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Effects of Metalloproteinase Inhibition in a Murine Model of Renal Ischemia-Reperfusion Injury

KATHERINE B. NOVAK, HAU D. LE, EMILY R. CHRISTISON-LAGAY, VANIA NOSE, ROBERT J. DOIRON, MARSHA A. MOSES, AND MARK PUDER

Department of Surgery and the Vascular Biology Program [K.B.N., H.D.L., E.R.C.-L., R.J.D., M.A.M., M.P.], Children's Hospital Boston, Harvard Medical School, Boston, MA 02115; Department of Pathology [V.N.], Brigham and Women's Hospital, Boston, Massachusetts 02115

ABSTRACT: Ischemia-reperfusion injury (IRI) is a leading cause of acute tubular necrosis (ATN) and delayed graft function in transplanted organs. Up-regulation of matrix metalloproteinases (MMPs) propagates the microinflammatory response that drives IRI. This study sought to determine the specific effects of Marimastat (Vernalis, BB-2516), a broad spectrum MMP and TNF-α-converting enzyme inhibitor, on IRI-induced ATN. Mice were pretreated with Marimastat or methylcellulose vehicle for 4 d before surgery. Renal pedicles were bilaterally occluded for 30 min and allowed to reperfuse for 24 h. Baseline creatinine levels were consistent between experimental groups; however, post-IRI creatinine levels were 4-fold higher in control mice ($p < 0.0001$). The mean difference between the post-IRI histology grades of Marimastat-treated and control kidneys was 1.57 ($p = 0.003$), demonstrating more severe damage to control kidneys. Post-IRI mean ($\pm$SEM) MMP-2 activity rose from baseline levels in control mice (3.62 ± 0.99); however, pretreated mice presented only a slight increase in mean MMP-2 activity (1.57 ± 0.72) ($p < 0.001$). In conclusion, these data demonstrate that MMP inhibition is associated with a reduction of IRI in a murine model. **(*Pediatr Res* 67: 257–262, 2010)**

Ischemia-reperfusion injury (IRI) affects post-transplant renal function and largely determines short- and long-term graft survival. Although some ischemia is unavoidable, IRI continues to be the leading cause of acute renal injury and delayed graft function in transplanted organs (1). The development of acute tubular necrosis (ATN), one of the leading causes of intrinsic acute renal injury, is a hallmark of IRI. IRI induces the release of proinflammatory cytokines which, in turn, contribute to graft fibrosis (2,3). There is no clinically acceptable therapy that directly addresses the cellular damage induced by IRI (4,5). Because of its intrinsic nature, IRI remains a challenge in the field of organ transplantation.

Ischemia reperfusion initiates a cascade of events that involve tubular epithelial injury, inflammation, and altered microvascular function (6). The initial ischemic event depletes ATP reservoirs, which subsequently impairs cellular processes such as protein synthesis, lipogenesis, and membrane transport. The succeeding biochemical abnormalities disrupt the cytoskeleton, damage cellular proteins, and degrade DNA, leading to apoptosis and/or necrosis of tubular epithelial cells (7). The cellular damage induces a vigorous inflammatory response, which further propagates post-ischemic tissue damage (8,9).

Matrix metalloproteinases (MMPs) are active components of inflammation that are also involved in other processes in which tissue remodeling maintains proper function such as embryonic development, angiogenesis, and wound healing (10,11). Up-regulation of MMPs propagates the microinflammatory response that drives IRI. Evidence of MMP activation has been inferred microscopically from loss of junctional complexes and altered expression and distribution of cellular adhesion molecules, suggesting a disruption of cell-cell and cell-extracellular matrix (ECM) interactions (12,13). MMPs are sequestered by neutrophils and are activated by TNF-α, which is up-regulated in animal models of ischemic injury (14,15). TNF-α is a critical mediator of both the physiologic defensive response to and pathogenesis of IRI and exists in a biologically active, secreted form, and in an inactive membrane-anchored precursor. Cleavage of the TNF-α proform into its soluble form is mediated by TNF-α-converting enzyme (TACE, also known as ADAM17 and CD156b), which belongs to the disintegrin and metalloproteinase family of zinc metalloproteinases (16,17). The TACE-induced increase in TNF-α availability is thought to promote a more extensive inflammatory response, which would directly impact the severity of tissue injury.

