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Protective effect of milk fat globule-EGF factor VIII after renal ischemia-reperfusion injury in mice

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Abstract

Objectives—Renal ischemia-reperfusion (I/R) injury causes acute renal failure and the hallmarks of renal I/R injury are inflammation, apoptosis, necrosis, and capillary dysfunction. Milk fat globule-EGF factor VIII (MFG-E8), a membrane-associated secretory glycoprotein, is produced by immune cells and reported to participate in multiple physiological processes associated with tissue remodeling. We have recently shown that MFG-E8 treatment attenuates organ injury, inflammatory responses, and survival after sepsis through the enhancement of phagocytosis of apoptotic cells. The purpose of this study was to determine whether administration of MFG-E8 attenuates renal I/R injury.

Design—Prospective, controlled, and randomized animal study.

Setting—A research institute laboratory.

Subjects—Male C57BL/6J mice (20–25g).

Interventions—Renal I/R injury with bilateral renal pedicle clamping for 45 minutes, followed by reperfusion. A recombinant murine MFG-E8 (rmMFG-E8; 0.4 µg/20g) was given intraperitoneally at the beginning of reperfusion.

Measurements and Main Results—MFG-E8 levels, organ injury variables, inflammatory responses, histology, apoptosis, and capillary functions were assessed at 1.5 and 20 hours after reperfusion. A 60-hour survival study was conducted in MFG-E8^{−/−} and rmMFG-E8-treated wild-type (WT) mice. After renal I/R injury, MFG-E8 mRNA and protein expressions were significantly decreased in the kidneys and spleen. Treatment with rmMFG-E8 recovered renal dysfunction, significantly suppressed inflammatory responses, apoptosis, necrosis, and improved capillary functions in the kidneys. In the survival study, MFG-E8^{−/−} mice showed a significant deterioration and, in contrast, rmMFG-E8-treated WT mice showed a significant improvement of survival compared with vehicle-treated WT mice.

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Conclusions—MFG-E8 can be developed as novel treatment for renal I/R injury. This protective effect appears to be mediated through the enhancement of apoptotic cell clearance and improvement of capillary functions in the kidneys.

Keywords

MFG-E8; renal ischemia-reperfusion injury; inflammation; apoptosis; necrosis; capillary function

INTRODUCTION

Renal ischemia-reperfusion (I/R) injury causes acute renal failure (ARF) in various clinical settings, including kidney transplantation and cardiopulmonary and aortic bypass surgery. Renal I/R injury is associated with high mortality and morbidity (1, 2). Ischemia typically damages renal tubular epithelial cells and also glomerular cells and is characterized by several hallmark features at the cellular level: Profound intracellular ATP depletion and a fall in tissue oxygen and glucose content with a concomitant rise in intracellular calcium (3, 4). Although ischemic events alone may lead to necrosis and apoptosis in the kidneys, reperfusion occurs upon restoration of blood flow and is associated with production of reactive oxygen species (ROS), inflammatory mediators, and increased apoptosis as well as necrosis (5, 6). It has been shown that renal I/R injury can be ameliorated by inhibiting molecules involved in apoptosis, necrosis, or inflammation, suggesting that multiple injury and death mechanism may be involved in renal I/R injury (7). Among these, the coexistence of apoptosis and necrosis in renal tissues is a characteristic feature of renal I/R injury. Both types of cell death have been implicated significantly in the pathogenesis of ARF, which is marked by a loss of tubular epithelial cells and subsequent renal dysfunction (8, 9).

Moreover, there is growing evidence that additional mechanisms which contributes to the ongoing pathogenesis of I/R injury-induced ARF has been reported. For instance, renal vascular endothelial injury and dysfunction, due to increases in renal vascular resistance and persistent reductions in renal blood flow, exacerbates hypoxia and play an important part in extending renal tubular epithelial injury and subsequent cell death (10).

