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# Inhibition of matrix metalloproteinases reduces ischemia-reperfusion acute kidney injury

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Matrix metalloproteinases (MMPs) are endopeptidases that degrade extracellular matrix and involved in ischemic organ injuries. The present study was designed to determine the role of MMP-2 in the development of ischemic acute kidney injury (AKI). AKI was induced in MMP-2 wild-type (MMP-$2^{+/+}$) mice by 30, 60, 90, and 120 min renal ischemia and reperfusion. Renal histology, expression and activity of MMP-2 and MMP-9, and renal function were examined during the development of AKI. AKI was also induced in MMP-2-deficient (MMP-$2^{-/-}$) mice and MMP-$2^{+/+}$ mice treated with inhibitor of MMPs (minocycline and synthetic peptide MMP inhibitor). In MMP-$2^{+/+}$ mice, MMP-2 and MMP-9 activities increased significantly at 2 to 24 h, peaked at 6 h, after reperfusion. Immunohistochemical analysis identified MMP-2 in the interstitium around tubules and peritubular capillaries in the outer medulla. Acute tubular injury (ATI), including apoptosis and necrosis, was evident in the outer medulla at 24 h, along with renal dysfunction. As ischemia period increases, MMP-2 and MMP-9 activities at 6 h and severity of AKI at 24 h increased depending on the duration of ischemia between 30 and 120 min. However, the kidneys of MMP-$2^{-/-}$ mice showed minimal ATI; serum creatinine 24 h after reperfusion was significantly low in these mice. Inhibitors of MMPs reduced ATI and improved renal dysfunction at 24 h. We conclude that MMPs, especially MMP-2 have a pathogenic role in ischemia-reperfusion AKI, and that inhibitors of MMPs can protect against ischemic AKI.

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**KEYWORDS:** acute kidney injury; acute tubular injury; ischemia/reperfusion; MMP-2; MMP-9; MMP inhibitor

Ischemia-reperfusion acute kidney injury (AKI) remains a major cause of morbidity and mortality.[1–5] The pathophysiology of AKI is complex; the initial ischemia and reperfusion events rapidly lead to energy loss, which ultimately triggers a wide and intricately linked cascade of tubular epithelial cell death pathways. Over the past decade, various molecular mechanisms have been implicated, including activation of $Ca^{2+}$-dependent proteases or other enzymes, oxidative stress, and even programmed cell death signals, such as apoptosis.[6–8] In addition to these primarily intracellular events, evidence for the importance of intercellular signaling is beginning to emerge, all cells in the renal tubular and microvasculature are also affected, not just tubular epithelial cells.[6–10] Renal tubulovascular perturbations in AKI lead to tubular damage, back leak, obstructive cast formation, leukocyte infiltration, and altered renal microvascular function that contribute to the development of AKI over hours or days after ischemia-reperfusion.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases responsible for extracellular matrix turnover, as well as degradation of bioactive proteins.[11,12] This family includes collagenases, gelatinases, stromelysins, and membrane-type MMPs. Recently, MMPs, especially gelatinases (MMP-2 and MMP-9) have been demonstrated to have major roles in the pathophysiology of ischemia-reperfusion injury in several organs, including the central nervous system, heart, liver, lung, and other tissues.[13–20] In the kidney, several studies have demonstrated that MMP-2 and MMP-9 are upregulated after ischemia-reperfusion, and that MMP activation modulates renal microvascular permeability.[21–24] However, the role of MMPs during the development of AKI is still uncertain, because contradictory results have been reported by several studies.[25–27]

The present study was designed to determine whether MMP-2 and MMP-9 are activated during the development of ischemic AKI. After demonstrating this to be the case, experiments were then performed to determine the therapeutic effect of inhibition of MMP activity on ischemic AKI using MMP-2 knockout mice, minocycline, and a synthetic peptide MMP-2/MMP-9 inhibitor, in reducing the development of ischemic AKI.

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## MATERIALS AND METHODS

### Mice

Gelatinase A (MMP-2) knockout (MMP-$2^{-/-}$) mice were generated by gene targeting,[28] and MMP-$2^{-/-}$ mice generated in C57BL/6 genetic background were kindly provided by Shigeyoshi Itohara (Laboratory for Behavioral Genetics, RIKEN Brain Science Institute, Saitama, Japan). Mice were used at 3 to 4 months of age. All experimental procedures described here were approved by the Nippon Medical School Animal Studies Committee.

### Renal Ischemia-Reperfusion Model

We performed three experiments using MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice. In experiment 1, AKI was induced in MMP-$2^{+/+}$ mice by 30, 60, 90, or 120 min of ischemia using complete occlusion of both renal arteries by Sugita aneurysmal clips (Mizuho Ikakogyo, Tokyo, Japan). The mice were anesthetized with intraperitoneal (IP) pentobarbital sodium (40–70 mg/kg) and placed on a homeothermic table to maintain their body temperature at 37°C. After reperfusion, the mice ($n=5$ at each time point) were killed at 0, 2, 6, 12, and 24 h after reperfusion. In experiment 2, AKI was induced in MMP-$2^{-/-}$ mice by 60 min ischemia. After reperfusion, mice ($n=5$ at each time point) were sacrificed at 0, 2, 6, 12, or 24 h. In experiment 3, MMP-$2^{+/+}$ mice with 60 min ischemia-reperfusion received minocycline (Sigma, St Louis, MO, USA) or synthetic peptide MMPs inhibitor (MMP-2/MMP-9 Inhibitor III, Calbiochem, Germany) from 36 h before renal ischemia to 24 h after reperfusion. Minocycline 45 mg/kg in dimethyl sulfoxide and 0.9% NaCl or an equal volume of dimethyl sulfoxide and 0.9% NaCl (placebo) was injected IP. This was administered 36 h before renal ischemia and was followed by 22.5 mg/kg IP every 12 h for a total of six doses, based on previously established protocols.[22,29] The synthetic peptide MMP-2/MMP-9 inhibitor III 2.5 mg/kg in 0.9% NaCl or an equal volume of 0.9% NaCl (placebo) was injected IP. This was administered 36 h before renal ischemia and followed by 2.5 mg/kg IP every 12 h for a total of six doses. The dose of MMP-2/MMP-9 Inhibitor III was based on the *in vivo* anti-cancer therapies reported in the literature.[30] Mice ($n=5$ at each time point) treated with minocycline or MMP-2/MMP-9 inhibitor III were killed at 6 h or 24 h. To assess renal function, blood samples were collected for measurement of plasma creatinine and blood urea nitrogen using an autoanalyzer (SRL, Tokyo, Japan).

### Histopathological and Immunohistochemical Examination

After removal of the kidney, renal tissues were fixed in 20% buffered formalin and embedded in paraffin for light microscopy. Tissues were stained with hematoxylin and eosin, periodic acid-Schiff and periodic acid-methenamine Silver for histopathological examination.

