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Growth Arrest-Specific Protein 6 Protects Against Renal Ischemia-Reperfusion Injury

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Abstract

Background—Renal injury caused by ischemia-reperfusion (I/R) often occurs following shock or transplantation. Growth arrest-specific protein 6 (Gas6) is a secreted protein that binds to the TAM-family tyrosine kinase receptors which modulate the inflammatory response and activate cell survival pathways. We hypothesized that Gas6 could have a protective role in attenuating the severity of renal injury after I/R.

Materials and methods—Adult mice were subjected to 45 min of bilateral renal ischemia. Recombinant mouse Gas6 (rmGas6, 5 µg/mouse) or normal saline (vehicle) was administered intraperitoneally 1 h before ischemia and all subjects were sacrificed at 23 h after I/R for blood and tissue analysis. The expression of protein and mRNA was assessed by Western blotting and qPCR, respectively.

Results—Treatment with rmGas6 significantly decreased serum levels of creatinine and BUN by 29% and 27%, respectively, improved the renal histological injury index and reduced the apoptosis in the kidneys, compared to the vehicle. Renal mRNA levels of IL-1 β , IL-6, TNF- α , KC and MIP-2 were decreased significantly by 99%, 60%, 53%, 58%, and 43%, with rmGas6 treatment, respectively. After I/R, renal I κ B- α levels were reduced by 40%, while they returned to sham levels with rmGas6 treatment. The mRNA levels of iNOS and COX-2 were reduced by 79% and 70%, respectively, while the expression of cyclin D1 was increased by 2.1-fold in the rmGas6-treated group, compared to the vehicle.

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Author contributions:

W.-L.Y., E.M. and P.W. conceived and designed the experiments. M.G., S.R. and M.K. performed the experiments. M.G. analyzed the data and drafted the manuscript. W.-L.Y., J.N. and G.F.C. interpreted the results. W.-L.Y. critically revised the manuscript. P.W. reviewed the manuscript and supervised the whole project.

Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Conclusions—Gas6 suppresses the NF- κ B pathway and promotes cell proliferation, leading to reduction of inflammation and protection of renal injury induced by I/R.

Keywords

Gas6; Renal; Ischemia-reperfusion injury; Inflammation; Apoptosis; Cell proliferation

1. Introduction

Renal ischemia-reperfusion (I/R) is a severe complication of profound hypotension during shock or transplantation procedures and accounts for the majority of acute kidney injury (AKI) in post-surgical patients with high mortality in intensive care units [1–3]. Despite the generous blood supply which the kidney receives, it is still prone to ischemia as its functional cells exist in a low oxygen tension environment [4,5]. The effect of I/R upon the kidney does not only effect the kidney itself, but distant organs such as the lung, heart and brain [6–8]. Allograft rejection after renal transplantation is enhanced due to I/R as it can worsen the immunologic response towards the transplanted organ itself and other organ systems [9,10]. Given the detrimental consequences of this pathology, we have guided our research efforts in investigating new therapeutic targets to ameliorate I/R injury.

During the reperfusion phase of I/R, a proinflammatory response occurs in order to protect viable tissue and clear dead or dying cells from the previously ischematized area [11]. This response becomes exaggerated and the intended defense mechanisms can harm local tissue. Multiple studies reflect the relative increase of IL-1 β , IL-6 and TNF- α in the affected tissue as well as different organ systems after I/R injury [12]. Nitrogenous stress is also a well-characterized feature of I/R injury and can be used to assess the degree of tissue damage [13]. To blunt, or to some extent limit, the hyperinflammatory response seen after I/R would be of clinical use so to decrease the incidence and/or severity of injury.

The TAM (Tyro3, Axl, Mer) family is the receptors of the growth arrest-specific protein 6 (Gas6), which has a recognized role in decreasing the innate immune response and function to dampen acute inflammation [14,15]. Gas6, via its Gla domain, is capable of binding phosphatidylcholine, encouraging phagocytosis [16]. Through the stimulation of the TAM receptors, multiple downstream pathways lead to decreasing inflammatory responses, stimulating phagocytosis, limiting hypoxic cell injury and encouraging cell proliferation [17,18]. Moreover, the Mer receptor, once bound by Gas6, is able to decrease inflammation due to LPS and during the septic process [19,20]. Conversely, the inhibition of Gas6 expression by toll-like receptors as well as the loss of the Mer receptor, leads to enhanced inflammation [21,22]. During inflammation, Gas6 is expressed at higher levels than at baseline values; however the efficacy of Gas6 is reduced dramatically as it is bound to the soluble form of its receptor sAxl [23]. This receptor shedding and subsequent inactivation of Gas6 may preclude the physiologic benefit of endogenous Gas6 [23]. Much focus upon the Gas6-Axl receptor prosurvival capabilities has been given in the study of various neoplastic diseases, specifically acute myeloid leukemia [24]. Thus, we hypothesized that free, unbound, Gas6 could active its natural receptors and induce the protective pathways aforementioned.

