Original Paper

ADAMTS-7 Expression Increases in the Early Stage of Angiotensin II-Induced Renal Injury in Elderly Mice

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Key Words

ADAMTS-7 • Angiotensin II • Renal injury • Early stage • Inflammatory • Elderly

Abstract

Background/Aims: We investigated the recently described family of proteinases, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), and matrix metalloproteinases (MMPs) as inflammatory mediators in inflammatory kidney damage by studying ADAMTS-1, -4, and -7 and MMP-9 expression in elderly mouse kidneys after angiotensin II (Ang II) administration. **Methods:** Ang II (2.5 µg/kg/min) or norepinephrine (8.3 µg/kg/min) was subcutaneously infused in old mice. Renal injury was assessed by hematoxylin-eosin staining, 24-h albuminuria, and immunohistochemistry to evaluate inflammatory cell markers. The mRNA and protein expression of ADAMTS-1, -4, and -7 and MMP-9 were determined using real-time PCR, Western blot, and immunohistochemistry 3 days after Ang II or norepinephrine administration. **Results:** Elderly mice in the Ang II group developed hypertension and pathological kidney damage. The mRNA and protein levels of ADAMTS-7 in the Ang II group were 3.3 ± 1.1 ($P = 0.019$) and 1.6 ± 0.1 ($P = 0.047$) vs. 1.0 ± 0.1 and 1.0 ± 0.1 in the control group on day 3. In contrast, treatment with the hypertensive agent norepinephrine did not lead to obvious renal damage or an increase in renal ADAMTS-7 expression. **Conclusions:** Renal ADAMTS-7 expression was induced by Ang II in elderly mice. The overexpression of ADAMTS-7 might contribute to early inflammatory kidney damage associated with aging.

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Introduction

Kidney disease has emerged as a worldwide health problem. The incidence of chronic kidney disease (CKD) and end-stage renal disease (ESRD) in the general population has increased enormously over the past several decades, becoming a significant burden for healthcare systems. To adequately address this issue, detecting kidney disease patients early and optimizing their treatment are crucial.

In most species, aging is associated with impaired adaptive and homeostatic mechanisms, leading to susceptibility to environmental or internal stressors with increasing rates of disease [1, 2]. Age-related changes lead to a functional decline of several organs, including the kidneys [3]. Clinical studies have shown that the development of CKD is associated with the aging process [4, 5]. Even adjustments for age-associated diseases do not explain the higher rate of morbidity observed in elderly patients [6]. The underlying mechanism that might explain this phenomenon remains unclear.

The renin-angiotensin system, especially angiotensin II (Ang II), plays a key role in the progression of chronic kidney damage, contributing to renal inflammation and fibrosis [7-9]. Ang II can both directly and indirectly activate different signaling pathways to trigger the inflammatory response in kidney disease [7, 10, 11]. Many *in vitro* and experimental studies have demonstrated that Ang II activates renal cells to produce inflammatory mediators.

A growing body of evidence suggests that inflammation plays a role in the development of CKD [5]. Increased interest in inflammation has led to the investigation of various inflammatory mediators, including extracellular matrix (ECM)-degrading proteinases. The most commonly studied ECM-degrading proteinase in kidney disease is matrix metalloproteinase-9 (MMP-9), which has been shown to contribute to the pathogenesis of renal fibrosis via osteopontin cleavage [12].

The recently identified metalloproteinase family of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) also degrades the ECM. ADAMTS have been implicated in the development and progression of many cardiovascular diseases and inflammation. ADAMTS-1 might facilitate atherogenesis by cleaving the ECM protein versican [13]. ADAMTS-4 was identified as an inflammatory-regulated enzyme in macrophage-rich areas of human atherosclerotic plaques [14]. ADAMTS-7 was shown to facilitate vascular smooth muscle cell migration, thereby promoting neointima formation following vascular injury [15].