The following primary antibodies or Lectin were used for immunohistochemistry. (a) Goat polyclonal anti-MMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect MMP-2-producing cells. (b) Goat polyclonal anti-TIMP-1 (Santa Cruz Biotechnology) to detect cells that express TIMP-1. (c) Goat polyclonal anti-TIMP-2 (Santa Cruz Biotechnology) to detect cells that express TIMP-2. (d) Texas red conjugated Lycopersicon esculentum (Tomato) Lectin (Vector Laboratories, Burlingame, CA, USA), which has been used as a marker for endothelial cells and some tubular epithelial cells.[31,32] For immunohistochemistry, frozen tissue sections were stained by the standard indirect technique, and observed with a fluorescence microscope. To detect MMP-2 expression on peritubular capillary (PTC) endothelial cells, double immunohistochemistry staining with MMP-2 and Tomato Lectin was performed. For this, 4 $\mu$m-thick frozen sections were stained with anti-MMP-2 antibody (goat IgG) and followed by FITC-labeled donkey anti-goat IgG antibody (Santa Cruz Biotechnology). Sections were then incubated with Texas-red conjugated Tomato Lectin. We used sections treated without primary antibodies as controls.

In histological sections, fragmented nuclear DNA associated with apoptosis was labeled by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method.[33]

For electron microscopic examination, the kidney tissue was fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide, dehydrated and embedded in Epok 812. Ultrathin sections were stained with uranyl acetate, lead citrate and then examined with an electron microscope (model H7100, Hitachi Corp., Tokyo, Japan).

To examine the severity of ATI, tissue damage was assessed in a blind manner and scored using a tubular damage score according to the percentage of damaged tubules (loss of brush border, tubular dilation, cast formation, and cell lysis);[34] 1, less than 25% damaged; 2, 25–50% damaged; 3, 50–75% damaged; and 4, more than 75% damaged. To examine the apoptotic tubular epithelial cells, the number of TUNEL + tubular epithelial cells, which were characterized by the tubular epithelial cells with TUNEL + nuclei, was counted in $\times 400$ high power field. For the tubular damage score and the number of TUNEL + tubular epithelial cells, a total of 40 high power fields in outer medulla from each mouse kidney were examined.

### Gelatin Zymography

As described previously,[35] supernatants of 10 $\mu$g of total protein from kidney homogenate from each animal was applied to gelatin zymography. Briefly, electrophoresis was carried out on 10% polyacrylamide gels containing 0.1% gelatin under nonreducing conditions. After electrophoresis, the gels were washed in 2.5% Triton X-100 to remove sodium dodecyl sulfate, incubated for 16 h at 37°C, and stained with 0.1% Coomassie Brilliant Blue R250 (Sigma). Densitometric analysis of the gels was performed using NIH image software (Image, v. 1.62; National Institutes of Health, Bethesda, Maryland). MMP-2 and MMP-9 activity was estimated from their gelatinolytic activities.

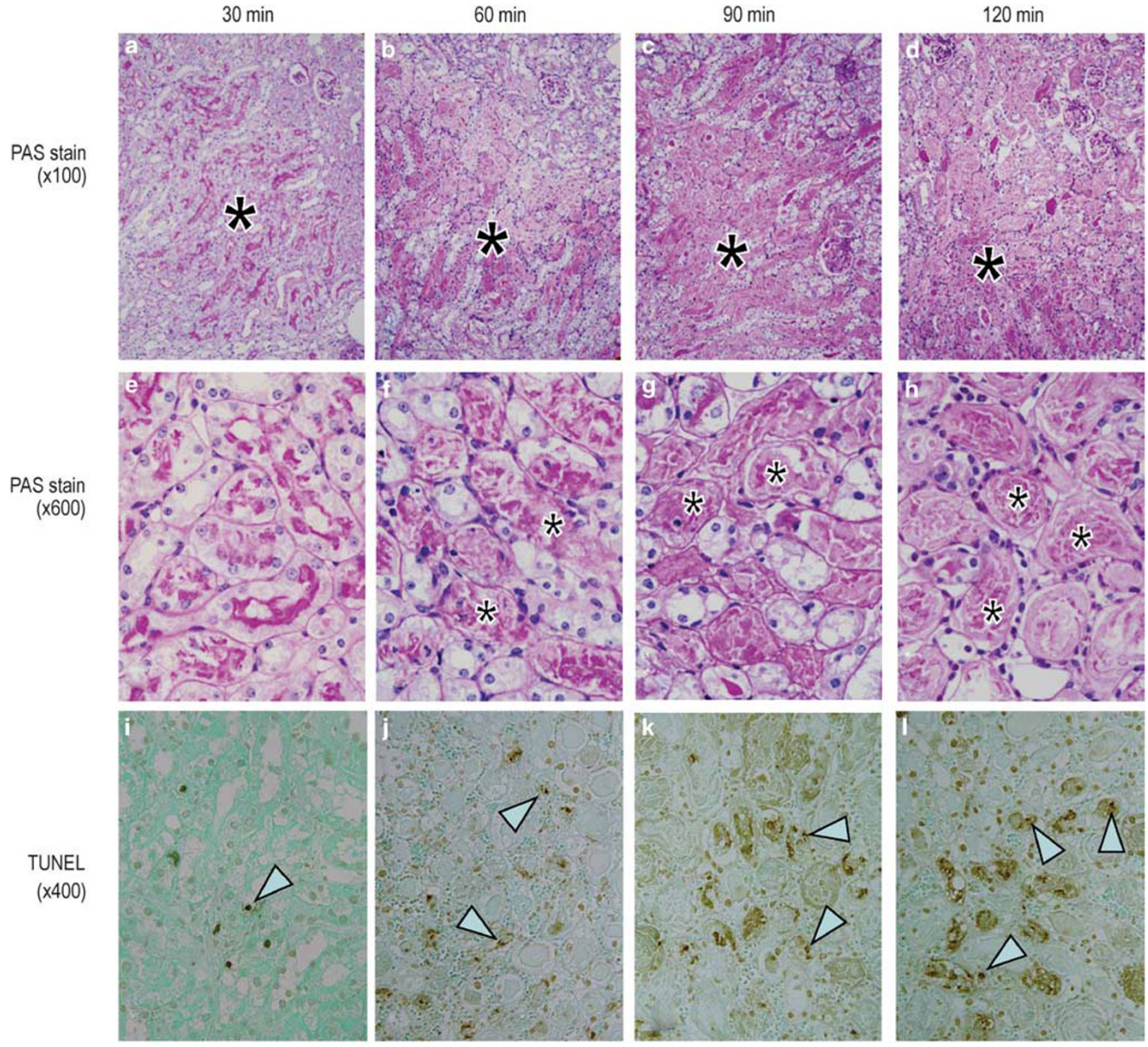


**Figure 1** Development of acute tubular injury (ATI) in MMP-2$^{+/+}$ mice at 24 h after 30 min ischemia (**a, e, i**), 60 min ischemia (**b, f, j**), 90 min ischemia (**c, g, k**), and 120 min ischemia (**d, h, l**) (**a–d**: periodic acid-Schiff (PAS) stain, × 100; **e–h**: PAS stain, × 600; **i–l**: TUNEL stain, × 400). ATI developed in the outer stripe of the outer medulla (* in **a–d**) in the kidney. Although tubular epithelial cell necrosis was not prominent at 24 h after 30 min ischemia, severe tubular epithelial cell necrosis (* in **f–h**) developed with TUNEL + dead cells (arrowheads in **i–l**) at 24 h after 60, 90, and 120 min ischemia.

