J Toxicol Pathol 2023; 36: 181–185

Short Communication

Analyses of damage-associated molecular patterns, particularly biglycan, in cisplatin-induced rat progressive renal fibrosis

Minto Nakagawa[1], Takeshi Izawa[1], Mitsuru Kuwamura[1], and Jyoji Yamate[1]*

[1] Laboratory of Veterinary Pathology, Osaka Metropolitan University, 1-58 Rinku-Ourai-Kita, Izumisano City, Osaka 598-8531, Japan

Abstract: Damage-associated molecular patterns (DAMPs) and their receptors (TLR-2 and -4) may play important roles in renal fibrosis, of which the pathogenesis is complicated. We used rat renal lesions induced by a single intraperitoneal injection of cisplatin at 6 mg/kg body weight; consisting of tissue damage of renal tubules on days 1 and 3, further damage and regeneration with inflammation mainly on days 5 and 7, and interstitial fibrosis on days 9, 12, 15, and 20. Microarray analyses on days 5 (the commencement of inflammation) and 9 (the commencement of interstitial fibrosis) showed that DAMPs increased by more than two-fold relative to control included common extra-cellular matrix (ECM) components such as laminin (Lamc2) and fibronectin, and heat shock protein family, as well as fibrinogen, although it was limited analysis; Lamc2, an element of basement membrane, may be regarded as an indicator for damaged renal tubules. In the real-time RT-PCR analyses, TLR-2 significantly increased transiently on day 1, whereas TLR-4 significantly increased on days 9 and 15, almost in agreement with the increased biglycan (a small leucine-rich proteoglycan as ubiquitous ECM component). As M1/M2 macrophages participated in renal lesions, such as inflammation and fibrosis, presumably, TLR-4, which may be expressed in immune cells, could play crucial roles in the formation of renal lesions in association with biglycan. (DOI: 10.1293/tox.2022-0148; J Toxicol Pathol 2023; 36: 181–185)

Key words: cisplatin, rat renal fibrosis, damage-associated molecular patterns, TLR-2, TLR-4, biglycan

Renal fibrosis is regarded as the common final pathway following renal tissue damage and may lead to chronic kidney disease (CKD) as the final stage. Macrophages and myofibroblasts play important roles in the development of renal fibrosis[1–4]. Transforming growth factor-β1 (TGF-β1), produced mainly by reactive macrophages, is an important factor for the activation and induction of myofibroblasts[5]. Partly, the myofibroblasts may be evoked via the epithelial-mesenchymal transition (EMT) in incompletely regenerating renal tubules[6]. Previously, we reported the participation of M1/M2-macrophages in cisplatin (CDDP)-induced rat renal lesions; $CD68^+$ M1 macrophages began to increase in injured areas, with increased expressions of M1-related inflammatory factors (interferon-gamma [IFN-ɤ], tumor necrosis factor-alpha [TNF-α], and interleukin [IL]-6), and, thereafter, $CD163^+$ M2 macrophages showed a gradual increase with interstitial fibrosis which was accompanied by increased TGF-β1 expression and myofibroblast development; M1/M2 macrophages cooperatively contribute to progressive renal fibrosis[1]. Macrophage infiltration may be induced by "damage-associated molecular patterns (DAMPs)" released by injured tissues or dying cells[7]. DAMPs activate Toll-like receptors (TLRs) expressed in immune cells such as macrophages[7–9]. TLRs are germline-encoded pattern-recognition receptors as a first line of innate defense by recognizing pathogen-associated molecular patterns as well as endogenous signals (such as DAMPs) of tissue injury[9]. Downstream effects of TLR engagement include the production of inflammatory cytokines and chemokines in affected cells[8]. DAMPs may be important factors in renal lesion development; however, the contribution of DAMPs and their TLRs to CDDP-induced rat renal fibrosis remains to be investigated.

Here, kidney samples, that we had previously investigated the participation of M1/M2-macrophages[1], were used; briefly, out of the 27 six-week old male F344/DuCrj rats, 24 rats were intraperitoneally injected once with CDDP (Nippon Kayaku Co. Ltd., Tokyo, Japan) at 6 mg/kg body weight (BW); three rats were sacrificed on each of days 1, 3, 5, 7, 9, 12, 15, and 20 after CDDP injection. The remaining three rats, served as controls, were injected with equivalent volumes of phosphate-buffered saline in the same manner, and were sacrificed on day 0. All the rats were euthanized under deep isoflurane anesthesia. The experimental protocols were performed according to the Institutional Guidelines for Animal Care and Use in our University.