Milk fat globule-EGF factor VIII (MFG-E8) is a membrane-associated glycoprotein originally found in milk and mammary epithelial cells (11). It has been shown that MFG-E8 is expressed in various cells, including macrophages, dendritic cells, and in several other epithelial cells, such as photoreceptor cells, retinal pigment epithelium cells, lamina propria epithelial cells of colonic mucosa, endometrial glandular and surface-luminal epithelial cells (12–17). Previous investigators have reported that MFG-E8 participates in multiple physiological processes associated with tissue remodeling (18). Among these, Hanayama et al. (15, 19) have found that MFG-E8 negatively regulates inflammation and autoimmunity by facilitating the clearance of apoptotic cells. Recently, we have shown that MFG-E8 has a similar impact on outcome in acute inflammatory conditions including sepsis and gut I/R injury. Exogenous administration of MFG-E8 ameliorated inflammatory responses and improved survival through the enhancement of phagocytosis of apoptotic cells (20–26).

Therefore, we hypothesized from these evidences that MFG-E8 attenuates tissue damage after renal I/R injury. In this report, we investigated the effects of renal I/R injury on MFG-E8 expression and whether administration of MFG-E8 beneficially affects associated inflammatory responses, tubular cell death, and capillary dysfunction. Some of the results of this study were previously reported in the form of an abstract (27).

MATERIALS AND METHODS

Animal Model of Renal I/R Injury

Male C57BL/6J mice (20–25g; Taconic, Albany, NY) were randomly assigned into different groups (n=11/group). Mice were anesthetized with isoflurane inhalation. Using a midline abdominal incision, renal I/R injury was induced by bilateral renal pedicle clamping with microvascular clamps for 45 min. Then the clamp was removed, and the reflow (reperfusion) was visually verified. At the beginning of reperfusion, mice were resuscitated with 0.5 ml saline intraperitoneally and were treated with recombinant murine MFG-E8 (rmMFG-E8; R&D Systems, Inc., Minneapolis, MN) (0.4 µg/20 g BW in 0.5 ml normal saline intraperitoneally) or normal saline (Vehicle). Control animals underwent the same surgical procedure, except for the clamping of the renal pedicle (Sham). At 1.5 and 20 hours after reperfusion, animals were anesthetized, and plasma and tissue samples were harvested and stored at –80°C until experiments. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research (Manhasset, NY).

Determination of Organ Injury Variables

Plasma levels of blood urea nitrogen (BUN), creatinine, and alanine aminotransferase (ALT) were determined at 20 hours after reperfusion by using commercial assay kits according to the manufacturer's instructions (Pointe Scientific, Lincoln Park, MI).

Water Content Determination and Histologic Examination

Kidney and liver edema was estimated at 20 hours after reperfusion by comparing wet-to-dry ratios. Tissues were dried in a 70°C oven for 72 hours. Tissue water content was calculated as percent H₂O = (1 - dry wt/wet wt) × 100%. Morphologic alterations in the kidneys were examined at 20 hours after reperfusion by light microscopy. The tissue was fixed in 10% formalin and later embedded in paraffin. The tissue paraffin sections (4-µm thick) were stained with hematoxylin and eosin. Histological changes in the cortex and in the outer stripe of the outer medulla (OSOM) were assessed by quantitative measurements of tissue damage by a blinded fashion. Tubular damage was defined as tubular epithelial swelling, loss of brush border, vacuolization, necrotic tubules, cast formation, and desquamation. The degree of kidney damage was estimated by the following criteria: 0, normal; 1, area of damage < 25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; and 4, 75–100% of the area being affected (28).