### *In situ* Zymography

To examine the localization of gelatinolytic activity in injured kidney, we applied *in situ* zymography using MMP *in situ* Zymo-Film (Wako Laboratory Chemicals, Osaka, Japan). MMP *in situ* Zymo-Film was developed for observation of the enzyme activity of MMPs in tissue specimens, and composed of a 7 $\mu$m-thick layer of special gelatin of a polyester base. Cryostat-cut kidney sections (4 to 6 $\mu$m thickness) were placed on the film, and incubated at 37°C for 6 to 32 h according to the instructions provided by the manufacturer. After incubation, films were stained in biebrich scarlet stain solution (Wako), and observed by light microscopy.

### Statistical Analysis

All data are expressed as mean ± s.d.. Statistical differences between groups were determined using Student's *t*-test. Significance was defined at $P<0.05$.

## RESULTS

### Ischemic AKI in MMP-2$^{+/+}$ Mice

We examined the development of ischemic AKI induced by 30, 60, 90, and 120 min ischemia-reperfusion in MMP-2$^{+/+}$ mice (Figure 1). At 24 h after reperfusion, although no apparent acute tubular injury (ATI) developed in the mice with 30 min ischemia, widespread necrosis of tubular epithelial

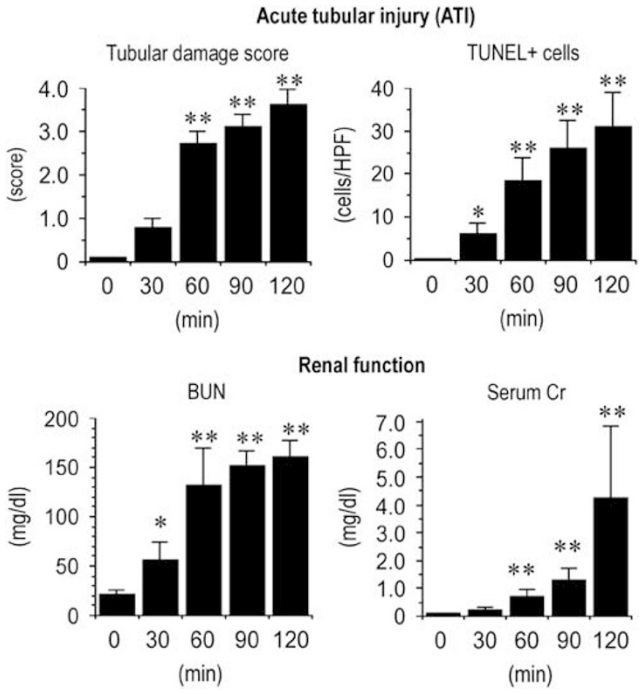


**Figure 2** The severity of acute tubular injury (ATI) and renal function at 24 h after 30, 60, 90, and 120 min ischemia. The score of tubular damage and the number of TUNEL + dead cells increased at 24 h after ischemia-reperfusion. Serum creatinine (Cr) and blood urea nitrogen (BUN) also increased at 24 h after ischemia-reperfusion. ATI and renal dysfunction at 24 h after reperfusion developed depending on the duration of ischemia between 30 min and 120 min. Data are mean ± s.d. of $n = 5$ mice. $^{*}P < 0.05$; $^{**}P < 0.01$.

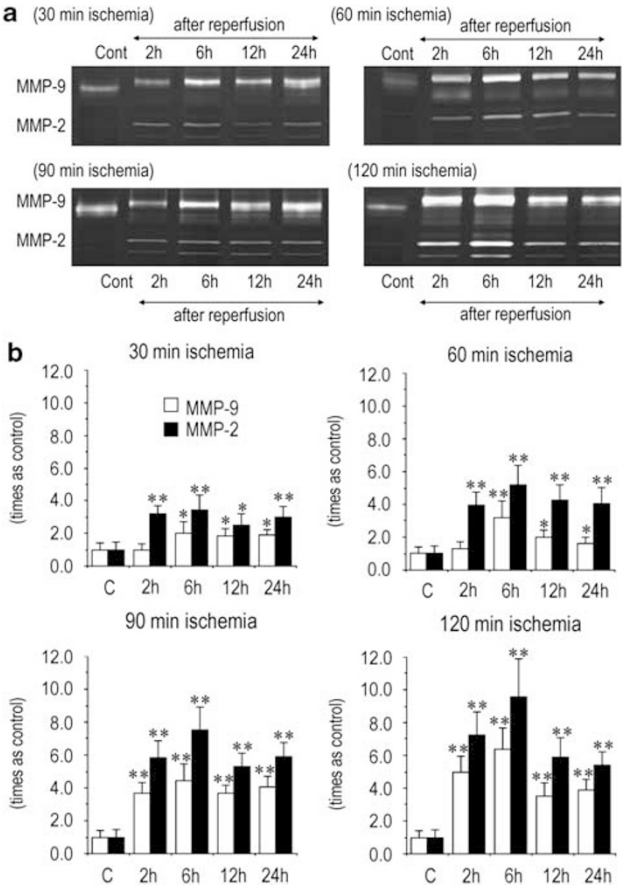


**Figure 3** MMP-2 and MMP-9 activities during ischemia-reperfusion injury after 30, 60, 90, and 120 min ischemia. (**a**) Representative gelatin zymography examples of five experiments during the development of ATI following 24 h after 30, 60, 90, and 120 min ischemia. (**b**) Quanititative data of MMP-2 and MMP-9 activities during the development of ATI following 24 h after 30, 60, 90, and 120 min ischemia. Data are mean ± s.d. of $n = 5$ mice. $^{*}P < 0.05$; $^{**}P < 0.01$. In gelatin zymography, MMP-2 and MMP-9 activities increased in injured kidney at 2 h after ischemia-reperfusion, reached its highest level at 6 h, before the full development of ATI. The activities gradually decreased thereafter. MMP-2 and MMP-9 activities at 6 h after reperfusion increased depending on the duration of ischemia between 30 min and 120 min.

cells was evident with flattened regenerating epithelial cells, especially in the straight portion of the proximal tubules in the outer medulla of the kidney of the mice with more than 60 min ischemia. In addition to the necrotic epithelial cells, apoptotic tubular epithelial cells were also present, indicated by TUNEL staining. ATI was maximal at 24 h after reperfusion, and renal dysfunction developed at 24 h (Figure 2). The severity of ATI, which was examined by tubular damaged score and the number of TUNEL + tubular epithelial cells, and the renal dysfunction developed depending on the duration of ischemia between 30 min and 120 min.

We also analyzed MMP-2 and MMP-9 activity in the renal cortex using gelatin zymography (Figure 3). Ischemia-reperfusion resulted in increased MMP-2 and MMP-9 activity in MMP-$2^{+/+}$ mice from the early period after reperfusion (at least 2 h). After reperfusion, the activity of both MMP-2 and MMP-9 increased and reached peak levels at 6 h. Subsequently, both activities gradually decreased but were still present at 24 h after reperfusion. The increase of MMP-2 and MMP-9 activities after reperfusion seemed to be dependent on the duration of ischemia between 30 min and 120 min.