Detailed histopathological findings are described in

Received: 30 December 2022, Accepted: 7 March 2023
Published online in J-STAGE: 22 March 2023
*Corresponding author: J Yamate (e-mail: yamate@omu.ac.jp)

our previous study; briefly, on days 1 and 3 after CDDP injection, renal proximal tubular epithelial cells, especially those at the S3 segment in the cortico-medullary junction, underwent swelling with nuclear degeneration; on days 5 (Fig. 1a) and 7, in addition to desquamation or regeneration of renal epithelial cells, infiltrating macrophages began to be seen in the affected areas; on days 9 (Fig. 1b), 12, 15, and 20, interstitial fibrosis gradually developed around variously dilated renal tubules, accompanied by an increase in myofibroblasts[1]. Interstitial fibrosis, demonstrable with Azan-Mallory staining for collagens and the appearance of α-smooth muscle actin-immunopositive myofibroblasts, was shown in our previous study[1]. In order to find out possible candidates of TLRs and DAMPs, first, microarray analysis was performed on days 5 and 9, because inflammation and interstitial fibrosis began to be seen on days 5 and 9, respectively. Total RNA was extracted from the cortico-medullary junction of the renal tissues using the SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA samples (n=1, pooled from three rats at each time point) were subjected to the following procedures: quality check using the Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), cDNA synthesis and cRNA labeling and amplification using the Low Input Quick Amp Labeling Kit (Agilent Technologies), hybridization at 65°C for 17 h with Whole Rat Genome 4×44K 1 color (Agilent Technologies), and signal scanning using the Microarray scanner (Agilent Technologies). On day 5 (the commencement of inflammation), the DAMPs listed in Table 1 were increased approximately 2- to 40-fold compared with those in the control, however no significant increases in TLRs mRNA expressions were observed. On day 9 (the commencement of interstitial fibrosis), along with increased expression of TLR-2 (3.1-fold) and TLR-4 (2.2-fold), the DAMPs listed in Table 2 were increased by approximately 2- to 20-fold compared with those in the control. Although this is a limited analysis, on days 5 and 9, extra-cellular matrix (ECM) components such as laminin (Lamc2) and fibronectin (Fn1), and the heat shock protein (Hsp) family (Hspa1b, Hspa1a, Hspb1 or Hspa2), as well as fibrinogen (Fgb), increased in common (Tables 1 and 2). Out of them, Lamc2 expression was remarkably high both on days 5 (39.6-fold) and 9 (12.6-fold). Specifically, on day 5, renal epithelial cells were injured by CDDP and desquamated, apparently leading to disruption of the tubular structure (Fig. 1a). When the basement membrane is disrupted, injured renal tubular epithelial cells are sloughed from the basement membrane and fall into the lumen, resulting in interstitial fibrosis, which is occasionally accompanied by abnormal epithelial regeneration based on the degree of disruption[10, 11]. Increased level of Lamc2 may be regarded as an indicator for the damaged renal tubules, followed by interstitial fibrosis. Increased expressions of S100 proteins (S100a8 and S100a9 at 11.3- and 9.1-fold, respectively) on day 5, HSPs (Hspa1b, Hspa1a, Hspb1 or Hspa2 at 2.1- to 2.9-fold) on days 5 and 9, and biglycan (Bgn at 3.9-fold) on day 9 were observed. S100 proteins and HSPs are regarded as cytoprotection proteins[12–14], and they are included in DAMPs[15].

Biglycan is a component of ECMs, and its detailed discussion is mentioned below. As DAMPs may act as triggers for inflammation or fibrosis in pathological lesions[16], the DAMPs listed in Tables 1 and 2 might have been related to the induction of inflammation on day 5 and fibrosis on day 9, although the more detailed analyses are needed.