In this study, we used a mouse model of bilateral renal ischemia to evaluate the effect of recombinant mouse Gas6 (rmGas6) upon the pathological process of I/R. After 23 h of I/R, kidney tissues were harvested for analysis as well as serum samples to assess common clinical markers of AKI. Additionally, we examined histological changes and degree of apoptosis in the kidneys after I/R and with rmGas6 treatment. In light of these findings, we proposed Gas6 as a potential therapeutic target for ischemia-reperfusion injury.

2. Materials and methods

2.1. Animal model of renal I/R injury

Male C75BL/6 mice (20–25 g; Taconic, Albany, NY) were allocated to three groups: sham, vehicle and treatment groups. Mice were anesthetized by isoflurane inhalation. Bilateral 1-cm flank incision was made to expose the respective kidney. The renal hilum was bluntly dissected and the vascular pedicle was cross-compressed with removable clamps. The kidney was visually inspected for adequate ischemic changes and returned to the retroperitoneum. The kidneys were ischemized for 45 min and then allowed to reperfuse with visual inspection. During this period of time, the flank of the mouse was covered with moist gauze under the lamp. Mice were resuscitated with 0.5 ml saline intraperitoneally after surgery. At 23 h after starting of ischemia, the animals were euthanized and blood and tissue samples were collected for various analyses. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of The Feinstein Institute of Medical Research.

2.2. Administration of rmGas6

One hour prior to performing I/R, rmGas6 or vehicle was given. Normal saline (vehicle) in 200 µl or rmGas6 (5 µg/mouse; R&D Systems, Minneapolis, MN) was delivered intraperitoneally via bolus injection. The dosage of rmGas6 was based on our previous study [25].

2.3. Serum levels of renal injury markers

Blood samples were centrifuged at 2,000g for 15 min to collect serum and then stored at –80°C. Creatinine and blood urea nitrogen (BUN) were determined by commercial assay kits from Pointe Scientific (Lincoln Park, MI), according to the instructions provided by the manufacturer.

2.4. Histological analysis

Renal tissue was fixed in 10% formalin and then embedded in paraffin. Tissue was sectioned at a thickness of 5 µm, placed onto glass slides and stained with hematoxylin and eosin (H&E). Examination of these tissue sections was evaluated under light microscopy in a blinded manner. Dilation or loss of Bowman's space, flattening of renal tubular epithelium, loss of tubular brush border, microhemorrhage, and tubular casts were assessed and graded (1+, < 10%; 2+, 10% to 25%; 3+, 26% to 75%; and 4+, > 75%) as previous described [26]. The scores from 10 high-powered fields per sample were averaged to represent each animal.

2.5. TUNEL assay

The renal paraffin-embedded sections were dewaxed and rehydrated in a graded series of ethanol. The TUNEL assay was performed by using an In Situ Cell Death Detection Kit from Roche (Indianapolis, IN), according to the manufacturer's instructions. Results were expressed as the average number of TUNEL-positive staining cells per 10 high-powered fields.

2.6. qPCR analysis

Total RNA was extracted from renal tissues using Trizol (Invitrogen, Carlsbad, CA) and was reverse-transcribed into cDNA using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was done in 25 μ l of final volume containing 0.08 μ mol of each forward and reverse primer, cDNA, and 12.5 μ l SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the temperature of 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 sec and 60°C for 1 min. Mouse β -actin was used for normalization. Expression of mRNA was represented as fold change in comparison to sham tissue levels. The primers used are listed in Table 1.

2.7. Western blotting

Renal tissue was homogenized in lysis buffer (10 mM Tris-HCl pH 7.5, 120 mM NaCl 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with protease inhibitor (Roche) by sonication. Total lysate was fractionated on Bis-Tris gels (4–12%) and transferred to membranes. The membranes were then blocked with 5% milk in 0.2 $\times$ PBS and then incubated with anti-cyclin D1, anti-I κ B- α or β -actin primary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, the membranes were incubated with a fluorescently-labeled secondary antibody. Scanning the membranes was completed with the Odyssey image system (LI-COR, Lincoln, NE) and the band intensity measured by the Odyssey densitometric software.

2.8. Statistical analysis

Data are expressed as mean $\pm$ standard error (SE) and compared via one way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) test. Significance was considered if $P < 0.05$.

3. Results

3.1. rmGas6 improves renal function after I/R

The degree of acute kidney injury is commonly measured by a rise in creatinine and BUN. In our previous study, the significant deterioration of the kidneys in mice was observed at 20 h after I/R operation [27]. To evaluate the effect of rmGas6 on the injury after I/R, we collected blood samples from sham animals as well as vehicle and rmGas6-treated mice at 23 h after starting of ischemia. In the vehicle group, levels of creatinine and BUN were significantly increased, yet after treatment with rmGas6 decreased by 29% and 27%, respectively (Fig. 1).