The degree and duration of MMPs elevation (protein or activity) has been shown to influence the extent of renal damage (11). Although IRI is a strong predictor of graft survival, no current therapies address the role of MMPs during kidney transplantation. This study was designed to determine the specific effects of pretreatment with Marimastat (Vernalis, BB-2516), a broad spectrum MMP-inhibitor, on IRI-induced ATN. Marimastat is a synthetic, low molecular weight succi-

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Correspondence: Mark Puder, M.D., Ph.D., 300 Longwood Ave, Fegan 3, Boston, MA 02115; e-mail: mark.puder@childrens.harvard.edu

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Abbreviations: ATN, acute tubular necrosis; **CMJ,** corticomedullary junction; **EMC,** extracellular matrix; **H&E,** hematoxylin and eosin; **HRP,** horseradish peroxidase; **IRI** ischemia-reperfusion injury; **MT,** Marimastat treatment; **MMPs,** matrix metalloproteinases; **TACE,** TNF-α-converting enzyme; **TN,** tubular necrosis

nate peptidomimetic inhibitor that covalently binds to the Zn^{2+} ion in the active site of MMP, thus inhibiting its action. Marimastat has been used in multiple oncologic clinical trials in the adult population. The most consistently reported toxicity concern is musculoskeletal pain is likely to occur after 2 mo of treatment (18,19). We previously demonstrated that Marimastat is capable of decreasing serum TNF-α receptor II levels (20). Although Marimastat is a broad-spectrum MMP inhibitor, this study focused on its ability to inhibit MMP-2 and MMP-9 (gelatinases), both of which play a role in ischemia-related inflammation. We propose that Marimastat will reduce kidney IRI, predominantly through its role as an inhibitor of MMP-2 and MMP-9.

MATERIALS AND METHODS

Animal model. Five- to seven-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed five animals to a cage in a barrier room with *ad libitum* access to food and water. Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. To ensure that Marimastat (half life 10 h) reaches a steady state plasma level, 96 h before surgery, the experimental group ($n = 7$) received 100 mg/kg of Marimastat treatment (MT) in 100 μL of 0.45% methylcellulose vehicle (Sigma Chemical Co.-Aldrich, St. Louis, MO) via orogastric gavage twice daily (21). Control mice ($n = 8$) received methylcellulose vehicle alone. A sham group ($n = 5$) was given methylcellulose vehicle alone. A dose-dependent relationship between Marimastat and TNF-α showed complete inhibition at 200 mg/kg (22).

IRI was performed through a flank incision after the animals were anesthetized with 2 to 4% isofluorane inhalation. IRI was induced as previously described (23). The kidney was isolated and a microvascular clamp (Roboz, Rockville, MD) was placed on the renal pedicle for 30 min, during which the kidney and clamp were gently placed back into the peritoneum. The contralateral kidney underwent the same procedure within 5 min. Treatment was continued for 24 h postoperatively. Sham mice underwent bilateral flank incisions without clamping the renal pedicles.

Specimen collection. Twenty-four hours after reperfusion, the experiment was ended. Animals was anesthetized, blood was collected, and centrifuged at 4°C at 8000 rpm for 10 min to separate the serum. Creatinine and blood urea nitrogen levels were measured using a Hitachi 917 analyzer (Roche, Branford, CT).

At the time of sacrifice, the kidneys were excised, weighed, and bisected transversely to their length. Approximately, one half of each kidney was fixed in 10% phosphate-buffered formalin at 4°C overnight, washed with PBS, and stored in 70% ethanol and then embedded in paraffin. Tissues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff reagent (PAS). Histologic sections were reviewed by a pathologist in blinded fashion and scored with a semiquantitative scale (Fig. 1) to evaluate the extent of tubular necrosis (TN): 0, normal kidney; 1, normal kidney with focal apoptosis; 2, focal TN <50% of the cortical-medullary junction (CMJ); 3, TN in the CMJ; 4, TN in the CMJ with focal extension into the cortex; and 5, TN extending through the cortex to the surface of the kidney. Histologic assessment and the scoring parameters were modified from previously described methods, with an emphasis on the location of TN (24,25).