Myeloperoxidase (MPO) Immunohistochemistry and Measurement of MPO Activity

Kidney samples were taken at 20 hours after reperfusion and the paraffin-embedded kidney sections were blocked and stained with rabbit anti-myeloperoxidase (MPO) antibody (Abcam, Cambridge, MA). After washing, the sections were reacted in biotinylated anti-rabbit IgG. Vectastain ABC reagent and DAB kit (Vector, Burlingame, CA) were used to reveal the immunohistochemical reaction. For the negative control, the primary antibody was substituted by normal rabbit IgG. Neutrophil accumulation within the kidneys was also estimated using the MPO activity assay, which is using the peroxidase catalyzed reaction, as we described previously (29).

Western Blotting

Tissue protein was fractionated on a Bis-Tris gel and transferred to a nitrocellulose membrane. The membranes were blocked by incubation in TBS with 0.1% Tween 20 (TBST) containing 5% bovine serum albumin or milk and incubated with primary anti-

MFG-E8, PARP, and VEGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the blots were incubated subsequently with horseradish peroxidase-labeled secondary antibody and washed. Bands were detected using a chemiluminescent peroxidase substrate (ECL Plus, Amersham, Little Chalfont, Buckinghamshire, UK).

Quantitative Real-Time PCR

Total RNA was extracted from tissue samples after 20 hours reperfusion using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 µg of total RNA using Oligo (dT)₁₂₋₁₈ primer (Invitrogen) and murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). The expressions of target mRNAs were detected by quantitative real-time PCR (7300 Real Time PCR System, Applied Biosystems) as SYBR Green as detection dye. Primer sequences were described in Table 1. Relative expression of each mRNA was calculated using $\Delta\Delta C_t$ threshold method.

TUNEL Assay

The presence of apoptotic renal tubular cells in the kidney sections were assessed after 1.5 hour reperfusion using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining kit (Roche Diagnostics, Indianapolis, IN) under a fluorescence microscope, as described previously (26).

Survival Study

To determine the role of MFG-E8 for survival benefit after renal I/R injury, C57BL/6/J wild-type (WT) mice with or without rmMFG-E8 treatment (a single dose, 0.4 µg/20g, intraperitoneal administration of rmMFG-E8 at the beginning of reperfusion), and male C57BL/6/J MFG-E8 knockout (KO) mice (a generous gift from Dr. Shigekazu Nagata, Kyoto University, Kyoto, Japan) were subjected to renal I/R injury and observed for 60 hours after the operation.

Statistical Analysis

Data are expressed as mean $\pm$ SEM and compared by one-way analysis of variance using Student-Newman-Keuls' test. Student *t* test was used for two-group analysis. The survival study was analyzed using Kaplan-Meier method and compared by log-rank test. Differences in values were considered significant at $p < .05$.

RESULTS

Suppression of MFG-E8 in Various Organs after Renal I/R Injury

To investigate whether MFG-E8 levels are altered after renal I/R injury, we measured its mRNA and protein levels at 20 hours after reperfusion. MFG-E8 is reported to be mainly produced in the spleen (19, 30). Therefore, we assessed the levels on the spleen, kidneys, and liver. Splenic MFG-E8 mRNA and protein levels decreased by an average 36% and 41%, respectively, after renal I/R injury ($p < .05$, Figs. 1A, B). Similarly, both MFG-E8 mRNA and protein levels in the kidneys decreased by 45% after renal I/R injury ($p < .05$, Figs. 1C, D). In the liver, MFG-E8 mRNA and protein levels decreased by 49% and 26%, respectively, although the decrease of protein levels was not statistically significant ($p < .05$ and $p = .18$, respectively, Figs. 1E, F).

Administration of rmMFG-E8 Attenuates Renal and Liver Dysfunction after Renal I/R Injury

In the measurement of renal excretory function, mice that were subjected to renal I/R injury caused renal dysfunction at 20 hours after reperfusion as expected by significant increase in plasma BUN and creatinine levels compared with sham-operated mice. In contrast,

treatment with rmMFG-E8 largely attenuated I/R injury-induced renal dysfunction ($p < .05$, Figs. 2A, B). Furthermore, treatment with rmMFG-E8 significantly suppressed the I/R injury-induced elevation of ALT which indicates the injury of the distant organ that is induced by this model ($p < .05$, Fig. 2C).

