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Full paper

A protease-activated receptor-1 antagonist protects against podocyte injury in a mouse model of nephropathy

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ABSTRACT

The kidney expresses protease-activated receptor-1 (PAR-1). PAR-1 is known as a thrombin receptor, but its role in kidney injury is not well understood. In this study, we examined the contribution of PAR-1 to kidney glomerular injury and the effects of its inhibition on development of nephropathy. Mice were divided into 3 groups: control, doxorubicin + vehicle (15 mg/kg doxorubicin and saline) and doxorubicin + Q94 (doxorubicin at 15 mg/kg and the PAR-1 antagonist Q94 at 5 mg/kg/d) groups. Where indicated, doxorubicin was administered intravenously and PAR-1 antagonist or saline vehicle by subcutaneous osmotic mini-pump. PAR-1 expression was increased in glomeruli of mice treated with doxorubicin. Q94 treatment significantly suppressed the increased albuminuria in these nephropathic mice. Pathological analysis showed that Q94 treatment significantly attenuated periodic acid–Schiff and desmin staining, indicators of podocyte injury, and also decreased glomerular levels of podocin and nephrin. Furthermore, thrombin increased intracellular calcium levels in podocytes. This increase was suppressed by Q94 and Rox4560, a transient receptor potential cation channel (TRPC)3/6 antagonist. In addition, both Q94 and Rox4560 suppressed the doxorubicin-induced increase in activities of caspase-9 and caspase-3 in podocytes. These data suggested that PAR-1 contributes to development of podocyte and glomerular injury and that PAR-1 antagonists have therapeutic potential.

1. Introduction

Chronic kidney disease is a substantial worldwide burden to patients and society.[1,2] Albuminuria is a typical characteristic of kidney disease and results from disruption of the glomerular filtration barrier. Podocytes are important cells for maintaining glomerular filtration barrier function. Thus, podocyte loss causes serious nephropathy.[3–5] For this reason, podocytes have been considered a principal therapeutic target for protecting the glomerular filtration barrier against plasma protein leakage into urine.[6,7]

The PAR-1 (protease-activated receptor 1), a Gi protein-coupled receptor,[8] is activated by thrombin-induced proteolytic cleavage. The N-terminal extracellular region of PAR-1 is cleaved by thrombin and the cleaved peptide then interacts with the PAR-1 receptor to induce transmembrane signaling.[8] Both human and rodent kidneys express PAR-1.[9] A recent review suggested that PAR-1 plays a functional role in rat glomeruli.[10] In a clinical study, urinary thrombin excretion in patients with glomerulonephritis was higher than in healthy subjects.[11] However, the contribution of PAR-1 to progression of glomerular injury has not been elucidated.

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In this study, we examined the role of PAR-1 on development of glomerular podocyte injury and its potential value as a therapeutic target. PAR-1 upregulation was confirmed in two glomerular injury models, doxorubicin-induced nephritis and anti-glomerular basement membrane (GBM) antiserum-induced nephritis model. The pharmacological inhibition of PAR-1[12] in doxorubicin model prevented development of podocyte injury. Moreover, to address biological changes in the podocyte after PAR-1 stimulation, we assessed PAR1-dependent regulation of apoptosis and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in podocytes. These processes were reported to represent a common pathway driving loss of podocyte foot processes.[13]

2. Materials and methods

2.1. Animal

All experiments were approved by the Institutional Animal Care and Use Committee of Kagawa University. BALB/c and C57BL/6 mice were from Clea Japan (Tokyo, Japan).

2.2. Materials

Q94, a PAR-1 antagonist, was purchased from Tocris Bioscience (Bristol, UK). Chemicals were from Sigma–Aldrich (St. Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan), unless otherwise specified. Rox4560, named 6228-0473 in a previous report,[14] was synthesized in the Faculty of Pharmaceutical Sciences, Kyushu University.