Next, we examined the expression of MMP-2, TIMP-1, and TIMP-2 in renal tissues from before ischemia and 6 h after reperfusion when MMP-2 activity was the highest during the experiment according to the results of gelatin zymography. At baseline, MMP-2 was negative in the outer medulla, whereas TIMP-1 and TIMP-2 were expressed on tubular epithelial cells (Figure 4). However, at 6 h after reperfusion, the expression of MMP-2 increased, and the expression of TIMP-1 and TIMP-2 decreased. MMP-2 was expressed on cells in the interstitium around tubules. Especially, double-immunostaining for MMP-2 and Tomato Lectin showed MMP-2 expression on endothelial cells in peritubular capillaries (PTCs) and cells in the interstitial areas around PTCs (probably pericytes or fibrocytes) (Figure 5). Examination of MMP-2 and MMP-9 activities by *in situ* zymography showed gelatinase activity mainly in the interstitium around tubules (probably PTCs) at 6 h. Moreover, TUNEL staining showed some TUNEL + endothelial cells in PTCs, before the development of tubular epithelial cell injury. In addition, congestion

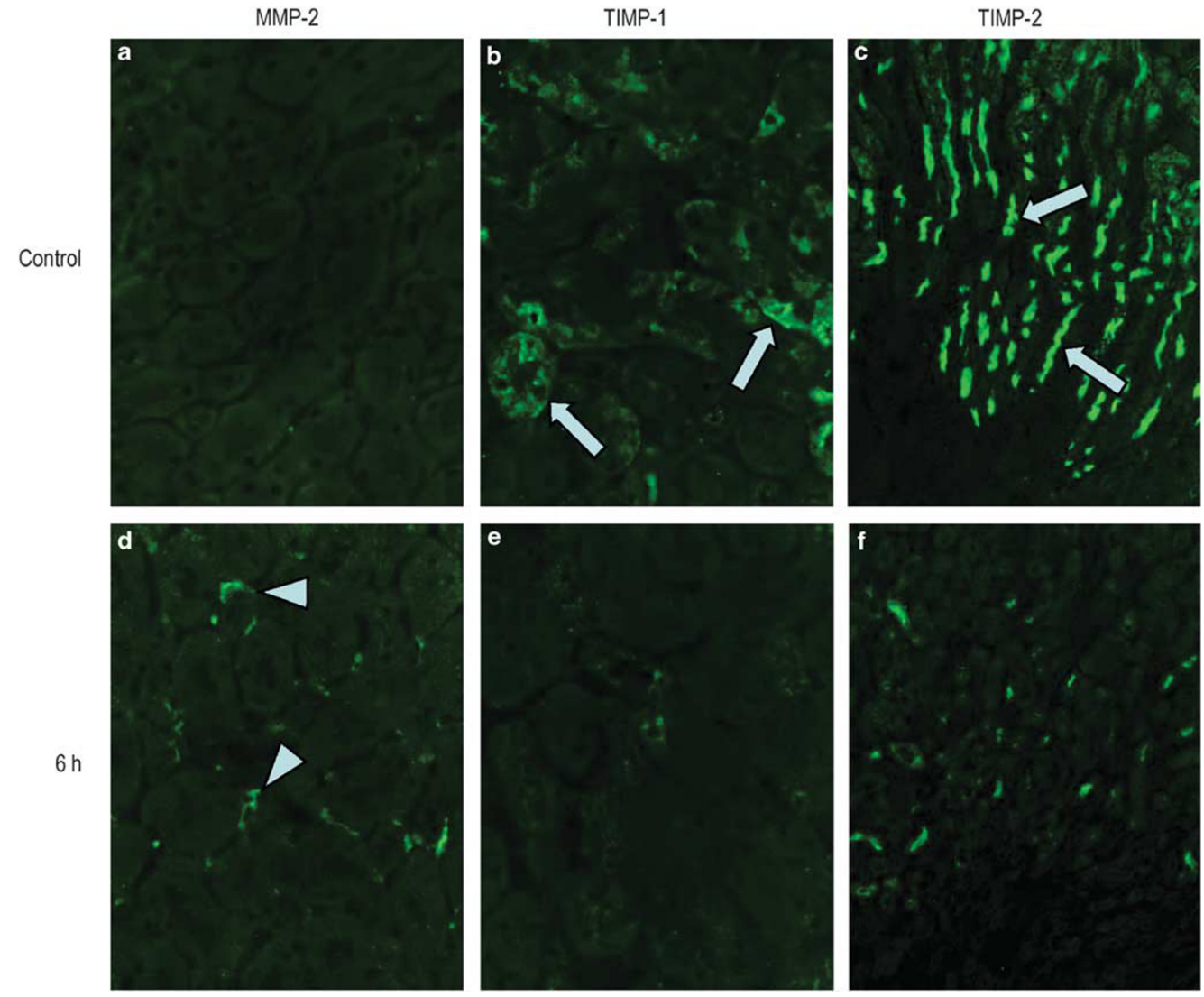


**Figure 4** Immunohistochemical analysis of the expression of MMP-2 (**a, d**: × 400), TIMP-1 (**b, e**: × 400), and TIMP-2 (**c, f**: × 200), before ischemia control (**a–c**) and 6 h (**d–f**) after 60-min ischemia-reperfusion in MMP-$2^{+/+}$ mice. At 6 h after ischemia-reperfusion when MMP-2 activities were highest, increased expression of MMP-2 (arrowhead) was evident in the interstitium around renal tubules. In contrast, tubular expression of TIMP-1 (arrow) and TIMP-2 (arrow) was decreased at 6 h after ischemia-reperfusion.

and interstitial hemorrhage were noted around the renal tubules at 6 h.

Electron microscopic findings showed that PTC injury developed at 6 h after reperfusion (Figure 6). Before the development of tubular epithelial injury, disruption of the basement membrane was evident in PTCs, resulting in the destruction of PTCs with interstitial edema and hemorrhage. Apoptotic endothelial cells, as well as swollen endothelial cells were noted in PTCs, suggesting apoptotic endothelial cell injury and endothelial cell activation after 60-min ischemia-reperfusion.

### Ischemic AKI in MMP-$2^{-/-}$ Mice

To clarify the role of MMP-2 in ischemia-reperfusion AKI, 60-min ischemia-reperfusion was induced in MMP-$2^{-/-}$ mice. The kidneys of MMP-$2^{-/-}$ mice showed minimal congestion and interstitial hemorrhage (probably associated with mild PTC injury) at 6 h, and mild renal tubular epithelial cell injury with preservation of renal blood urea nitrogen and Cr levels at 24 h (Figures 7 and 8). Gelatin zymography confirmed the lack of MMP-2 activity during the experiment (Figure 9). MMP-9 activity increased by 6 h, similar to MMP-$2^{+/+}$ mice. From 6 to 24 h after reperfusion, MMP-9 activity gradually decreased, but higher expression was evident at 24 h after reperfusion than pre-ischemic control rats (baseline). Comparison of MMP-$2^{-/-}$ and MMP-$2^{+/+}$ mice showed similar MMP-9 activity at both baseline and 6 h, and no upregulation of MMP-9 in MMP-$2^{-/-}$ mice before and after ischemia, compared with MMP-$2^{+/+}$ mice.