Administration of rmMFG-E8 Attenuates Renal Tubular Injury after Renal I/R Injury

Figure 3 shows the renal histology in cortex and OSOM area at 20 hours after reperfusion. Renal I/R injury induced remarkable tubular damage, including loss of brush border, necrotic tubules, and cast formation, compared with sham-operated mice which show the normal renal structures (Figs. 3A, B, D, E). As previous reports have demonstrated that tubular epithelial cells in the medulla are more susceptible to I/R injury than cortex, our results show that the tubular damage in OSOM is more severe compared with that in cortex (8, 31). In contrast, these tubular damage were remarkably attenuated in all rmMFG-E8-treated mice (Figs. 3C, F). Quantification of the tubular damage in cortex and OSOM area showed a significantly lower tubular injury score in rmMFG-E8-treated mice compared with vehicle-treated mice ($p < .05$, Figs. 3G, H).

Administration of rmMFG-E8 Attenuates Renal and Liver Edema after Renal I/R Injury

As shown in Figure 4, mice subjected to renal I/R had a significant increase of water content in the kidneys (Fig. 4A) and liver (Fig. 4B) at 20 hours after reperfusion as compared with sham-operated mice ($p < .05$). When renal I/R injury mice were treated with rmMFG-E8, both kidney and liver water contents were reduced and there were no statistical differences between sham-operated and rmMFG-E8-treated mice.

Administration of rmMFG-E8 Suppresses Inflammatory Response after Renal I/R Injury

Proinflammatory cytokines and chemokines are major contributors in the organ injury after renal I/R injury (32). We investigated whether interleukin-1 β (IL-1 β), IL-6, and macrophage inflammatory protein-2 (MIP-2) mRNA expressions in the kidneys were affected by the treatment with rmMFG-E8. As expected, kidney levels of the cytokines and chemokine increased significantly after renal I/R injury (IL-1 β by 19.9-fold, IL-6 by 3.3-fold, and MIP-2 by 12.3-fold). Treatment with rmMFG-E8 significantly reduced the proinflammatory response by 51, 40, and 37%, respectively ($p < .05$, Figs. 5A–C).

Administration of rmMFG-E8 Suppresses Neutrophil Infiltration after Renal I/R Injury

To determine the alterations in neutrophil infiltration, immunohistochemical staining of kidney sections for MPO was performed. Consistent with the increased expression of MIP-2, a potent chemoattractant for neutrophil, MPO immunostaining increased at 20 hours after reperfusion in vehicle-treated mice compared with sham-operated mice (Figs. 6A, B). Treatment with rmMFG-E8 after renal I/R injury reduced the MPO immunostaining (Fig. 6C). Likewise, MPO activity in the kidneys increased by 4.3-fold after renal I/R injury. Treatment with rmMFG-E8 markedly reduced the kidney MPO activity similar to sham level ($p < .05$, Fig. 6D).

Administration of rmMFG-E8 Suppresses Apoptosis after Renal I/R Injury

As a measure of tubular cell apoptosis, TUNEL staining of kidney sections was performed. At 20 hours after reperfusion, the majority of positive staining was derived from the fragmented nuclei in vehicle-treated mice (data not shown). Considering that this method detects not only apoptotic cells but extensive DNA fragmentation which is occurring in certain forms of necrotic cells (33, 34), fragmented positive stainings could be due to tubular necrosis. Therefore we chose earlier time point than 20 hours. At 1.5 hours after reperfusion, apoptotic tubular cells show positive staining of whole nuclei in both vehicle and rmMFG-

E8-treated mice. However, treatment with rmMFG-E8 suppressed the number of apoptotic cells in the kidneys after renal I/R injury (Figs. 7A–C). We also employed poly(ADP-ribose) polymerase (PARP) western blot as a marker of apoptosis. Mice subjected to renal I/R had a significant increase of cleaved PARP expression in the kidneys after 20 hours reperfusion. Treatment of rmMFG-E8 decreased kidney cleaved PARP levels significantly to levels similar to those in sham animals (Fig. 7D).