Zymography. Urine samples were collected before bilateral IRI and 24 h after IRI. Gelatin zymography was performed on urine samples as previously described (26,27) with modifications to determine MMP activity in the urine. Fifteen microliters of each urine sample was mixed with 10 μL buffer consisting of 4% SDS, 0.15 M Tris (pH 6.8), 20% (vol/vol) glycerol, and 0.5% (wt/vol) bromophenol blue. Samples were loaded into wells of a 10% SDS-PAGE gel containing 0.1% (wt/vol) gelatin (Bio-Rad Laboratories, Hercules, CA) on a mini gel apparatus. Gels were run at 200 V for 50 min then soaked in 2.5% TritonX-100 with gentle shaking for 30 min at ambient temperature. After incubating overnight at 37°C in substrate buffer (50 mM Tris-HCL buffer pH 8, 5 mM $CaCl_2$, and 0.02% NaN_3), gels were stained for 30 min in 0.5% Coomassie Blue R-250 in acetic acid, ethanol, and water (1:3:6) and destained for 1 h. As previously described, MMP levels were quantified by scoring the band strength of each type of MMP examined on the zymogram on a scale of zero to seven, with zero indicating no detectable MMPs and seven indicating strong bands (28).

Western blot analysis. Western blot analysis was performed on the lysate of the other half of each kidney. Protein extraction was performed using an ActiveMotif extraction kit according to the manufacture's protocol (ActiveMotif, Carlsbad, CA). Samples were normalized for protein content by the Bio-Rad protein assay (Bio-Rad Laboratories). Seventy-five micrograms of protein per sample were analyzed by SDS-PAGE. Membranes were then probed overnight at 4°C with either goat anti-mouse MMP-2 (1:500, R&D Systems), goat anti-mouse MMP-9 (1:500, R&D Systems), or rabbit polyclonical anti-human TACE (1:500, Novus Biologicals, Littleton, CO). The secondary antibodies used were either horseradish peroxidase (HRP)-linked donkey anti-goat IgG in a 1:5000 dilution (Santa Cruz, CA) or ECL donkey anti-rabbit IgG, HRP-linked whole antibody in a 1:5000 dilution (GE Healthcare, Piscataway, NJ). Equal protein loading was verified by incubating the

