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C5a/C5aR pathway accelerates renal ischemia-reperfusion injury by downregulating PGRN expression

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ABSTRACT

Recent reports indicate that the complement C5a/C5aR pathway and progranulin (PGRN) deficiency both contribute to ischemia-reperfusion (IR)-induced acute kidney injury. However, the underlying relationship between the C5a/C5aR signaling pathway and PGRN expression during acute kidney injury is poorly understood. In this study, we showed that C5aR expression was significantly upregulated after renal IR, and that C5aR deficiency led to a marked increase in PGRN expression and a significant reduction in tubular damage and production of inflammatory cytokines. In accordance with these results, recombinant C5a caused downregulation of PGRN protein and mRNA levels in renal tubular epithelial cells (HK-2 cells), which could be negated by disruption of C5a/C5aR signaling by the C5aR antagonist, as confirmed by immunofluorescence, western blotting, and quantitative real-time PCR. Moreover, C5aR deficiency resulted in attenuated NF-κB expression 24 h after IR, and recombinant C5a potentiated TNFα-induced NF-κB activation in HK-2 cells. Inhibition of NF-κB activation reversed C5a-induced downregulation of PGRN expression. Our results show for the first time that the complement C5a/C5aR pathway aggravates IR-induced acute kidney injury by suppressing PGRN expression and confirm that suppression of PGRN expression is associated with increased NF-κB activation induced by C5a.

1. Introduction

Renal ischemia-reperfusion (IR) is an unavoidable consequence of kidney, aortic, or liver surgery and a common cause of acute kidney injury (AKI) with high morbidity and mortality [1]. Without early intervention, patients with AKI can easily progress to chronic kidney disease [2,3]. It is therefore important to explore the mechanism of ischemia-reperfusion injury (IRI) and identify effective strategies for prevention and therapy.

Activation of the complement system occurs in various diseases, including cancer, inflammation and autoimmune diseases. After IR, the complement system is activated and components cleaved, which leads to the production of the complement components C3a, C5a, and/or the membrane attack complex (C5b-9) via three pathways (the classical, alternative, and/or lectin pathways) [4]. The complement component C5a is a major pro-inflammatory mediator and its specific receptor (C5aR) is expressed on monocytes, macrophages, and renal tubular epithelial cells. C5a/C5aR interactions cause recruitment of neutrophils and macrophages and exacerbate tubular cell injury in AKI [5,6]. C5aR deficiency on renal cells or circulating leukocytes can significantly ameliorate renal IR injury [7]. However, there is little data on the underlying mechanism for regulation of IR-induced AKI via the C5a/C5aR pathway. Progranulin (PGRN) is a 593-amino-acid multiple growth factor that is widely expressed on various cells (i.e., epithelial cells, neurons, and adipocytes) [8]. PGRN has anti-inflammatory properties that are relevant in neurodegenerative diseases, wound repair, arthritis, inflammatory bowel disease, acute lung injury, and AKI [9,10]. After IR, PGRN expression is significantly reduced and inflammation is upregulated. Interestingly, administration of recombinant PGRN after IR causes a marked reduction in renal pathological damage, infiltration of inflammatory cells and apoptosis of kidney cells [11]. In the lipopolysaccharide-induced AKI model, it was also confirmed that PGRN plays a protective role by reducing inflammatory responses and apoptosis of renal tubular epithelial cells [12]. In the present study, we investigate the relationship between the complement C5a/C5aR pathway and PGRN production during IR-induced AKI. Together, our study provides evidence for a strong negative correlation between C5a/C5aR signaling and PGRN expression during IR-induced renal injury, and confirm that

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suppression of PGRN expression is associated with increased NF-κB activation induced by C5a. These results indicate that blocking the C5aR signaling pathway to inhibit PGRN cleavage is a potential therapeutic strategy for IR-induced AKI.

2. Materials and methods

2.1. Mice and model of IRI

C5aR-knockout mice (C5aR KO, BALB/c background) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type mice (WT, BALB/c background) were purchased from the Animal Institute at the Academy of Medical Science (Beijing, China). Male mice (20–25 g, 8–12 weeks old) were used in all experiments. Renal IRI was induced as described previously [13]. Briefly, mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and then renal pedicles were bilaterally occluded for 35 min using vascular clamps. The vascular clamps were removed, 1.0 mL of warm saline was injected into the abdomen, and the incision was sutured. During surgery, the body temperature was maintained between 36 and 37.5 °C using a warm pad. After the mice awoke from anesthesia, they were placed into cages. Renal tissue and blood samples were harvested 24 h or 48 h after the surgery. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Third Military Medical University.

2.2. Renal function

Renal function was assessed by measuring blood urea nitrogen (BUN) concentrations using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA).