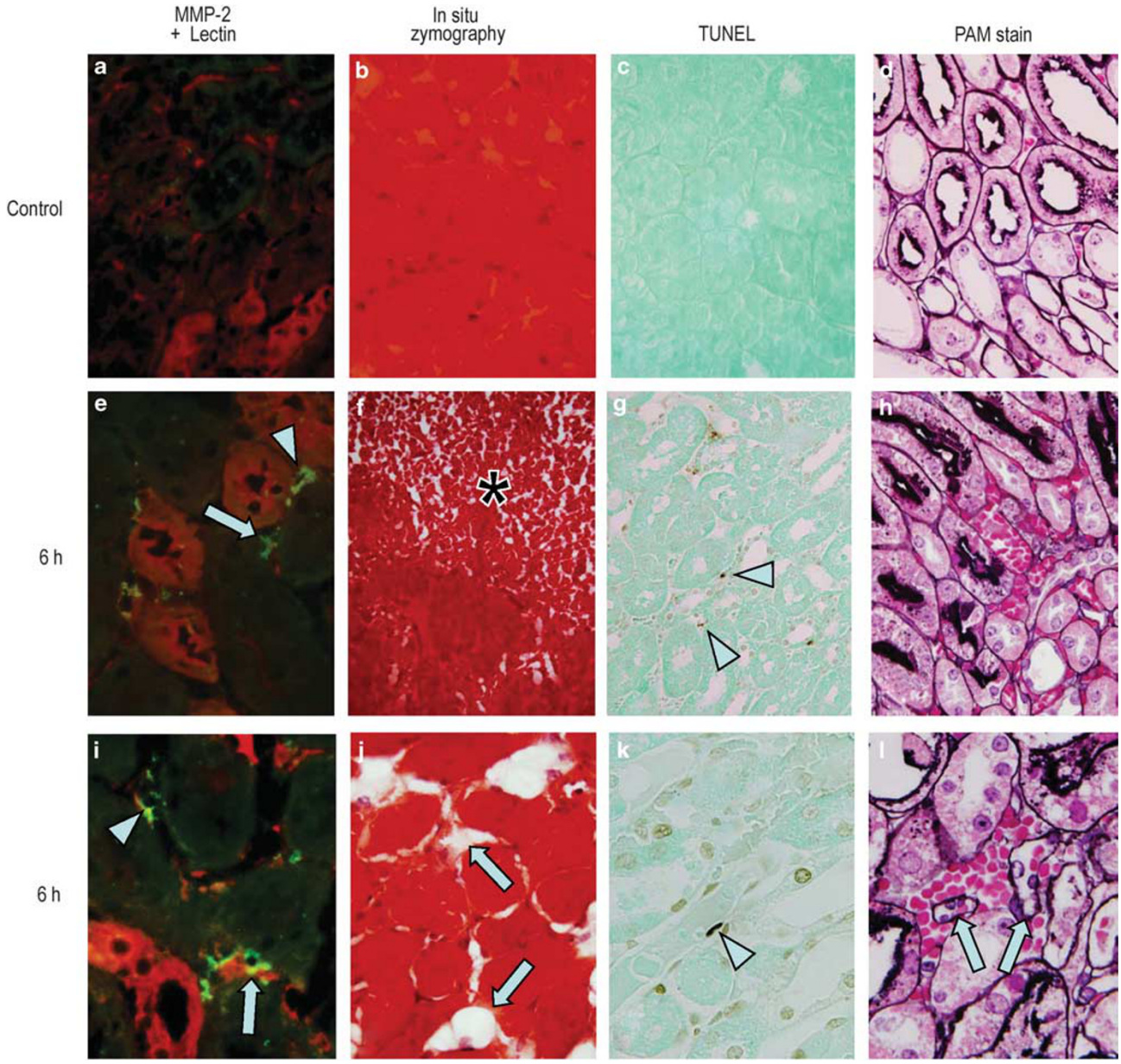


**Figure 5** Peritubular capillary (PTC) injury and interstitial hemorrhage before ischemia (control) (**a–d**) and 6 h (**e–l**) after 60-min ischemia-reperfusionin MMP-2$^{+/+}$ mice. MMP-2 + Lectin (**a, e**: × 600; **i**: × 800): double immunofluorescence studies of MMP-2 (FITC, green) and Tomato Lectin (Texas-red, red) showed expression of MMP-2 on endothelial cells in PTCs (arrowhead in **e, i**) and cells present around PTCs (probably pericytes or fibrocytes) (arrow in **e, i**). *In situ* zymography (**b, j**: × 600; **f**: × 100) (*) in (**f**) indicates the outer stripe of outer medulla in kidney. Gelatinase activity was evident in interstitium around tubules including PTCs in the outer medulla at 6 h after ischemia-reperfusion. TUNEL (**c, g**: × 600; **k**: × 800): TUNEL stain identified apoptotic cells with positive nuclear stain in the interstitium around renal tubules at 6 h after ischemia-reperfusion, before the development of tubular epithelial cell death. High magnification (**k**) clearly showed the TUNEL + apoptotic endothelial cell in PTC. Periodic acid-methenamine stain (**d, h**: × 600; **l**: × 800): interstitial hemorrhage around renal tubules at 6 h after 60-min ischemia-reperfusion, before the development of tubular epithelial cell necrosis. Arrow in (**l**) indicates PTCs with large and round endothelial cell nuclei in interstitial hemorrhage.

### Ischemic AKI in MMP-2$^{+/+}$ Mice Treated with Inhibitors of MMPs

To test the therapeutic effects of MMP inhibitors on ischemic AKI, MMP-2$^{+/+}$ mice subjected to 60-min ischemia-reperfusion were injected with minocycline or synthetic peptide MMP inhibitor (MMP-2/MMP-9 inhibitor III). Both inhibitors of MMPs reduced congestion and interstitial hemorrhage in the outer medulla in MMP-2$^{+/+}$ mice at 6 h, suggesting that this action is associated with inhibition of PTC injury (Figure 7). Furthermore, the two MMP inhibitors reduced ATI, which was examined by tubular damaged score and the number of TUNEL + tubular epithelial cells, and