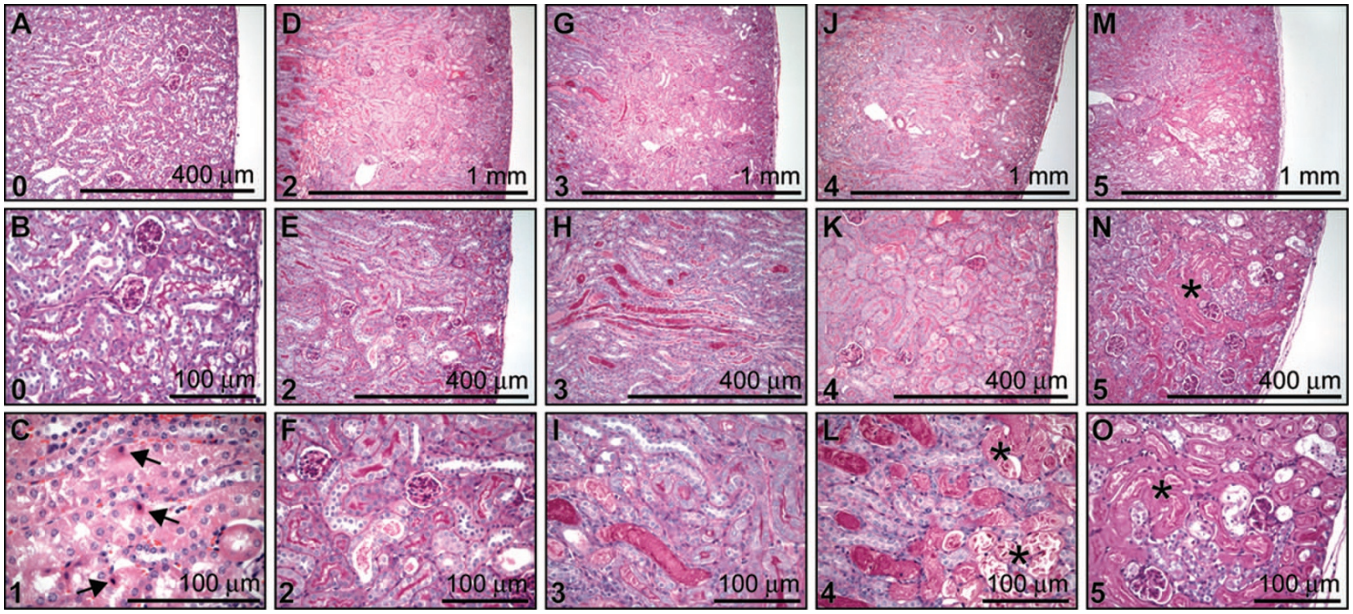

Figure 1. Histologic scoring criteria. (*A-O*) Representative PAS-stained kidney sections, unless otherwise stated, obtained 24 h after IR. Representative grade denoted at the top left corner of each frame. (*A* and *B*) Grade 0, normal kidney. (*C*) Grade 1, normal kidney (H&E) with focal apoptosis (denoted by arrow). (*D-F*) Grade 2, focal TN (<50% of CMJ). (*G-I*) Grade 3, TN in the CMJ. (*J-L*) Grade 4, TN in the CMJ with focal extension into the cortex. (*M-O*) Grade 5, TN extending through the cortex to the surface of the kidney. Necrosis is labeled with *.

same membrane with α-actin antibody in a 1:500 dilution (MS X Actin, Chemicon International, Temecula, CA). The probed proteins were developed with Pierce-enhanced chemiluminescent substrate for detection of HRP according to manufacturer's instructions (Pierce, Rockford, IL).

Statistical analysis. ANOVA with Tukey-Kramer multiple comparisons test was performed (unless otherwise indicated) using GraphPad InStat Version 3.05 (GraphPad Software, San Diego, CA). Results were obtained from two independent occasions and were expressed as mean ± SEM. For all experiments, probabilities of error (p values) were included; values for $p < 0.05$ were regarded as significant.

RESULTS

Histology grades. Histologic scores were determined by the presence and location of necrosis within the kidney. Both kidneys from each mouse received a separate, blinded grade. MT kidneys received a mean (±SEM) histologic score of 1.93 ± 0.34. Control kidneys demonstrated a significant degree of tubular damage and received a histologic score of 3.50 ± 20 (Fig. 2). The mean difference between MT and control kidneys was 1.57 ($p = 0.003$).

The injury seen in MT mice was mild and consistently limited to the CMJ (85.6% of the total number of kidneys). Extension of necrosis into the cortex denoted more severe tubular injury, frequently observed in control mice (56.3%). Injury severity increased in parallel to the proximity of necrotic extension toward the outer cortical surface, and more than half of control mice had necrotic extension into the cortex. One of the two kidneys from two MT mice had focal necrotic extension into the cortex (grade 4); however, neither mouse showed a significant increase in post-IRI plasma creatinine levels (0.0 and 0.4).

Serum creatinine. Serum creatinine levels were obtained from all experimental groups immediately before IRI and again 24 h after IRI. An additional draw was taken before the first Marimastat dose and showed that Marimastat did not affect creatinine levels before IRI (data not shown). Baseline creatinine levels before IRI were consistent between experimental groups ($p = 0.788$) (Fig. 3). Mean serum creatinine levels in mice pretreated with Marimastat (0.25 ± 0.03) were not statistically different from baseline levels (0.20 ± 0.00) ($p = 0.268$). In contrast, the post-IRI control mice demonstrated a significant increase to 1.01 ± 0.29 from the baseline levels of 0.21 ± 0.01 ($p = 0.003$). Post-IRI, the creatinine levels of control mice were 4-fold higher than in MT mice ($p < 0.0001$).