Next, using samples on days 0 (controls), 1, 3, 5, 7, 9, 12, 15, and 20, we investigated the kinetics of the mRNA expressions of DAMP genes such as nonhistone chromatin-binding protein high-mobility group box 1 (HMGB1), biglycan, Hspa1b, and S100a4, because they have been previously reported as influential DAMPs in kidney disease models[16]. These DAMPs and TLRs are related as follows: HMGB1 to TLR-2 and -4, Hsps to TLR-2 and -4 and S100

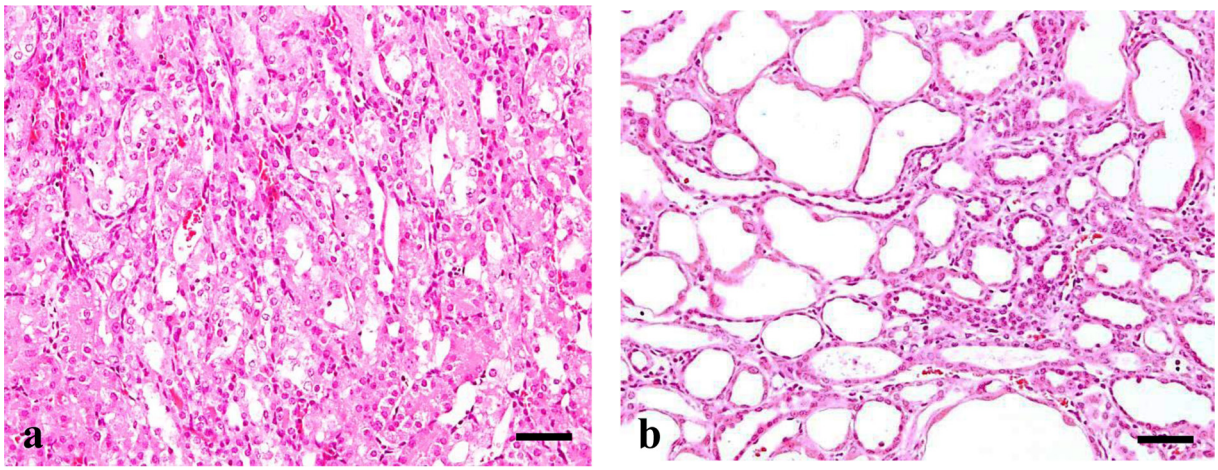

Fig. 1. Histopathology on days 5 and 9 after cisplatin injection in rats. (a) On day 5, renal epithelial cells of the affected tubules showed necrosis/swelling and desquamation, in addition to infiltrating macrophages. (b) On day 9, the variously dilated renal tubules were rimmed by regenerating epithelial cells, and interstitial fibrosis is seen, accompanied by inflammatory cells. Hematoxylin-eosin stain. Bar=50 μm.

proteins to TLR-4, as well as biglycan to TLR-2 and -4[7, 8]. Therefore, to further analyze the expressions of TLR-2 and TLR-4, as well as DAMPs (HMGB1, biglycan, Hspa1b, and S100a4), the real-time RT-PCR was performed. Total RNA, extracted from the corticomedullary junction as described above, was reverse-transcribed into cDNA using the Super Script® VILO ™ cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA)[1]. For TLR-2, TLR-4, HMGB1, biglycan, Hspa1b, and S100a4, the THUNDERBIRD™ Probe qPCR Mix (Toyobo, Co., Ltd., Osaka, Japan) and TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) were performed using the following probes (assay IDs): Rn02133647_s1 for TLR-2, Rn00569848_m1 for TLR-4, Rn02377062_g1 for HMGB1, Rn01529734_g1 for biglycan, Rn02532795_s1 for Hspa1b, Rn01451938_m1 for S100a4, and Rn00667869_m1 for β-actin. The amplification program consisted of one cycle at 95°C with a 1-min hold followed by 40 cycles at 95°C (denaturing) with a 15-sec hold, 60°C (extension) with a 30-sec hold, and lastly 20°C (cooling) with a 10-sec hold. The expression values of target genes were normalized to those of β-actin. RT-PCR data are expressed as mean ± standard deviation (SD). Differences between control and CDDP-injected groups were evaluated using Dunnett's test. Values of $p<0.05$ were considered significant.