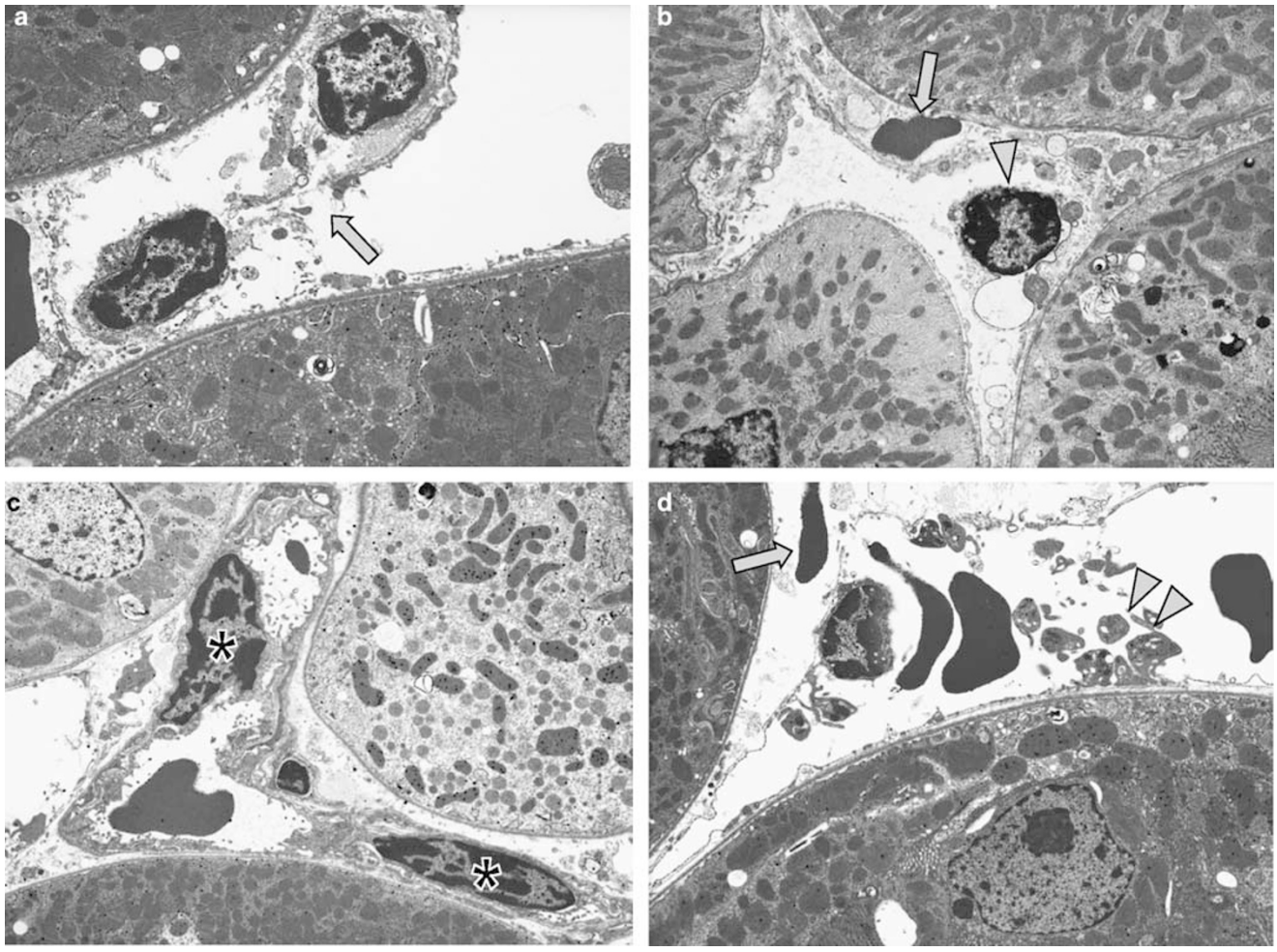


**Figure 6** Electron microscopy showing peritubular capillary (PTC) injury at 6 h after 60-min ischemia-reperfusion in MMP-$2^{+/+}$ mice (**a, d**: × 8,000; **b, c**: × 4,000). Disruption of the basement membrane (BM) (arrow in **a**) and destruction of PTCs were noted, together with interstitial edema and hemorrhage. Apoptotic endothelial cells (arrowhead in **b**) and swollen endothelial cells (* in **c**) were also seen in PTCs with intracapillary accumulation of platelets (arrowhead in **d**). Exudative red blood cells (arrow in **b** and **d**) were present aroud PTCs. Necrosis of tubular epithelial cells did not develop at 6 h after 60-min ischemia and reperfusion.

improved renal dysfunction at 24 h (Figures 7 and 8). These results suggest that inhibition of MMP-2 and MMP-9 could reduce ischemic AKI by protecting PTCs against injury.

## DISCUSSION

The present studies indicated that renal ischemia-reperfusion results in overexpression of MMP-2 at the site of renal injury (outer medulla). The expression of MMP-2 was detected on endothelial cells and pericytes and interstitial cells within or around the PTCs in the outer medulla. At 6 h after reperfusion when the activity of MMP-2 and MMP-9 was most increased during the experiment, destruction of PTCs developed with disruption of basement membrane of PTCs and interstitial hemorrhage. Subsequently, tubular epithelial cell injury, including apoptosis and necrosis developed in the outer medulla with renal dysfunction by 24 h. On the other hand, the kidneys of MMP-$2^{-/-}$ mice, or mice treated with minocycline or inhibitor of MMP-2 and MMP-9 showed minimal PTC injury and subsequent suppression of ATI with preservation of renal function by 24 h. Considered together, these findings indicate that overexpression of MMPs, especially MMP-2 mediated the degradation of basement membrane of PTCs and subsequent destruction of PTCs with interstitial hemorrhage, which could be associated with the development of severe ATI. Thus, MMP-2 and MMP-9 have a deleterious effect in ischemia-reperfusion AKI, and their inhibition protect against acute phase of AKI. The results also point to a potential therapeutic target for the development of new pharmacological agents aimed at reducing AKI.

There is a general agreement that MMPs have a pathologic role in ischemic organ failure by degrading ECM substrates that are essential for normal signalling and homeostasis within the ischemic site.[13–15] After the onset of ischemic-reperfusion, uncontrolled MMP activity is reported to mediate aberrant proteolysis thus leading to microvascular dysfunction and cell death.[22,36] Many groups have demonstrated that MMPs,