For these reasons, the purpose of the present study was to investigate whether ADAMTS is involved in the early phase of inflammatory renal injury induced by Ang II in elderly mice. Our results might contribute to the early diagnosis and treatment of kidney disease.

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of China-Japan Friendship Hospital. All of the surgeries were performed in sodium pentobarbital-anesthetized mice, and all efforts were made to minimize suffering. Thirty-six-week-old male C57BL/6 mice were purchased from the Animal Center of Peking University Health Science Center (Beijing, China) and maintained in a specific pathogen-free facility at the Experimental Center of China-Japan Friendship Hospital (accredited for animal care by the Chinese Association for Accreditation of Laboratory Animal Care) under a 12 h/12 h light/dark cycle with controlled room temperature and free access to standard chow and tap water.

Animal experiments

Two/three groups of mice ($n = 6$ per group) received continuous Ang II (2.5 $\mu\text{g/kg/min}$; Sigma Chemical, St Louis, MO, USA) [16], norepinephrine (8.3 $\mu\text{g/kg/min}$; Sigma Chemical, St Louis, MO, USA) [17], or saline via subcutaneous osmotic minipumps (Alza, Palo Alto, CA, USA) for 3 days. The mice in each

group underwent renal biopsy under anesthesia on day 3 and were then sacrificed by sodium pentobarbital overdose.

Cell culture

Human embryonic kidney 293 (HEK293) cells (ATCC CRL-1573 line) were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in 5% CO₂ at 37°C. For treatment, HEK293 cells were cultured at 40-50% of confluence and then incubated for 24 h with different doses of Ang II or 1 μM Ang II for different times.

Blood pressure measurement

Arterial systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), and heart rate (HR) were measured in conscious, restrained mice using a noninvasive computerized tail-cuff system (Muromachi Kikai, Tokyo, Japan). Measurements were performed every day, beginning 2 days before the placement of the minipumps. The blood pressure value for each mouse was calculated as the average of three separate measurements in each session.

Urine albumin measurement

To measure 24-h proteinuria, the mice were placed in individual mouse metabolic cages with free access to food and water. Urinary albumin excretion was assayed using a mouse albumin enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories, Montgomery, TX, USA).

Histology and immunohistochemistry

Paraformaldehyde-fixed tissue was embedded with optimum cutting temperature compound or paraffin and processed, and 5 μm sections were stained with hematoxylin-eosin. Additional sections were immunostained using an indirect horseradish peroxidase immunoperoxidase method using specific antibodies for the following antigens: ADAMTS-7 (ImmunoWay, Newark, DE, USA) and CD68 and neutrophil elastase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative controls for the immunostaining consisted of replacing each of the primary antibodies with an equivalent concentration of irrelevant rabbit polyclonal antibody. At least three different specimens were analyzed. The area of positive staining or tubular lumen diameter was analyzed using ImagePro-Plus 6.0 software (Media Cybernetics, Singapore, Singapore). For each kidney, six randomly selected fields were analyzed in a blinded manner by a technician who had no knowledge of the specific background of the study.

Quantitative real-time RT-PCR

Total RNA from kidney or HEK293 cells was isolated using Trizol reagent (Applygen Technologies, Beijing, China) and reverse-transcribed with a reverse transcription system (Promega, Madison, WI, USA). The reaction mixture then underwent PCR. The amount of PCR products formed in each cycle was evaluated by SYBR Green I fluorescence. Amplification reactions were performed using the ABI Prism 7500 PCR System. All of the amplification reactions underwent 35 cycles and were performed in duplicate (an initial stage of 5 min at 95°C, followed by a three-step cycle of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C). The accuracy of the PCR products was confirmed by sequencing the amplicons. The relative target mRNA levels were normalized to the internal control, β-actin. The following primers were used: ADAMTS-1 (forward, TTTGGCCAGCAGCTACATCT; reverse, CACACCTTCACAGAGGCTGA), ADAMTS-4 (forward, GCCTTCCTCCTGTCCTTAGC; reverse, TAGCAACATCTCCCAAAGG), ADAMTS-7 (forward, AACCAGGAACGCCTACCTTT; reverse, CGGGTCCTTGCTACTGTTA), MMP-9 (forward, CCAGATATGGGAGAGAAGC; reverse, GGCCTTTGAAGGTTTGAAT), β-actin (forward, ATCTGGCACCACACCTTC; reverse, AGCCAGGTCCAGACGCA).