Administration of rmMFG-E8 Improves Capillary Function after Renal I/R Injury

As a measure of capillary function, vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1) expressions in the kidneys after 20 hours after reperfusion were assessed. VEGF mRNA levels were decreased significantly by 61% and protein levels by 32% after renal I/R injury. Treatment with rmMFG-E8 significantly increased VEGF mRNA and protein levels in renal I/R injury mice ($p < .05$, Figs. 8A, B). Furthermore, ET-1 mRNA levels were significantly increased by 55% after renal I/R injury and the treatment with rmMFG-E8 significantly reduced them as similar as sham-operated mice ($p < .05$, Fig. 8C).

MFG-E8 Has Survival Benefit after Renal I/R Injury

The above results suggest that rmMFG-E8 treatment is beneficial in renal I/R-mediated organ injury. We therefore conducted a survival study to prove the long-term benefit of MFG-E8 using MFG-E8 KO (MFG-E8^{-/-} group) and WT mice with (rmMFG-E8 group) or without (Vehicle group) rmMFG-E8 treatment. Figure 9 shows the survival curves in each groups, the survival rate at 60 hours after renal I/R injury in the MFG-E8^{-/-} group was significantly lower than that in the Vehicle group (11.1% vs. 43.8%; $P < .05$). The rmMFG-E8 group showed a significant improvement of the survival rate to 73.3% compared with that in the Vehicle group ($p < .05$).

DISCUSSION

In this study, we show that MFG-E8 expression was decreased and treatment with MFG-E8 attenuated renal tissue damage and renal excretory dysfunction in the mice after renal I/R injury. These beneficial effects of MFG-E8 were associated with suppression of inflammatory responses, tubular cell death induced by apoptosis and necrosis, and improvement of capillary function. To our knowledge, this is the first study to demonstrate effects of MFG-E8 against I/R injury in the kidneys.

MFG-E8 participates in various physiological functions associated with tissue remodeling and maintenance of homeostasis (18). Hanayama et al. (15) have shown that MFG-E8 is one of the bridging molecules between apoptotic cells and phagocytes by binding to phosphatidylserine (PS) exposed on apoptotic cells and to $\alpha_v\beta_3/\alpha_v\beta_5$ integrins on phagocytes. Additionally, MFG-E8 deficient mice showed various characteristics of inflammation and autoimmunity and suffered from glomerulonephritis that are specifically due to defects in apoptotic cell engulfment by phagocytes (19). This may raise the possibility that the kidney is one of the sensitive organs associated with the abnormality of MFG-E8 production. In this report, we demonstrated that MFG-E8 productions in various tissues were decreased after renal I/R injury. We have previously shown that cecal ligation and puncture (CLP) induced-sepsis and gut I/R injury suppressed MFG-E8 production in vital organs. Stimulation with lipopolysaccharide (LPS), which plays a pivotal role for the pathogenesis of CLP sepsis, *in vitro* and *in vivo* also suppressed MFG-E8 production (20, 21, 23, 24, 35). Given the fact that renal I/R injury has less chance in exposure to LPS, other candidate pathways besides LPS should exist for the down-regulation of MFG-E8 after renal

I/R injury. Moreover, which cell type is affected by the decline of MFG-E8 under renal I/R injury conditions is currently not known and is subject to further investigation.