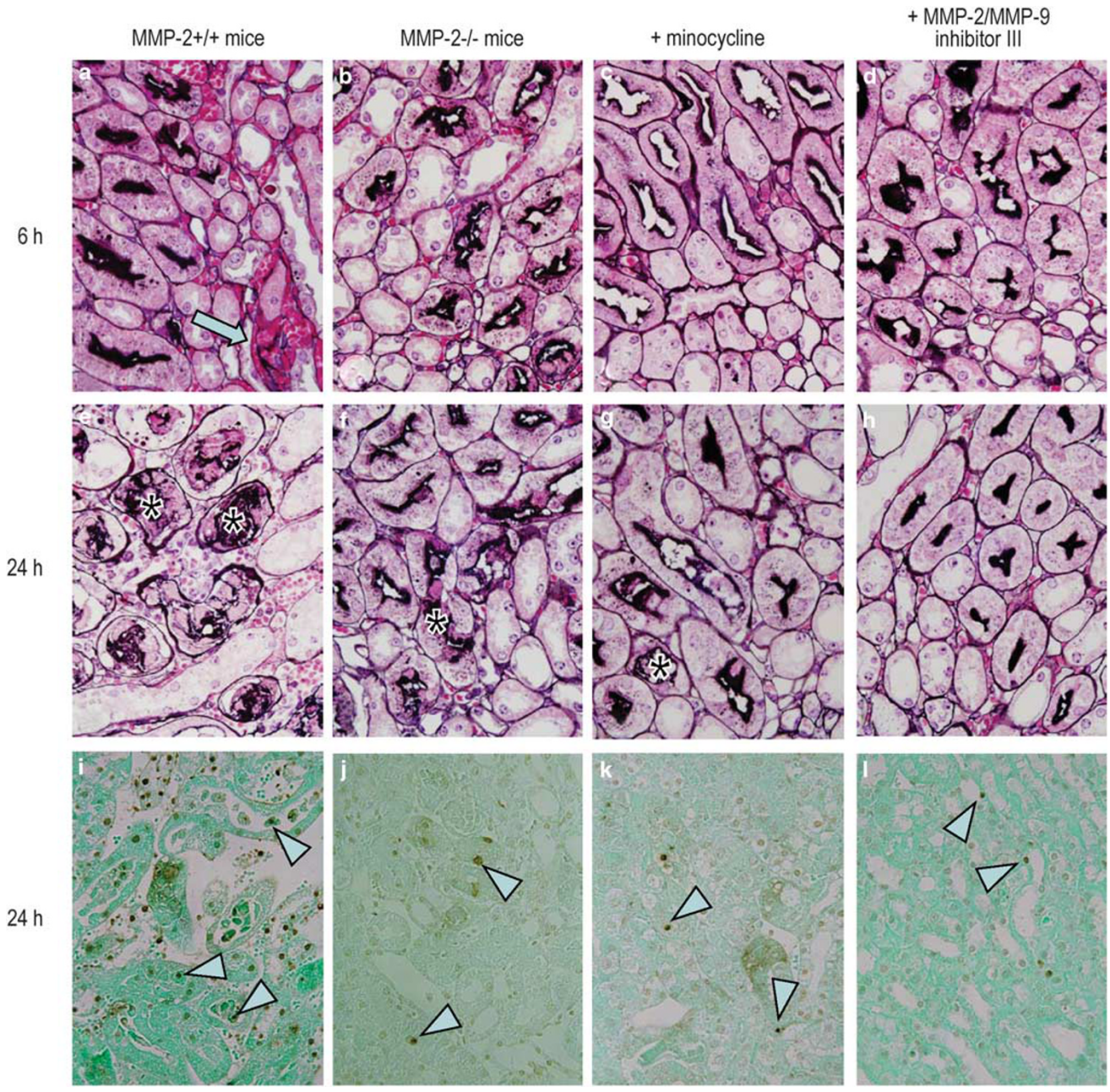


**Figure 7** Interstitial hemorrhage at 6 h (**a–d**) and acute tubular injury (AKI) at 24 h (**e–l**) after 60-min ischemia-reperfusion in MMP-$2^{+/+}$ mice (**a, e, i**), MMP-$2^{-/-}$ mice (**b, f, j**), and MMP-$2^{+/+}$ mice treated with inhibitor of MMPs, minocycline (**c, g, k**) and synthetic peptide (MMP-2/MMP-9 inhibitor III) (**d, h, l**) (**a–h**: periodic acid-methenamine stain, ×600; **i–l**: TUNEL stain, ×400). Congestion of peritubular capillaries and interstitial hemorrhage at 6 h and ATI including TUNEL + apoptotic cells (arrowhead in **i** to **l**) at 24 h were diminished in MMP-$2^{-/-}$ mice and MMP-$2^{+/+}$ mice treated with MMP inhibitors, compared with MMP-$2^{+/+}$ mice.

especially MMP-2 and MMP-9, rapidly increase in cerebral, heart, liver, lung, and other tissues after ischemia or ischemia-reperfusion in experimental animals.[13–20]

Renal ischemia or ischemia-reperfusion is also reported to increase MMP-2 and MMP-9 protein levels or activities.[21–24] Overexpression of MMP-2 and MMP-9 is localized to the interstitium or tubulointerstitium, and may have an important role in renal microvascular injury with leakage of higher molecular weight substances and capillary loss after ischemia.[22] In the present study, the expression of MMP-2 and MMP-9 was detected much earlier, at least 2 h after reperfusion and MMP-2 appeared to stain with endothelial cells in PTCs and interstitial cells. When the activities of MMP-2 and MMP-9 reached peak levels at 6 h after reperfusion, before the full development of tubular epithelial cell injury, disruption of the basement membrane of PTCs was noted with endothelial cell death and interstitial edema and hemorrhage, indicating that renal microvascular

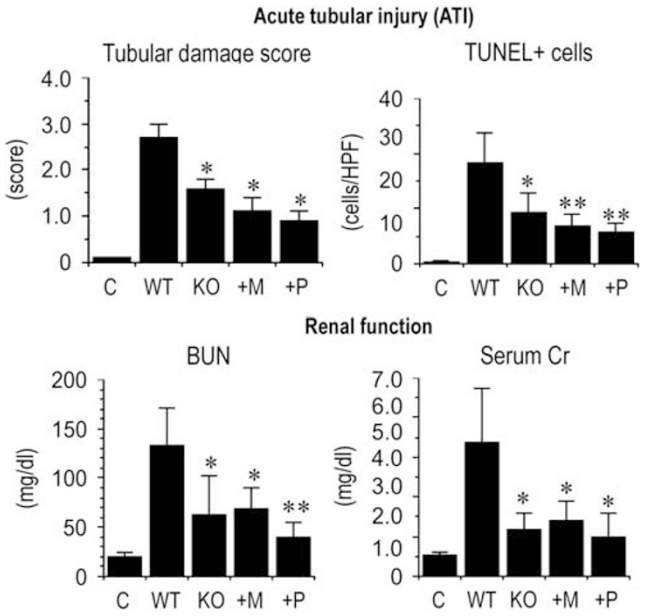


**Figure 8** The severity of acute tubular injury (ATI) and renal function before ischemia (C) and 24 h after 60 min ischemia in MMP-$2^{+/+}$ mice (WT), MMP-$2^{-/-}$ mice (KO), MMP-$2^{+/+}$ mice treated with inhibitors of MMPs, minocycline (+M) and synthetic MMP-2/MMP-9 inhibitor (+P). The development of ATI, which was determined by tubular damage score and TUNEL + tubular epithelial cells at 24 h after reperfusion, was inhibited in MMP-$2^{-/-}$ mice or by the treatment of minocycline and synthetic MMP-2/MMP-9 inhibitor. Renal dysfunction (BUN and serum Cr) at 24 h after reperfusion was also prevented in MMP-$2^{-/-}$ mice or by the treatment of minocycline and synthetic MMP-2/MMP-9 inhibitor. The degree of tubular damage score, the number of TUNEL+ tubular epithelial cells, and levels of serum Cr and BUN seemed to be lower in both MMP inhibitors than in MMP-$2^{-/-}$ mice. Data are mean ± s.d. of $n = 5$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