TLR-2 transiently increased significantly on day 1 (5.0-fold; Fig. 2a), whereas TLR-4 mRNA expression increased significantly (3.0- to 5.0-fold) on days 9 and 15 (Fig. 2b). Biglycan significantly increased on days 9, 15, and 20 (2.5- to 4.0-fold) in the progressive renal fibrosis stage (Fig. 2c) compared with those in the control, which is consistent with TLR-4 mRNA expression. However, there was no significant change in the expression of HMGB1, Hspa1b, or S100a4 expressions. RT-PCR analyses showed that TLR-2 was expressed at a very early stage on day 1. TLR-2 is reported to be markedly up-regulated in the tubular and tubulointerstitial cells of patients with chronic renal injury[17]. However, the absence of TLR-2 using TLR2−/− mice does not affect the development of chronic renal injury and subsequent fibrosis in unilateral ureteral obstruction-injured kidneys[17]. Thus, TLR-2 may not be important in progressive renal fibrosis, however may contribute to the inflammation of renal tissues only at the early stages[17]. The ligands for TLR-2 in early stages require further investigation.

It is very interesting to note that TLR-4 significantly increased at the fibrosis progression stages on days 9 to 20, particularly with significant increases on days 9 and 15. A study using CDDP-treated TLR-4−/− mice revealed that the mutants had less renal damages (evaluated by histopathological findings, leukocyte infiltration and cytokine production levels) than did the wild type mice[9]. It is reported that CDDP injection to mice had no effect on the expression of TLR-4 ligands such as HMGB1, Hsp60, and Hsp70[9]. Increased expressions in HMGB1, Hspa1b, and S100a4 also were not confirmed in the present CDDP-injected rats (data not shown). However, TLR-4 expressions apparently corresponded to a significant increase in biglycan on days 9, 15, and 20. Biglycan, a small leucine-rich proteoglycan, is a ubiquitous ECM component. Biglycan, which has been considered to be released from ECMs in damaged tissues, may boost inflammation by signaling through TLR-2 and TLR-4[18]. Despite no significant change, the TLR-2 gene showed

Table 1. Microarray Analysis on Day 5 (the Commencement of Inflammation) after Cisplatin Injection

Gene name	Description	Foldchange vs. control
Lamc2	ref|Rattus norvegicus laminin, gamma 2 (Lamc2), mRNA [NM_001100640]	39.6
Fgb	ref|Rattus norvegicus fibrinogen beta chain (Fgb), mRNA [NM_020071]	24.7
S100a8	ref|Rattus norvegicus S100 calcium binding protein A8 (S100a8), mRNA [NM_053822]	11.3
S100a9	ref|Rattus norvegicus S100 calcium binding protein A9 (S100a9), mRNA [NM_053587]	9.1
Fga	ref|Rattus norvegicus fibrinogen alpha chain (Fga), transcript variant 1, mRNA [NM_001008724]	6.6
Hspa1b	ref|Rattus norvegicus heat shock 70kD protein 1B (mapped) (Hspa1b), mRNA [NM_212504]	2.9
Hspa1a	ref|Rattus norvegicus heat shock 70kD protein 1A (Hspa1a), mRNA [NM_031971]	2.2
Fn1	ref|Rattus norvegicus fibronectin 1 (Fn1), mRNA [NM_019143]	2.1
Hspb1	ref|Rattus norvegicus heat shock protein 1 (Hspb1), mRNA [NM_031970]	2.1

Table 2. Microarray Analysis on Day 9 (the Commencement of Interstitial Fibrosis) after Cisplatin Injection

Gene name	Description	Foldchange vs. control
Fgb	ref|Rattus norvegicus fibrinogen beta chain (Fgb), mRNA [NM_020071]	20.0
Lamc2	ref|Rattus norvegicus laminin, gamma 2 (Lamc2), mRNA [NM_001100640]	12.6
Fn1	ref|Rattus norvegicus fibronectin 1 (Fn1), mRNA [NM_019143]	4.1
Bgn	ref|Rattus norvegicus biglycan (Bgn), mRNA [NM_017087]	3.9
Tlr2	ref|Rattus norvegicus toll-like receptor 2 (Tlr2), mRNA [NM_198769]	3.1
Hspa1b	ref|Rattus norvegicus heat shock 70kD protein 1B (mapped) (Hspa1b), mRNA [NM_212504]	2.3
Hspa2	ref|Rattus norvegicus heat shock protein 2 (Hspa2), mRNA [NM_021863]	2.2
Tlr4	ref|Rattus norvegicus toll-like receptor 4 (Tlr4), mRNA [NM_019178]	2.2