Western blot

The extracts of mouse tissue or HEK293 cells were collected using tissue/cell lysis buffer (Beyotime, Jiangsu, China) plus 1 mM phenylmethylsulfonyl fluoride. Total protein was quantified using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes, which were incubated with an anti-β-actin antibody (MBL, Nagoya, Japan) or anti-ADAMTS-1, ADAMTS-4, ADAMTS-7, or MMP-9 antibody (Abcam, Cambridge, MA, USA), washed, and incubated

with an appropriate IRDye800-conjugated second antibody (Rockland, Gilbertsville, PA, USA). Specific immunofluorescence bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All of the results are expressed as the mean $\pm$ SEM or original data that represented one of at least three independent experiments. The data were analyzed using an unpaired Student's *t*-test and two-way analysis of variance (ANOVA) with Prism software (GraphPad, La Jolla, CA, USA) followed by the Student-Newman-Keuls *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Ang II-induced blood pressure elevations and renal injury

Blood pressure in the control and Ang II mice were in the normotensive range before Ang II administration (Fig. 1a-c). After placement of the subcutaneous osmotic minipumps that delivered Ang II (2.5 μ g/kg/min) [16], hypertension developed in the old mice. The SBP, DBP, and MBP in Ang II-infused mice were significantly higher after Ang II administration, whereas blood pressure in vehicle-infused mice remained normal during the study (Fig. 1a-c). Mean SBP in the control and Ang II mice was 108 ± 5 mmHg vs. 141 ± 5 mmHg ($P < 0.001$) on day 3 (Fig. 1a). No difference in HR was detected between Ang II-infused and control mice (Fig. 1d).

The renal histopathological findings are shown in Fig. 1e-i. Marked tubular injury, consisting of both tubular atrophy and tubular dilatation in the renal cortex and medulla, was found in Ang II-infused mice; none of the control mice had evidence of tubular injury on day 3. In contrast, Ang II exerted no marked effect on glomerular volume or cellularity.

Tubular injury in Ang II-infused mice was associated with inflammatory cell infiltration and significant albuminuria. Twenty-four-hour albuminuria in the control and Ang II mice was 163 ± 40 μ g vs. 487 ± 94 μ g ($P < 0.01$) on day 3 (Fig. 4o). Immunohistochemical staining was performed based on neutrophil elastase and CD68 expression, markers of neutrophils and macrophages, respectively. Both neutrophil elastase-positive cells and CD68-positive cells were found in renal tissue in Ang II-infused mice (Fig. 4h-m).

Renal ADAMTS-7 mRNA and protein expression in Ang II-infused mice

Elevated mRNA and protein expression of ADAMTS-7 was observed in the kidneys in Ang II-infused mice (Fig. 2c, 3a). The mRNA and protein levels of ADAMTS-7 were much higher in Ang II-infused mice compared with the control group (3.3 ± 1.1 vs. 1.0 ± 0.1 , $p = 0.019$, and 1.6 ± 0.1 vs. 1.0 ± 0.1 , $p = 0.047$; Fig. 2c, 3d) on day 3 after Ang II administration. No differences in the mRNA and protein expression of ADAMTS-1, ADAMTS-4, and MMP-9 were detected in the kidneys in the two groups (Fig. 2a, b, d, 3a-c, e). Additionally, immunohistochemistry indicated an increase in ADAMTS-7 expression in the kidneys in Ang II-infused mice compared with controls on day 3 (Fig. 3f-h).