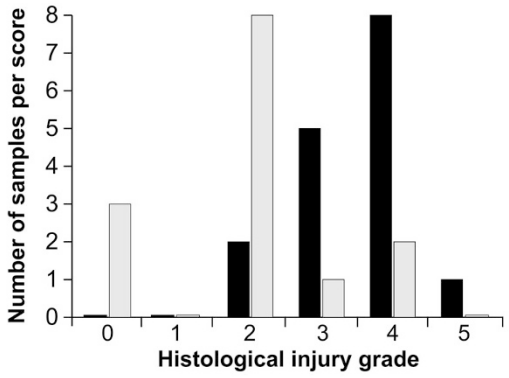

Figure 2. Distribution of Histologic injury scores. Vehicle (100 μL of 0.45% methylcellulose), denoted in black, and Marimastat (100 mg/kg in 100 μL of vehicle), denoted in gray, were administered twice daily. Scores were determined 24 h post-IRI by a masked pathologist.

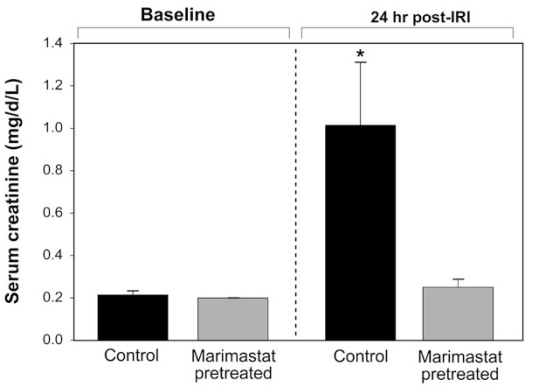

Figure 3. Creatinine levels 24 h post-IRI. *In vivo* creatinine levels were measured before injury and before euthanasia (24 h post-IRI). Vehicle (100 μL of 0.45% methylcellulose) and Marimastat (100 mg/kg in 100 μL of vehicle) were administered 96 h before surgery and was continued 24 h postoperatively for a total of 10 doses. *$p = 0.003$ compared with baseline levels.

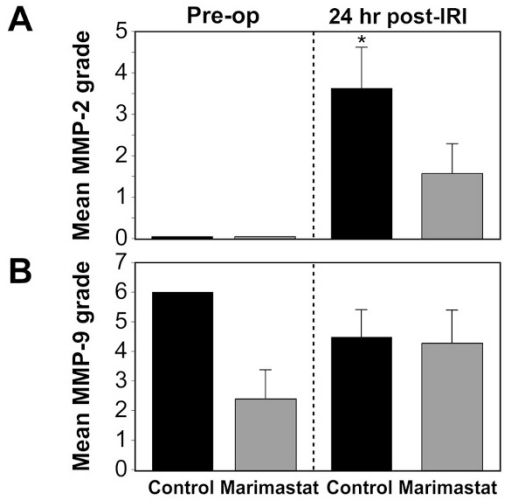

Figure 4. MMP activity. Gelatin zymography was performed on mouse urine collected before and after IRI; both proenzyme and activated proteinases appear as zones of substrate clearing. (*A*) Graph of MMP-2 and (*B*) MMP-9 activity before injury and before euthanasia (24 h post-IRI) in control and Marimastat-treated mice. *$p < 0.001$ compared to pre-op.

Gelatin zymography. Urine was screened for MMP activity by gelatin zymography before bilateral IRI and 24 h after IRI. In contrast to blood specimen collection, urine collection is noninvasive and will not influence detectable MMP levels. The band strength visualized by gelatin zymography provided an estimation of relative activity of the gelatinases MMP-2 and MMP-9 (Fig. 4*A* and *B*).