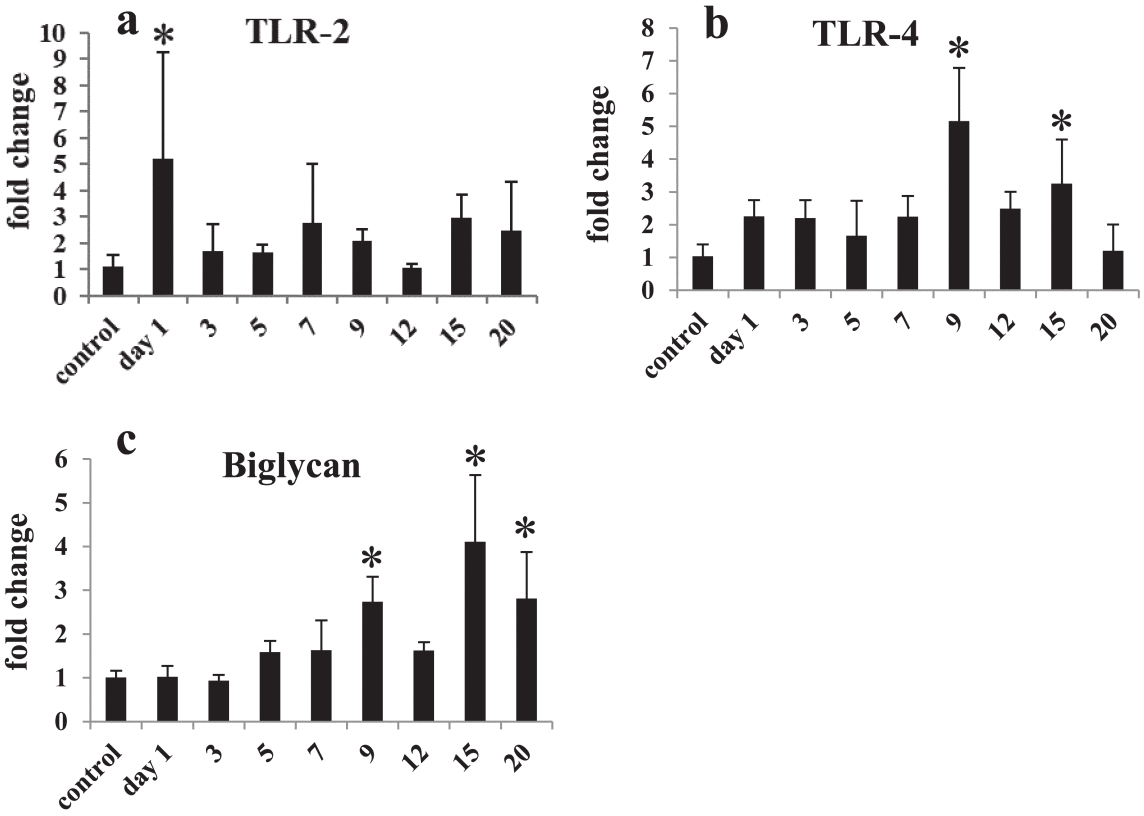

Fig. 2. Messenger ribonucleic acid (mRNA) expressions of toll-like receptor (TLR)-2 (a) and TLR-4 (b), as well as biglycan (c) by real-time reverse transcription polymerase chain reaction (RT-PCR) method using renal lesion samples on days 0 (control), 1, 3, 5, 7, 9, 12, 15, and 20 after cisplatin injection. Expression levels were normalized to β-actin RNA levels. Bar represents the mean ± standard deviation. *, significantly different from controls at $p<0.05$ by Dunnett's test.

more than 2.0-fold increase relative to that in control on days 9, 15, and 20, and the microarray on day 9 revealed that TLR-2 was 3.1-fold higher than in the control. In the present CDDP-induced rat renal fibrosis, mainly on days 9 to 20, biglycan might function as an endogenous ligand, in correlation with increased TLR-4 levels at the late stages of interstitial fibrosis, although the relationship in the kinetics between TLRs and DAMPs listed in Tables 1 and 2 requires further investigation.

