (SD) rats were randomly assigned to the subtotal nephrectomy (SNX) group (n = 54) or control group (n = 36). SD rats were subjected to subtotal nephrectomy surgery in the SNX group and sham surgery in the control group as reported previously [10,11]. Briefly, the right kidneys of rats in the SNX group were removed under intraperitoneal anaesthesia with pentobarbital sodium. Seven days later, the upper and lower cortex of the left kidney (approximately 60%–70% of the right kidney *by weight*) was removed, and 1/3 of the left kidney was *preserved*. Rats in the control group underwent renal decapsulation *only*.

2.3. Drug administration

Z-VAD-fmk (Z-VAD, MP Biomedicals, Solon, OH, USA) and Necrostatin-1 (Nec-1, Sigma–Aldrich, *St. Louis., MO, USA*) were dissolved in 10% dimethyl sulfoxide (DMSO, Sigma, USA). Rats underwent the SNX surgery, recovered for 4 weeks, were injected intraperitoneally with Z-VAD (1.0 mg/kg/d [12]) or Nec-1 (1.65 mg/kg/d [9]) or an equal volume of 10% DOSM (vehicle) and were maintained for 4 more weeks. At 8 weeks after surgery, the rats were sacrificed.

2.4. Renal function

Serum urea nitrogen and creatinine concentrations were used to assess renal function and *were* assayed using an automatic biochemical analyser (Roche Hx-49, Mannheim, Germany).

2.5. Renal morphology

The remnant kidney tissue was fixed in 10% formalin buffer and embedded in paraffin. Four-micrometre-thick sections of the kidney were stained with periodic acid–Schiff (PAS) for morphological analysis in a double-blind fashion to determine the extent of renal injury. The glomerular sclerosis index (GSI) and the *tubulointerstitial* damage scores (TIS) *were* assessed [13,14].

2.6. Transmission electron microscopy (TEM)

Several pieces of 1-mm^3 renal tissue fragments were fixed in 2.5% glutaraldehyde phosphate buffer (pH 7.4) overnight at 4 °C. Then, the tissue fragments were fixed in 2% osmium tetroxide for 1 h and block-stained with 2% uranyl acetate. Next, the kidney tissues were embedded in epoxy resin and dehydrated. Then, ultrathin sections were used and stained with uranyl acetate and subsequently stained with lead citrate. The ultrastructure of kidney tissues was observed by electron microscopy (Hitachi-7500, Japan).

2.7. Quantitative real-time PCR (qPCR)

Total RNA was extracted from kidney tissue with RNAiso Plus (Total RNA extraction reagent, TaKaRa BIO INC. Japan). Approximately 1000 ng of total RNA was reverse transcribed with the Prime Script® ™ RT Reagent Kit with gDNA Erase (TaKaRa BIO INC. Japan) and amplified in triplicate using SYBR® Premix Ex TaqTM II (TaKaRa BIO INC. Japan) with a CFX96TM Real-Time PCR detection system (Bio-Rad, California, USA), according to the manufacturer's instructions. The following primer sequences were used (forward and reverse): RIP1, forward 5′-AGGTACAGGAGTTTGGTATGGGC-3′ and reverse 5′-GGTGGTGCCAAGGAG ATGTATG-3'; RIP3, forward 5′-TAGTTTATGAAATGCTGGACCGC-3′ and reverse 5′-GCCAAGGTGTCAGATGATGTCC-3'. Gene expression relative to the housekeeping gene GAPDH was determined using the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blotting

Kidney tissue was lysed with RIPA lysate buffer (Beyotime, Jiangsu, China), and the protein concentration was determined using a BCA protein quantitative kit (Beyotime, Jiangsu, China). Equal amounts of protein were separated on a SDS-PAGE gel, and the proteins were transferred to a PVDF membrane (EMD Millipore, USA). Immunoblotting was performed according to standard procedures with the following primary antibodies: anti-RIP1 monoclonal antibody (R&D Systems, Minneapolis, MN, USA), anti-RIP3 polyclonal antibody (Abcam Inc., Cambridge, USA), anti-PGAM5 polyclonal antibody and anti-MLKL polyclonal antibody (Santa Cruz Biotechnology, California, USA), anti-Drp1 polyclonal antibody (Cell Signalling Technology, Boston, USA), and anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, California, USA). Western *blotting* was performed using conventional methods as described previously [15].

2.9. Immunofluorescence staining

Kidney paraffin sections (4 μm) were deparaffinised and incubated with a mouse anti-RIP1 antibody (R&D Systems, Minneapolis, MN, USA) and rabbit anti-RIP3 antibody (Abcam Inc., Cambridge, USA). The secondary antibodies were Alexa *Fluor* 488-labelled goat anti-mouse *and* Cy3-labelled donkey anti-rabbit (Beyotime, Jiangsu, China). The sections were counterstained with mounting medium containing DAPI. Confocal images were acquired using *laser* confocal microscopy (Leica TCP SP5, Leica Microsystems GmbH, Wetzlar, Germany).

2.10. Reactive oxygen species (ROS) level

The ROS level in kidney tissue was tested as previously described [16]. Briefly, the kidney tissue was homogenised with ice-cold PBS buffer to obtain a concentration of 5 mg tissue/ml and centrifuged to collect the supernatant. Then, the fluorescence probe DCFH-DA (NanJing JianCheng Bioengineering, Nanjing, China) was added to the supernatant, and the two were incubated together at 37 °C for 30 min. The fluorescence intensity of the DCF product was measured using a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Parts of the homogenate supernatant were used to test the protein concentration with a BCA protein quantitative kit (Beyotime, Jiangsu, China). Fluorescence intensity/protein concentration was calculated.