Norepinephrine-induced blood pressure elevations and renal injury

To investigate whether Ang II-induced renal injury was attributable simply to elevated blood pressure, we used norepinephrine, which acts through hypertensive mechanisms that are distinct from Ang II [18]. Norepinephrine infusion (8.3 μ g/kg/min) over a 3-day period induced blood pressure elevations that were comparable to Ang II infusion in elderly

Fig. 1. Angiotensin II (Ang II)-induced renal injury in elderly mice on day 3. a-d Arterial systolic blood pressures (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), and heart rate (HR). Representative micrographs of the renal cortex (e, g) and medulla (f, h) subjected to hematoxylin-eosin (HE) staining and quantification of tubular lumen diameter (i) (200 $\times$ magnification). The data are expressed as mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$, vs. controls ($n = 6$).

Fig. 3.

3. ADAMTS-7 protein expression was increased by Ang II infusion in elderly mice on day 3. a-e Representative Western blots of ADAMTS-1, ADAMTS-4, ADAMTS-7, and MMP-9 protein expression and quantification. f-h Representative immunohistochemical staining of ADAMTS-7 expression and quantification (200× magnification). The data are expressed as mean ± SEM. * $P < 0.05$ ($n = 6$).

mice on days 1-3 (Fig. 4a-c) [17]. A certain degree of tubular atrophy and tubular dilatation was found in the renal cortex in norepinephrine-infused mice (Fig. 4e-g, n), but none of the norepinephrine mice exhibited evidence of albuminuria or neutrophil elastase- or CD68-positive cell infiltration (Fig. 4h-m, o).

Renal ADAMTS-7 mRNA and protein expression in norepinephrine-infused mice

To investigate the mechanism of the increase in ADAMTS-7 expression, we detected ADAMTS-7 expression in the kidneys in norepinephrine-infused mice. The mRNA and protein expression of ADAMTS-7 did not obviously change in the kidneys in norepinephrine-infused mice (Fig. 5a-c). Additionally, immunohistochemical studies indicated similar ADAMTS-7 expression in the kidneys in norepinephrine-infused mice compared with controls on day 3 (Fig. 5d-g). Thus, we conclude that Ang II itself and not hypertension was involved in renal injury and the increase in ADAMTS-7 expression in the early stage of renal injury.

ADAMTS-7 mRNA and protein expression in Ang II-stimulated HEK293 cells

To further investigate the effect of Ang II on ADAMTS-7 expression, we examined ADAMTS-7 expression in HEK293 cells incubated with different doses of Ang II for 24 h or 1 μ M Ang II for different times. Compared with controls, ADAMTS-7 mRNA and protein expression was concentration- and time-dependently upregulated by Ang II (Fig. 6a-d). The

Fig. 4. Renal injury in elderly mice was induced by Ang II but not norepinephrine (NE) on day 3. a-d SBP, DBP, MBP, and HR. The figure shows representative micrographs of renal tissue subjected to HE staining (e-g) and quantification of tubular lumen diameter (n). h-m Representative immunohistochemical staining of neutrophil elastase or CD68 expression (400× magnification). The arrows indicate neutrophil elastase- or CD68-positive cells. o Twenty-four-hour proteinuria. The data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. controls; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. controls ($n = 6$).

Fig. 5. ADAMTS-7 expression in the kidneys in elderly mice is regulated by Ang II but not NE on day 3. **a** mRNA levels of ADAMTS-7 measured by quantitative PCR. **b, c** Representative Western blot of ADAMTS-7 protein expression and quantification. **d-g** Representative immunohistochemical staining of ADAMTS-7 expression and quantification (400× magnification). The data are expressed as mean ± SEM. * $P < 0.05$ ($n = 6$).

protein levels of ADAMTS-7 were much higher in Ang II-stimulated cells compared with controls (1.5 ± 0.1 vs. 1.0 ± 0.01 , $P = 0.047$; Fig. 6f) 24 h after 1 μ M Ang II incubation.