Based on results in our previous study[1] and present study, the possible pathogenesis on the participation of M1/M2-macrophages and DAMPs in CDDP-induced rat renal fibrosis is shown in Fig. 3. Renal lesions induced by a single injection of CDDP at 6 mg/kg BW consisted of damage such as necrosis and apoptosis in renal tubular epithelial cells on days 1 and 3, further damage and regeneration of the affected tubules with inflammation mainly on days 5 and 7, and progressive interstitial fibrosis on days 9, 12, 15, and 20. Although the observation period was limited to 20 days, renal interstitial fibrosis was considered to be progressed until the end of observation[1]. $CD68^+$ M1 macrophages for inflammation/injury began to be seen on day 5, accompanied by increased inflammatory cytokines (IFN-ɤ, TNF-α, and IL-6). On day 9 onwards, $CD163^+$ M2 macrophages showed a gradual increase with interstitial fibrosis, accompanied by

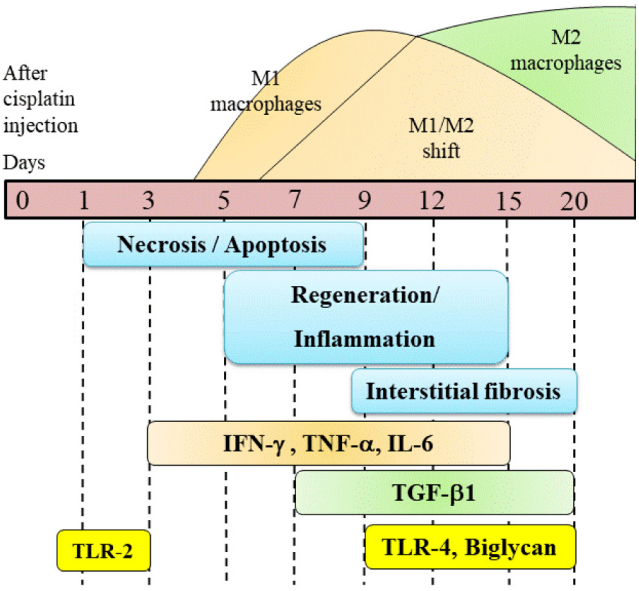

Fig. 3. A possible pathogenesis of cisplatin-induced rat renal fibrosis, based on M1/M2-macrophages[1], and TLR-2/-4 and biglycan. IFN-ɤ: interferon-gamma; TNF-α: tumor necrosis factor-alpha; IL: interleukin; TGF-β1: transforming growth factor-β1; TLR: toll-like receptor.

increased TGF-β1 expression and myofibroblast development. Interestingly, 62.0–78.0% of $CD68^+$ M1 macrophages co-expressed CD163, indicating that M1/M2 macrophages contribute to progressive renal fibrosis, presumably via a functional shift from M1 to M2. Increased TLR-2 on day 1 might be related to renal tissue injury at the very early stage, whereas TLR-4 increased on days 9 and 15 could be associated with fibrosis progression at the late stages, in relation to increased biglycan as a DAMP.

Finally, because it is known that the administration of chemotherapeutic agent, cisplatin, leads to acute kidney injury of which the process has a complex pathophysiological map[19], the present data may provide useful information on the possible roles of DAMPs in the mechanism of CDDP-induced renal lesions.

Disclosure of Potential Conflicts of Interest: The authors declare no conflicts of interest.

Acknowledgments: This work was supported partly by the JSPS KAKENHI Grant Number 19H03130 (to Yamate); the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number JP21am0101123 (to Yamate).