Active MMP-2 was undetectable in the preoperative samples of MT and control mice. MMP-2 activity rose significantly from baseline levels 24 h post-IRI in control mice (3.62 ± 0.99); however, MT mice demonstrated only a slight increase in MMP-2 activity (1.57 ± 0.72) (Fig. 4*D*). The post-IRI rise in MMP-2 activity between control and MT mice was significant ($p < 0.001$). There was no statistical difference in MMP-9 activity between groups and time points (Fig. 4*E*).

Western blot. Western blot analysis was conducted to determine the levels of MMP-2, MMP-9, and TACE in kidney

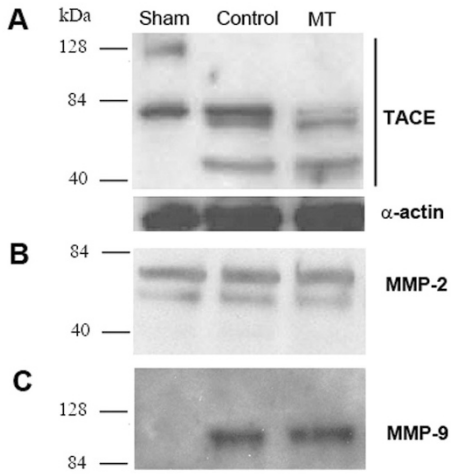

Figure 5. Western Blots of tissue TACE, MMP-2 and MMP-9. Western Blots were performed on sham, control, and MT kidneys. TACE expressed as 120 and 85 kD bands in sham kidney; 85 and 45 kD bands in untreated (control) kidney; and 85 and 45 kD bands in MT kidney (*A*). MMP-2 was expressed as a 65 kD band in the sham, control, and MT kidneys (*B*). MMP-9 was not expressed in sham kidney but expressed as 90 kD in control and MT kidneys (*C*).

tissue. In normal kidney, TACE is expressed constitutively as an 85 kD protein. Another 120 kD band has also been described under reducing conditions in the normal kidney (17). In normal kidneys, both bands were present (Fig. 5*A*). Twenty-four h after IRI, control mice showed a decrease in expression of the 85 kD band, whereas the 120 kD band was no longer present. Instead, a smaller, ~45 kD band was noted. Mice in the MT group showed reduced expression of the 85 kD band.

MMP-2 was expressed in normal kidneys (29) as a 65 kD band, and there was no change in its level in control and MT kidneys postoperatively (Fig. 5*B*). On the other hand, MMP-9 (90 kD band) was not expressed in normal kidney. IRI kidneys expressed MMP-9 in the control and MT group after 24 h (Fig. 5*C*).

DISCUSSION

Inflammation plays an important role in the progression from IRI to ATN and ultimately compromised renal function. Post-IR inflammation is a pivotal phase, which heavily dictates the severity of tubular injury. Up-regulation of MMPs propagates this microinflammatory response (30). The limited research regarding MMP expression in post-IRI of the kidneys has focused on its later role in tubulointerstitial fibrosis and chronic allograft nephropathy (31,32). This study is directed at MMP participation in earlier stages of injury.

MMP activity is most pronounced and their inhibition is most effective in the early stage of disease. Animal models studying the efficacy of MMP inhibition in IRI and various cancers have suggested that MMP inhibition initiated before or in the initial period of disease progression significantly reduced the severity and degree of tissue damage (33,34). Lutz *et al.* (35) demonstrated that MMP-2 inhibition reduced allograph nephropathy if initiated in the early, prefibrotic stages of disease but produced more severe nephropathy once fibrosis was established. In clinical trials, however, MMP inhibition has been typically implemented at more advanced stages of disease (36,37). The therapeutic modalities appropriate at the early stages may be misdirected once significant injury has been established. The disparity of outcome between experimental models and clinical trials demonstrates that the point of therapeutic intervention may be a critical factor determining the relevance and efficacy of MMP inhibition.

The murine model of renal IRI used in this study represents the renal injury observed during early graft dysfunction in the transplanted kidney (23). Tubular dysfunction begins shortly after the onset of ischemia, initiating a dynamic interplay between proinflammatory mediators that will direct subsequent inflammation.