As an attempt to reveal a mechanism of renoprotection by MFG-E8 treatment, we investigated the apoptotic cell death. It is because several studies have been reported in which MFG-E8 plays an important role at the interface between phagocytosis of apoptotic cells and inflammation in chronic and acute inflammatory diseases (19, 24, 25). Induction of apoptosis is one of the major causes of tissue damage after renal I/R injury. Inhibition of certain elements of the apoptotic pathway seemed to ameliorate renal I/R injury (36, 37). Recently, it has been shown that not only necrotic cells but apoptotic cells which were not engulfed appropriately by phagocytes, a process termed 'secondary necrosis', release potentially cytotoxic and antigenic intracellular contents which can promote an inflammatory response (38, 39). It is also reported that increases in apoptosis after renal I/R injury were directly associated with increased renal inflammation and following further tissue damage (40, 41). Our results showed that treatment with MFG-E8 reduced the number of apoptotic cells and suppressed local inflammation after renal I/R injury. Although we have no direct data showing the impairment of phagocytosis, it is suggested from previous studies (15, 19, 24, 25, 42) that decreased MFG-E8 expression in our model is associated with impaired phagocytosis of apoptotic cells and that the beneficial effect of MFG-E8 treatment is partially due to its recovery.

The histological amelioration of overall tubular disruption in rmMFG-E8 treated mice was a highly significant observation (Figure 3). Apparently, in addition to apoptosis, tubular necrosis was suppressed in these animals as well. Considering our result, despite the increased amount of necrotic tubular cells in histology, apoptosis was seen in a small minority of tubular cells as evidenced by the TUNEL stains in our renal I/R injured mice. Previous reports have shown that necrosis of proximal tubules is more predominant than apoptosis in renal I/R injury (43, 44). Based on these observations, we speculate that necrotic tubular cell death and subsequent hyper-inflammatory responses should contribute to renal injury in this model and that the beneficial effect of MFG-E8 treatment is attributed to more towards a decrease of necrosis instead of apoptosis.

Increasing number of evidences has been reported that I/R injury induces renal vascular damage which may prolong the hypoperfused state of the kidney and exacerbate renal injury following I/R (10, 45). Moreover, recent studies have focused on rapid changes in the blood flow as the initiation or maintenance of injury in the settings of I/R injury. Yamamoto et al. (46) reported that *in vivo* renal perfusion in peritubular vessels was decreased within minutes of reperfusion. It is well known that the reduced blood flow and hypoxic conditions that occur in renal I/R injury lead to deprivation of vital nutrients and loss of ATP in the vascular and tubular cells causing necrotic cell death (7).

Recent findings indicate that VEGF is not only a potent vascular permeability and angiogenic regulator but also regulates various capillary functions, including endothelial cell proliferation, vasodilatation, by preventing endothelial apoptosis, and exertion of anti-inflammatory effects (47, 48). So far, the alteration of VEGF expressions after renal I/R injury is controversial (49, 50). However, in consistent with our result, Basile et al. (51) reported that VEGF expression was repressed in early phase of renal I/R injury. The loss of VEGF expression was due to the reduction of its content in proximal tubules and to increased production of molecules that inhibit VEGF activity. The mechanism involving the recovery of VEGF expression by MFG-E8 treatment is unknown. However, a previous report has suggested a direct interaction between VEGF and MFG-E8. Silvestre et al. (52) have reported that MFG-E8 is required for VEGF-dependent blood vessel growth and

increase in blood flow not only under physiological but ischemic condition. Furthermore VEGF induces an increase of MFG-E8 production in endothelial cells.

One of the hallmark features in renal vasculature after I/R injury is prolonged vasoconstriction, so that functional recovery may be prevented or delayed (53). ET-1 is a potent vasoconstrictor produced by endothelial cells, which plays a crucial role in the dysregulation of tissue microvascular perfusion (54). ET-1 has been implicated in the pathogenesis of renal I/R injury, in which ET-1 expression is elevated in post-ischemic kidneys (55, 56). Modulating enhanced ET-1 expressions by 17 β -estradiol is reported to exhibit the protective effects after renal I/R injury. This suppression of ET-1 is suggested to be derived from the regulation of nuclear factor- κ B (NF κ B) activation at a transcriptional level of the ET-1 gene (57, 58). It is likely that MFG-E8 can suppress the I/R injury-induced ET-1 overexpression through its regulatory effect of NF- κ B activation (12). Furthermore a recent report has shown that platelet membrane PS is essential for hemostasis and thrombosis, and that MFG-E8 has an anti-coagulant property through its blockade of PS exposure on platelets (59). Taken together, our results suggest that MFG-E8 treatment improves the deteriorated capillary functions after renal I/R injury through various pathways, including VEGF-dependent vascular protection, inhibition of vasoconstriction by ET-1, and suppression of thrombosis.