2.3. Histology

Histological assessments were performed as described previously [7]. Briefly, sections stained with hematoxylin and eosin were observed by microscopy at 200 × magnification using a blinded method. Epithelial injury in the corticomedullary junction (i.e., loss of proximal tubule brush border, protein casts, and cellular necrosis) was scored on a six-point scale according to the percentage of injured tubules: 0, none; 1, 0–10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, > 75%. At least 10 fields per section were examined.

2.4. Immunohistochemistry

Renal tissues were rapidly fixed with 4% paraformaldehyde 24 h after reperfusion and then paraffin-embedded. Epitopes were unmasked using EDTA (1 mmol/L, pH 8.0). Renal sections were incubated with primary antibodies against PGRN (Santa Cruz Biotechnology, Dallas, TX, 1:50 dilution), KIM-1 (Bioss, Wuhan, China, 1:100 dilution), C5aR (Abcam, Cambridge, MA, USA, 1:100 dilution), or NF-κB p65 (Beyotime, Shanghai, China, 1:50 dilution), then conjugated with anti-rabbit secondary antibodies (Beyotime, Shanghai, China, 1:1000 dilution). Stained renal tissues were imaged by microscopy (Olympus BX51, Tokyo, Japan).

2.5. Immunofluorescence staining

Renal tissues were frozen rapidly after reperfusion. Renal sections were incubated with primary antibodies against PGRN (Santa Cruz Biotechnology, Dallas, TX, 1:50 dilution), then conjugated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibodies (Bioss, Wuhan, China, 1:100 dilution). Stained renal tissues were imaged by fluorescence microscopy (Olympus BX51, Tokyo, Japan).

2.6. Cell culture and treatments

Immortalized human renal proximal tubule cells (HK-2) were cultured in DMEM medium (10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine) in 5% CO_2 at 37 °C. HK-2 cells were treated with 50 nM C5a (Hycult, Uden, Netherlands), 20 ng/mL caffeic acid phenethyl ester (CAPE, a NF-κB inhibitor) (Selleckchem, Houston, USA), 2.5 μg/mL C5a receptor antagonist (C5aRA) (GL Biochem, Shanghai, China), or 50 ng/mL TNF-α (Novoprotein, Shanghai, China) for the indicated time period.

2.7. Cell immunofluorescence staining

HK-2 cells were treated with C5a or a combination of C5a and C5aRA for 24 h as described above. In some experiments, HK-2 cells were treated with C5a, TNF-α, a combination of C5a and C5aRA, or a combination of C5a, TNF-α and C5aRA for 2 h. Cells were stained with antibodies against PGRN (Santa Cruz Biotechnology, Dallas, TX, 1:50 dilution) or antibodies against NF-κB p65 (Beyotime Institute of Biotechnology, Shanghai, China, 1:200 dilution). Stained cells were observed by fluorescence microscopy (Olympus BX51, Tokyo, Japan).

2.8. RNA extraction and quantitative real-time PCR

Total RNA was extracted from renal tissues or cells using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's protocol. mRNA levels were measured by quantitative real-time PCR (qRT-PCR) using a DyNAmo HS SYBR Green qPCR kit (Thermo Fisher, Epsom, United Kingdom) using the primers listed in Table 1 and the following thermocycling protocol: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

2.9. Western blotting

Renal tissue or HK-2 cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China). A polyclonal antibody against PGRN (Santa Cruz Biotechnology, Dallas, TX, 1:200 dilution) was used to measure PGRN levels by western blotting, as described previously [10]. The levels of PGRN relative to the housekeeping protein β-actin (Beyotime, Shanghai, China) were calculated using ImageJ 1.46 r software.

Table 1
Primers used for quantitative real-time PCR.

Gene	Forward primer	Reverse primer
Mus C5aR	5′-GACCACCACCGAGTAGATGA-3′	5′-TGGACCCCATAGATAACAGCAG-3′
Mus TNF-α	5′-AGGCACTCCCCCAAAAGATG-3′	5′-TTTGCTACGACGTGGGCTAC-3′
Mus IL-6	5′-TAGTCCTTCCTACCCCAATTTCC-3′	5′-TTGGTCCTTAGCCACTCCTTC-3′
Mus IL-1β	5′-GCTCTCCACCTCAATGGACA-3′	5′-TTGGGATCCACACTCTCCAG-3′
Mus PGRN	5′-GGTTGATGGTTCGTGGGGATGTTG-3′	5′-AAGGCAAAGACACTGCCCTGTTGG-3′
Mus GAPDH	5′-CTCTGCTCCTCCTGTTCGAC-3′	5′-GCGCCCAATACGACCAAATC-3′
Homo PGRN	5′-AAGGCAAAGACACTGCCCTGTTGG-3′	5′-GTAGCGCTCAGACTACAGACC-3′
Homo β-actin	5′-GAAGTGTGACGTGGACATCC-3′	5′-CCGATCCACACGGAGTACTT-3′