MMP activity is mediated by several mechanisms and at several levels. TNF-α plays a role in the induction and propagation of inflammation by directly activating MMPs and initiating an influx of neutrophils to the site of injury (9). Cleavage of the cell-associated TNF-α proform into its soluble, biologically active form is mediated by TACE (16,17). Activation of TACE after ischemia-reperfusion has been shown to directly contribute to the pathogenesis of brain, lung, heart, and liver tissue injury (38–40). The upstream inhibition of TACE potentially abrogates the inflammatory cascade by reducing the amount of activated TNF-α. Results obtained from western blot analysis showed a change in the pattern of expression of TACE between controls and mice that underwent IRI. Twenty-four h after IRI, a new band with molecular weight 45 kD was identified with anti-TACE antibody. Although further investigation is warranted, this molecule could be the catalytic domain of TACE that has been described elsewhere (41).

When administered before IRI, Marimastat-reduced IRI as evidenced by both histologic and biochemical analysis. Morphologically, Marimastat diminished the scope and severity of ATN. Although apoptosis does contribute to ischemic injury, necrosis is more often associated with IRI. In contrast to apoptosis, which occurs under normal physiologic conditions and requires ATP, necrosis does not require ATP to proceed and only occurs after gross cellular injury (42). The location of necrosis was an important consideration in the histologic analysis because different metabolic demands within the kidney may determine areas most affected by ischemia. Necrosis was most prominent in the CMJ, which is presumably due to its high metabolic activity and its limited ability to sustain anaerobic metabolism amid decreased oxygen tension (43,44). Not only do cortical proximal tubules require very low oxygen extraction to support tubule metabolism but also blood flow in the postischemic kidney is more concentrated in the cortex rather than the medulla, which could explain the initiation and predominance of injury in the CMJ (44). TN is associated with elevated serum creatinine levels as seen in control mice. In contrast, serum creatinine in Marimastat mice demonstrated a nonsignificant increase over baseline indicating that Marimastat reduced TN.

Although MMP-2 is constitutively expressed in renal tissue, the absence of MMP-2 activity in urine samples at baseline of both MT and control mice might reflect its low urinary level

until IRI occurs. A recent study found that although MMP-2 activity increased 24 h after ischemia, an increase in MMP-9 activity was not detected until 48–72 h postischemia (45). Because we only measured MMP levels 24 h after IRI, our findings are consistent with this study demonstrating that urine MMP-2 is a prominent initial participant in post-IRI.

Results from the western blot showed increased MMP-9 protein levels after renal IRI. Gelatin zymography, however, demonstrated that MMP-9 activity remained unchanged between groups and time points. MMP-9 has been shown to form a complex with other molecules such as neutrophil gelatinase-associated lipocalin, which protects it from degredation (46) and is a marker of renal injury (47). Therefore, it is likely that increased MMP-9 protein level was partially due to a decrease of MMP-9 degradation.

Inhibition of MMPs has become a widely studied topic because of their involvement in many pathologic states. Pretreatment with MMP inhibitors may be applicable clinically. It may benefit those awaiting transplantation in which viable, fully functional organs are expected to undergo transient ischemia, an important one being pediatric transplantation. A crucial element of graft health and sustainability is the degree of ischemic injury, which may be effectively reduced if MMP inhibition is initiated before insult. Pretreatment with Marimastat may be a preferable alternative to current therapies in pediatric transplantation, such as tetracyclines, due to its more favorable side effect profile. Despite being protective against IRI in a variety of tissues, the use of tetracyclines in the pediatric population is complicated by noncompliance, enamel discoloration, stunted growth, and nephrotoxicity (48,49). The period immediately after reperfusion is a critical point in the prevention of renal injury (6).

In conclusion, as we have shown that urine MMPs can be used as biomarkers to follow a disease process (50), we demonstrate here that prevention of MMP-2 activation may be a possible mechanism for renal post-transplant protection. Prophylactic MMP inhibition in both donor and recipient before and after renal transplant may decrease post-transplant morbidity and foster graft function.

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