Interestingly, we have found a significant increase of MFG-E8 expression in the kidney after rmMFG-E8 treatment compared to Vehicle group (data not shown). Although, the detailed mechanism of this restoration is unclear, our histology data have demonstrated the maintenance of normal renal structures by rmMFG-E8 treatment. Therefore, it implies that the kidney epithelial cells produce considerable amount of MFG-E8, which were lost/damaged due to I/R injury resulting in the decreased levels of MFG-E8; however, the treatment of rmMFG-E8 maintained the normal kidney structures and thereby plays a role for the restoration of endogenous levels of MFG-E8.

In summary, renal I/R injury suppressed MFG-E8 levels and induced considerable organ injury, as characterized by renal dysfunction, increased inflammatory responses, induction of apoptosis and necrosis, and decreased capillary functions in the kidneys. The administration of MFG-E8 ameliorated kidney injury and improved survival. Thus, MFG-E8 can be developed as novel treatment for renal I/R injury. This protective effect appears to be mediated through the enhancement of apoptotic cell clearance and improvement of capillary functions in the kidneys.

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FIGURE 1. Alterations in MFG-E8 mRNA and protein expression in the spleen (A, B), kidneys (C, D), and liver (E, F) at the end of 20-hour reperfusion after 45 minutes renal ischemia-reperfusion (I/R) injury or sham operation (Sham). Representative blots are also presented. Data are expressed as means $\pm$ SE (n=5–8/group) and compared by Student *t* test; **p* < .05 versus Sham group.

FIGURE 2.

Alterations in plasma organ injury variables (BUN (A), creatinine (B), ALT (C)) in sham-operated mice (Sham), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8) at 20 hours after reperfusion. Data are expressed as means $\pm$ SE (n=8–11/group) and compared by one-way ANOVA and Student-Newman-Keuls test; * $p < .05$ versus Sham group; # $p < .05$ versus Vehicle group.

FIGURE 3.

Alterations in renal histology in sham-operated mice (Sham), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8) at 20 hours after reperfusion. Representative photomicrographs with hematoxylin-eosin stain were collected from cortex (A, Sham; B, Vehicle; C, rmMFG-E8) and the outer stripe of outer medulla (OSOM) (D, Sham; E, Vehicle; F, rmMFG-E8). Original magnification $\times 200$. Tubular injury in cortex (G) and OSOM (H) was scored by the method in methods. Data are expressed as means $\pm$ SE ($n=6$ /group) and compared by one-way ANOVA and Student-Newman-Keuls test; * $p < .05$ versus Sham group; # $p < .05$ versus Vehicle group.

FIGURE 4.

Alterations in water content in the kidneys (A) and liver (B) in sham-operated mice (Sham), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8) at 20 hours after reperfusion. Data are expressed as means $\pm$ SE (n=6–8/group) and compared by one-way ANOVA and Student-Newman-Keuls test; * p < .05 versus Sham group; # p < .05 versus Vehicle group.

FIGURE 5.

Alterations in kidney IL-1 β (A), IL-6 (B), and MIP-2 (C) mRNA expressions in sham-operated mice (Sham), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8) at 20 hours after reperfusion. Data are expressed as means $\pm$ SE (n=7–10/group) and compared by one-way ANOVA and Student-Newman-Keuls test; * p < .05 versus Sham group; # p < .05 versus Vehicle group.