References

1. Nakagawa M, Karim MR, Izawa T, Kuwamura M, and Yamate J. Immunophenotypical characterization of M1/M2 macrophages and lymphocytes in cisplatin-induced rat progressive renal fibrosis. Cells. **10**: 257. 2021. [Medline] [CrossRef]
2. Ricardo SD, van Goor H, and Eddy AA. Macrophage diversity in renal injury and repair. J Clin Invest. **118**: 3522–3530. 2008. [Medline] [CrossRef]
3. Henderson NC, Mackinnon AC, Farnworth SL, Kipari T, Haslett C, Iredale JP, Liu FT, Hughes J, and Sethi T. Galectin-3 expression and secretion links macrophages to the promotion of renal fibrosis. Am J Pathol. **172**: 288–298. 2008. [Medline] [CrossRef]
4. Yamate J, Sato K, Ide M, Nakanishi M, Kuwamura M, Sakuma S, and Nakatsuji S. Participation of different macrophage populations and myofibroblastic cells in chronically developed renal interstitial fibrosis after cisplatin-induced renal injury in rats. Vet Pathol. **39**: 322–333. 2002. [Medline] [CrossRef]
5. LeBleu VS, Taduri G, O'Connell J, Teng Y, Cooke VG, Woda C, Sugimoto H, and Kalluri R. Origin and function of myofibroblasts in kidney fibrosis. Nat Med. **19**: 1047–1053. 2013. [Medline] [CrossRef]
6. Terada N, Karim MR, Izawa T, Kuwamura M, and Yamate J. Expression of β-catenin in regenerating renal tubules of cisplatin-induced kidney failure in rats. Clin Exp Nephrol. **22**: 1240–1250. 2018. [Medline] [CrossRef]
7. Rosin DL, and Okusa MD. Dangers within: DAMP responses to damage and cell death in kidney disease. J Am Soc Nephrol. **22**: 416–425. 2011. [Medline] [CrossRef]
8. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, Alexander SI, Sharland AF, and Chadban SJ. TLR4 activation mediates kidney ischemia/reperfusion injury. J Clin Invest. **117**: 2847–2859. 2007. [Medline] [CrossRef]
9. Zhang B, Ramesh G, Uematsu S, Akira S, and Reeves WB. TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity. J Am Soc Nephrol. **19**: 923–932. 2008. [Medline] [CrossRef]
10. Saberi K, Pasbakhsh P, Omidi A, Borhani-Haghighi M, Nekoonam S, Omidi N, Ghasemi S, and Kashani IR. Melatonin preconditioning of bone marrow-derived mesenchymal stem cells promotes their engraftment and improves renal regeneration in a rat model of chronic kidney disease. J Mol Histol. **50**: 129–140. 2019. [Medline] [CrossRef]
11. Nakatsuji S, Yamate J, and Sakuma S. Relationship between vimentin expressing renal tubules and interstitial fibrosis in chronic progressive nephropathy in aged rats. Virchows Arch. **433**: 359–367. 1998. [Medline] [CrossRef]
12. Trøstrup H, Holstein P, Christophersen L, Jørgensen B, Karlsmark T, Høiby N, Moser C, and Ågren MS. S100A8/A9 is an important host defence mediator in neuropathic foot ulcers in patients with type 2 diabetes mellitus. Arch Dermatol Res. **308**: 347–355. 2016. [Medline] [CrossRef]
13. Ikemoto M, Murayama H, Itoh H, Totani M, and Fujita M. Intrinsic function of S100A8/A9 complex as an anti-inflammatory protein in liver injury induced by lipopolysaccharide in rats. Clin Chim Acta. **376**: 197–204. 2007. [Medline] [CrossRef]
14. Toivola DM, Strnad P, Habtezion A, and Omary MB. Intermediate filaments take the heat as stress proteins. Trends Cell Biol. **20**: 79–91. 2010. [Medline] [CrossRef]
15. Foell D, Wittkowski H, and Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. Nat Clin Pract Rheumatol. **3**: 382–390. 2007. [Medline] [CrossRef]
16. Anders HJ, and Schaefer L. Beyond tissue injury-damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. J Am Soc Nephrol. **25**: 1387–1400. 2014. [Medline] [CrossRef]
17. Leemans JC, Butter LM, Pulskens WP, Teske GJ, Claessen N, van der Poll T, and Florquin S. The role of toll-like receptor 2 in inflammation and fibrosis during progressive renal injury. PLoS One. **4**: e5704. 2009. [Medline] [CrossRef]
18. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Götte M, Malle E, Schaefer RM, and Gröne HJ. The matrix component biglycan is proinflammatory and signals through toll-like receptors 4 and 2 in macrophages. J Clin Invest. **115**: 2223–2233. 2005. [Medline] [CrossRef]
19. McSweeney KR, Gadanec LK, Qaradakhi T, Ali BA, Zulli A, and Apostolopoulos V. Mechanisms of cisplatin-induced acute kidney injury: pathological mechanisms, pharmacological interventions, and genetic mitigations. Cancers (Basel). **13**: 1572. 2021. [Medline] [CrossRef]