2.3. Mouse model for podocyte injury

Nephropathy with podocyte injury was induced by a single intravenous injection of anti-GBM antiserum[15] or by a single intravenous injection of doxorubicin, at 15 mg/kg (D1515, Sigma–Aldrich), in 0.9% saline.[16,17] Preparation of anti-GBM antiserum in rabbits was performed based on the Spiro's method[18] with some modification for mice experiment.[19] In brief, glomeruli were isolated by differential sieving from mouse renal cortex and disrupted by sonication. The GBM was collected by centrifugation, emulsified with complete Freund's adjuvant (Difco, Detroit, MI, USA) and immunized in rabbits. C57Bl/6 mice, at 8 weeks of age, were immunized by an intraperitoneal injection of 25 mg/kg normal rabbit IgG (MP Biomedicals, Santa Ana, CA, USA) emulsified with complete Freund's adjuvant (Difco), given 5 days before either vehicle (normal rabbit serum) or anti-GBM antiserum (0.3 mL, i.v.). The kidneys were removed and preserved for later processing for mRNA at week 4 after the anti-GBM antiserum injection. C57Bl/6 mice in the control group received the same volume of control antiserum. To investigate effects of PAR-1 inhibition on kidney damage, BALB/c mice were divided into the following groups: control ($n = 4$), doxorubicin + vehicle (15 mg/kg doxorubicin and saline, $n = 5$) and doxorubicin + Q94 (doxorubicin at 15 mg/kg and Q94 at 5 mg/kg/d, $n = 7$) groups. Where indicated, doxorubicin was administered intravenously and PAR-1 antagonist or saline vehicle by subcutaneous osmotic mini-pump. Albuminuria and podocyte injury were analyzed at 2 wk after injections. Urine samples were collected for 24 h in a metabolic cage before sacrifice. The kidneys were removed and preserved for later processing for mRNA, histology and immunohistochemistry (IHC) analyses.

2.4. Assay for albuminuria

Albumin levels were measured using commercially available assay kits (Mouse Albumin ELISA kit, Shibayagi, Gunma, Japan).

2.5. Assays for urinary and plasma creatinine

Commercially available assay kits were used to measure creatinine (LabAssay Creatinine kit; Wako, Osaka, Japan).

2.6. Histology

Kidneys were fixed with 15% formalin (pH 7.4), embedded in paraffin, cut into 2 μm sections and mounted on slides. The sections were then stained with periodic acid–Schiff (PAS) reagent and sirius red. Images were evaluated using light microscopy (BX-51/DP-72; Olympus, Tokyo, Japan). Glomerular sclerotic and sirius red positive areas were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Immunohistochemistry

Kidney tissues were fixed with 15% formalin (pH 7.4), embedded in paraffin, cut into 2 μm sections and mounted on slides. IHC staining for desmin[20] and 4-hydroxynonenal (4-HNE)[21] was performed with Histofine Simple Stain MAX-PO MULTI (Nichirei Biosciences, Tokyo, Japan). After deparaffinization with xylene, sections were incubated with 0.3% hydrogen peroxide, for 15 min for desmin or 30 min for 4-HNE, to block endogenous peroxidases. For desmin antigen retrieval, sections were incubated for 30 min in 0.01 mol/L citrate buffer (pH 6.0) at 100 °C. Proteinase K (DAKO Cytomation, Glostrup, Denmark) was used for 4-HNE antigen retrieval, with incubation for 10 min. After blocking with 10% goat serum, sections were incubated overnight at 4 °C with primary antibodies, each at 1:200 dilution (anti-human desmin mouse monoclonal antibody, D33, DAKO Cytomation; anti-hydroxynonenal antibody, ab46545, Abcam, Cambridge, UK). After washing sections and incubating them with secondary antibody for 1 h at room temperature, DAB substrate (DAKO Cytomation) was used to visualize IHC staining. Finally, counterstaining was performed with hematoxylin (DAKO Cytomation). Positively stained areas were analyzed using ImageJ software.

2.8. Laser-capture microdissection

Laser-capture microdissection (LCM) was performed as previously described.[22] Frozen tissues embedded in OCT were subsequently cryosectioned into 20 μm sections and fast-stained using Arcturus histogene frozen section staining kit (Ambion Inc., Austin, TX, USA). For each sample, 300–400 glomeruli were captured under direct visualization with CapSure HS Laser-capture microdissection tubes, using a laser microdissector pressure-catapulting device (Arcturus® LCM, Applied Biosystems, Waltham, MA, USA). Glomerular mRNA, for podocin and nephrin measurements, was extracted using an RNAqueous-Micro kit (Ambion Inc.).

2.9. Cell culture

Conditionally immortalized mouse podocytes were cultured as previously described.[20] Under growth permissive conditions, cells were seeded on type I collagen coated dishes at 33 °C. The growth medium was RPMI 1640 (Sigma–Aldrich) with 10% fetal bovine serum and 20 U/mL mouse interferon-γ (Sigma–Aldrich) to drive the expression of a thermosensitive T-antigen. To induce differentiation, cells were maintained at 37 °C without interferon-γ for 10–14 d. All experiments were performed with differentiated podocytes.