FIGURE 6.

Alterations in myeloperoxidase (MPO) immunohistochemical stainings of the kidney. Photomicrographs of kidney sections from sham-operated mice (Sham) (A), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) (B) or recombinant murine MFG-E8 (rmMFG-E8) (C) at 20 hours after reperfusion. Representative photomicrographs are shown. Dark brown staining represents the positive cells (original magnification $\times 200$). Alterations in kidney MPO activity (D) in Sham, Vehicle or rmMFG-E8 treated mice. Data are expressed as means $\pm$ SE ($n=7$ /group) and compared by one-way ANOVA and Student-Newman-Keuls test; * $p < .05$ versus Sham group; # $p < .05$ versus Vehicle group.

FIGURE 7.

Alterations in apoptosis in the kidneys. The kidney sections were stained with TUNEL (green fluorescent). Photomicrographs of kidney sections from sham-operated mice (Sham) (A), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) (B) or recombinant murine MFG-E8 (rmMFG-E8) (C) at 1.5 hours after reperfusion.

Representative photomicrographs are shown (original magnification $\times 200$). Alterations in kidney cleaved poly(ADP-ribose) polymerase (PARP) (D) in Sham, Vehicle or rmMFG-E8 treated mice. Data are expressed as means $\pm$ SE ($n=5-6$ /group) and compared by one-way ANOVA and Student-Newman-Keuls test; * $p < .05$ versus Sham group; # $p < .05$ versus Vehicle group.

FIGURE 8.

Alterations in kidney vascular endothelial growth factor (VEGF) mRNA (A), protein (B), and endothelin-1 (ET-1) mRNA (C) expressions in sham-operated mice (Sham), renal ischemia-reperfusion (I/R) injury mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8) at 20 hours after reperfusion. Representative blots are also presented. Data are expressed as means $\pm$ SE (n=6–9/group) and compared by one-way ANOVA and Student-Newman-Keuls test; * p < .05 versus Sham group; # p < .05 versus Vehicle group.

FIGURE 9.

Alterations in the survival rate after renal ischemia-reperfusion (I/R) injury in MFG-E8^{-/-} mice and Wild-type mice treated with normal saline (Vehicle) or recombinant murine MFG-E8 (rmMFG-E8). Each group of mice received normal saline or a single dose (0.4 μg/20g) rmMFG-E8 intraperitoneally at the beginning of reperfusion and observed for 60 hours. The survival rate was estimated by the Kaplan-Meier method and compared by using the log-rank test (n=15–18/group); **p* < .05 versus Vehicle group.

TABLE 1**Primers for Quantitative Real-Time PCR**

Gene (Accession no.)	Sequence (5'-3')
MFG-E8 (NM_008594)	
Forward:	CGCACAGGGATCGTCAATG
Reverse:	CGCAGAAGGTTACCTGGAT
IL-6 (NM_031168)	
Forward:	CGCTATGAAGTTCCTCTCTGCAA
Reverse:	CACCAGCATCAGTCCCAAGA
IL-1 β (NM_008361)	
Forward:	TTTAGCTCACCCATGGCTTCA
Reverse:	TGCATCTTGCAGGGTCTTTTC
MIP-2 (NM_009140)	
Forward:	CCCTGGTTCAGAAAATCATCCA
Reverse:	GCTCCTCCTTTCCAGGTCAGT
VEGF (M95200.1)	
Forward:	CAAGGCGAGGCAGCTTGA
Reverse:	CTCACCGCCTTGGCTTGT
ET-1 (NM_010104)	
Forward:	TGATGGACAAGGAGTGTGTCTACTTC
Reverse:	GCGCTCGGGAGTGTTGAC
β -actin (NM_007393)	
Forward:	CGTGAAAAGATGACCCAGATCA
Reverse:	TGGTACGACCAGAGGCATACAG