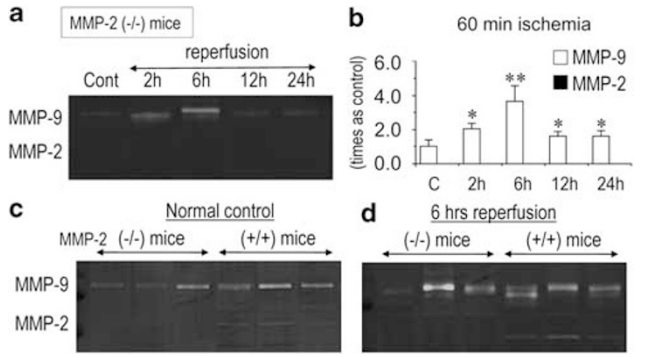


**Figure 9** MMP-2 and MMP-9 activities in gelatin zymography in MMP-$2^{-/-}$ mice. (**a**) Representative gelatin zymography examples of five experiments during the development of ATI in MMP-$2^{-/-}$ mice following 24 h after 60 min ischemia. (**b**) Quanititative data of MMP-2 and MMP-9 activities during the development of ATI following 24 h after 60 min ischemia. Data are mean ± s.d. of $n = 5$ mice. $^{*}P < 0.05$; $^{**}P < 0.01$. (**c, d**) Representative gelatin zymography examples of three experiments each in MMP-$2^{-/-}$ and MMP-$2^{+/+}$ mice at preischemia baseline (**c**) and at 6 h (**d**) after 60 min ischemia. Gelatin zymography using tissues of MMP-$2^{-/-}$ mice showed no MMP-2 activity during the experiment. MMP-9 activity increased at 6 h in these mice, similar to MMP-$2^{+/+}$ mice. MMP-9 activity gradually decreased at 12 and 24 h after reperfusion. MMP-9 activity was similar in MMP-$2^{-/-}$ and MMP-$2^{+/+}$ mice at baseline and at 6 h after 60-min ischemia-reperfusion.

destruction developed in the outer medulla, before the full progression of ATI.

In stroke, MMPs are rapidly upregulated after cerebral ischemia in animal models and patients.[13,36,37] By degrading the neurovascular matrix, MMPs could explain the blood-brain barrier leakage, edema and hemorrhage.[36] By disrupting cell-matrix homeostasis, MMPs may trigger cell death.[38,39] Similarly, the present study demonstrated that, in the development of ischemic ATI, MMP-2, and MMP-9 may have an important role in renal microvascular destruction, and may contribute to the development of tubular epithelial cell death and renal dysfunction. Although MMP-2 and MMP-9 could contribute to disease progression, an important role for MMP-9 in particular has been suggested in ischemic injury of several organs.[13–15,36,37] In patients with AKI, MMP-9 is a useful urinary biomarker for the early diagnosis of AKI.[40] MMP-$9^{-/-}$ mice and animals treated with MMP-9 inhibitors are protected against ischemia-reperfusion injury in several organs.[13–15,36,37,41–44] However, MMP-2 has an important role in the development of ischemic ATI, because prevention of PTC destruction and renal dysfunction after ischemia-reperfusion were evident in MMP-$2^{-/-}$ mice in the present study. Furthermore, in AKI, one recent study indicated that overexpression of MMP-9 after folic acid injection or 45-min ischemia-reperfusion protected renal tubules against apoptosis, and severe ATI developed in MMP-$9^{-/-}$ mice.[27] Therefore, in AKI, MMP-2 may have a more strong influence than MMP-9 on the development of ischemic AKI, although, in the present study, the ameliorative effects of inhibitors of both MMP-2 and MMP-9 on the severity of ATI were more pronounced than those of MMP-$2^{-/-}$ mice. MMP-2 was expressed on endothelial cells and pericytes or interstitial cells within or around PTCs. In several organs, the most abundant source of MMP-9 is infiltrating neutrophils.[36,37,45] However, in AKI at 6 h after reperfusion, at the time of peak activity of MMP-9, only few neutrophils had infiltrated at the site of renal injury (data not shown).

The function of MMPs is also regulated by enzyme inhibitors. The endogenous inhibitors of MMP-9 and MMP-2, are TIMPs, with TIMP-1 being more selective for MMP-9, and TIMP-2 being more selective for MMP-2.[11,12] In diseases characterized by profound matrix degradation, the balance between MMPs and TIMPs is often offset, resulting in an overall net increase in MMP activity. Immunohistochemical analysis in the present study demonstrated downregulation of TIMP-1 and TIMP-2 at 6 h after reperfusion. Thus, the ischemia-reperfusion-induced MMP expression is expected to shift the protease–protease inhibitor balance toward proteolytic activity and ECM degradation.

To examine the possible use of MMP inhibitor therapy for AKI, ischemic AKI in MMP-$2^{+/+}$ mice was examined further by administering minocycline or MMP-2/MMP-9 inhibitor. The results of this experiment demonstrated that

MMP-2 and MMP-9 inhibitors significantly reduced the severity of PTC damage, suppressed the development of AKI, and preserved renal function after ischemia-reperfusion, clearly demonstrating the importance of this enzyme in the development of ischemic AKI. Although inhibition of MMP-2 and MMP-9 mitigates the PTC destruction and the development of AKI, our observation does not exclude the possible role of the inflammatory cascade to renal microvascular injury after ischemia. MMP activity is usually linked to inflammation.[46–48] MMPs participate in complex injury responses through interactions with and activation of cytokines, chemokines and other pericellular, and cell surface substrates, and inhibition would limit the action of these pro-inflammatory agents. Furthermore, minocycline is known for its broad anti-inflammatory actions, in addition to its action as an MMP inhibitor. Indeed, minocycline has been demonstrated to inhibit tubular cell apoptosis, diminish inflammation, and provide an overall protective effect on renal function following ischemia.[29] Thus, minocycline may function by inhibiting MMPs' activities and/or a number of other mechanisms.

In the present study, ischemia-reperfusion renal injury resulted in overexpression of MMPs, especially MMP-2 at the site of injury in the kidney. The development of ischemic AKI was associated with upregulation of MMPs, which subsequently mediated acute microvascular disruption and progression of ATI. Data from MMP-$2^{-/-}$ mice and pharmacological experiments suggest that MMP-2 and MMP-9 may be attractive therapeutic targets for ischemic AKI. However, our results in the present study contradicts with findings of the other studies, which states that MMP-2 is elevated in late (8 weeks) post-ischemia-reperfusion injury,[25] that renal MMP activity is unaffected by ischemia-reperfusion, or that inhibition of MMPs does not alter the outcome of renal function.[26] We assume these discrepancies may be related to the animal/strain used, and moreover the severity of injury/length of ischemia used in the studies held by us and the studies done by them. We are therefore planning on doing a pre-clinical study using non-human primates to test the effects of MMP inhibition on AKI. Accumulating evidence also suggests that MMPs may have beneficial roles during remodeling and/or recovery from the several diseases and organ injuries, including ischemia-reperfusion.[13,37,49,50] We are therefore now examining the roles of MMP-2 and MMP-9, and the influences of inhibitors of these MMPs, in the recovery from ischemic AKI.

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**DISCLOSURE/CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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