Discussion

Despite the technical advances in and resources dedicated to the treatment of CKD, it is a growing problem worldwide, especially in the aging population. Clarification of kidney disease mechanisms and the early detection of kidney damage are clearly needed.

Fig. 6. Ang II concentration- and time-dependently promoted ADAMTS-7 expression in HEK293 cells. a, b mRNA levels of ADAMTS-7 measured by quantitative PCR. c, d Representative Western blot of ADAMTS-7 protein expression and quantification. The data are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

Hypertension is one of the most important causes of CKD. In the present study, Ang II induced modest hypertension and evident renal injury, including tubular atrophy, tubular dilatation, inflammatory cell infiltration, and albuminuria, in elderly mice in the early stage. To some extent, our results perhaps explain why sustained hypertension may develop several weeks to months after a short series of daily Ang II infusions [19] because kidney damage can exacerbate the development of hypertension.

Matrix metalloproteinases are candidates in the pathological molecular mechanisms of CKD. These enzymes degrade the ECM, modulate inflammation, and regulate tissue remodeling. One study demonstrated that MMP-9 was elevated in the pathogenesis of renal fibrosis [12], and MMP-2 and MMP-10 were elevated in ESRD [20]. Cheng *et al.* demonstrated that MMP-2 overexpression in transgenic mice leads to structural alterations in the tubular basement membrane, triggering the tubular epithelial-mesenchymal transition and resulting in tubular atrophy, fibrosis, and renal failure [21].

ADAMTS is a recently identified family of extracellular metalloproteinases that has been shown to participate in tissue destruction [22]. The ADAMTS family of proteins comprises 20 members. ADAMTS-1, ADAMTS-4, and ADAMTS-7 are particularly well-studied members of this family [23, 24]. Accumulating studies have shown that ADAMT-7 plays an important role in cardiovascular disease [25]. Our previous studies also showed that ADAMTS-7 mediated injury-induced vascular remodeling and calcification [15, 26]. The role of ADAMTS-7 in inflammatory renal injury has not yet been studied. In the present study, we found that renal ADAMTS-7 expression was induced in old mouse kidneys on day 3 after Ang II administration, with no difference in MMP-9, ADAMTS-1, and ADAMTS-4 expression between the two groups.

To exclude the possibility that Ang II-induced renal injury and ADAMTS-7 expression might be simply attributable to increased blood pressure, we compared the effects of norepinephrine and Ang II infusion on renal function and renal ADAMTS-7 expression. Norepinephrine infusion caused blood pressure elevations, tubular atrophy, and tubular dilatation that were comparable to Ang II infusion in elderly mice on day 3, but only Ang II-infused mice had evidence of albuminuria, inflammatory cell infiltration, and increased ADAMTS-7 expression (Fig. 4, 5).

We also histologically analyzed ADAMTS-1, -4, and -7 and MMP-9 expression in the kidneys in 20-week-old male C57BL/6 mice with Ang II-mediated kidney damage (data not shown). Adult mice in the Ang II group developed more severe hypertension. However, the histological examination identified fewer renal histopathological changes in adult mice than in elderly mice. No significant differences in the mRNA and protein levels of ADAMTS-1, -4, and -7 and MMP9 were detected between the two groups of adult mice. This may indicate that the aging kidney is more sensitive to inflammation, which may be because of differential ADAMTS-7 induction between adult and aging mice.

Conclusion

In summary, we found renal injury and increased renal ADAMTS-7 expression in the early stage of Ang II-mediated inflammatory kidney impairment in old mice. ADAMTS-7 may play important pathogenic roles in the progression of elderly kidney damage.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (URL: www.nsf.gov.cn/Portal0/default152.htm) [81170287 and 30770865 to J. Zheng]. The funding agency had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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