3.2. rmGas6 attenuates renal injury and apoptosis after I/R

We used a validated scoring system which has a highest possible score of 4 and accounts for alterations in Bowman's capsule spacing, compromised convoluted tubule epithelium, intratubular cast formation and hemorrhage to evaluate the renal tissue damage. At 23 h after I/R, mice treated with vehicle demonstrated major disruptions in architecture as compared with sham (Fig. 2A). The vehicle group had an injury score of 3.5, whereas the rmGas6-treated group displayed a significantly less severity of damage, scored at 2.1 or a 40% reduction in comparison with the vehicle (Fig. 2C). We also conducted a TUNEL assay to detect the apoptotic cells in the kidneys. After I/R, the apoptotic cells stained with green fluorescence were well observed in the vehicle (Fig. 2B). The number of countable apoptotic cells in the vehicle group was 27.2 ± 5.5 , while it significantly reduced to 10.1 ± 3.3 in the rmGas6-treated group (Fig. 2D).

3.3 rmGas6 decreases proinflammatory cytokine and chemokine expression after I/R

I/R injury is mediated not only by the initial ischemia and reperfusion, but the generation of proinflammatory cytokines contributes to the overall injury [12]. At 23 h after I/R, the mRNA levels of renal IL-1 β , IL-6 and TNF- α were markedly increased in the vehicle group, as determined by qPCR (Fig. 3). However, with treatment of rmGas6, the levels of their mRNA expression fell by 99%, 60% and 53%, respectively (Fig 3).

The chemotaxis of inflammatory cells contributes significantly to injury after I/R [28]. Since the production of these molecules is under similar regulation as the aforementioned cytokines, we then evaluated the expression of specific chemokines. As expected, there was an upregulation of mRNA expression of KC and MIP-2, the mouse analog of human IL-8, as determined by qPCR (Fig. 4). In contrast, rmGas6 administration showed a 58% and 43% reduction of KC and MIP-2, respectively, compared to the vehicle (Fig. 4).

3.4. rmGas6 suppresses the expression of inflammatory mediators after I/R

The production of inflammatory mediators via the downstream events of I/R is well studied. Two pathways which are triggered by inflammation involve the enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [29]. The mRNA levels of both enzymes were elevated in the kidneys of the vehicle group, as determined by qPCR (Fig. 5). Treatment with rmGas6 reduced the levels of iNOS and COX-2 by 79% and 70%, respectively (Fig. 5).

3.5. rmGas6 inhibits the activation of NF- κ B in the kidneys after I/R

To determine the effect of rmGas6 on the activation of NF- κ B signaling, we measured the protein levels of its inhibitor, I κ B- α , by Western blotting. At 23 h after I/R, the I κ B- α expression in the whole renal lysate of the vehicle group was decreased by 40%, compared to the sham (Fig. 6). However, in rmGas6-treated animals, the expression of renal I κ B- α was restored toward the sham level (Fig. 6). This result suggested that rmGas6 could prevent the degradation of I κ B- α and subsequent inhibition of NF- κ B translocation and activation.

3.6. rmGas6 promotes renal cell proliferation after I/R

Cell cycle progression leading towards new cell growth and repair is a key component of tissue healing and regeneration [30]. In the vehicle group, the expression of renal cyclin D1, a cell cycle promoter, was decreased to 33% of the sham level, as determined by Western blotting (Fig. 7). In contrast, in the rmGas6-treated group, cyclin D1 was restored to 68% of the sham level, approximately double the amount in the vehicle group (Fig. 7).

4. Discussion

The impact of I/R injury in the clinical setting is very significant. Investigation and eventual utilization of novel mechanisms to avoid preventable injury is still in high demand. Towards this goal, we have elucidated a potential candidate for the treatment strategy and further research in Gas6. By using a mouse model of bilateral renal ischemia followed by a period of restoration of blood flow, we have demonstrated that pretreatment with rmGas6 can be protective to the kidneys underwent I/R-induced injury. The clinical markers of renal function (creatinine and BUN), histological renal injury index, apoptosis, proinflammatory cytokines, chemokines and mediators were decreased with rmGas6 treatment. Such beneficial role of Gas6 in protecting organ injury from I/R stress has also been reported in an animal model of hepatic I/R injury [31].

In our model, it clearly indicates that the renal tissues are severe damaged after I/R. As judged from histology, the integrity of renal morphology is lost in the vehicle group, showing large areas of tubular dilation and necrosis, which is corresponding to the elevation of serum renal injury markers, creatinine and BUN. In contrast, with rmGas6 treatment, the renal morphology is much well preserved with less necrotic area after I/R and the levels of serum renal injury markers are also decreased. In the mean while, we also observed a significant reduction of apoptotic cells in the kidneys after I/R with rmGas6 treatment. One of the biological functions of Gas6 is to enhance the uptake of apoptotic cells by macrophages for clearing away dying cells [16]. In addition, it has been indicated that failure of removing the apoptotic cells will progress to secondary necrosis, leading to induction of the inflammatory responses and tissue injury [32]. Thus, the enhancement of the phagocytosis can be one of the mechanisms for exogenous supplement of rmGas6 to reduce renal injury after I/R.

Dampening the inflammatory responses is another activity of rmGas6 treatment that we have demonstrated, showing the reduction of the expression of IL-1 β , IL-6 and TNF- α . The high levels of those proinflammatory cytokines has been well recognized as a crucial factor in contribution of organ injury after I/R [12]. The induction of these cytokines is regulated by NF- κ B signaling pathway [33]. By measuring the protein levels of I κ B- α , we have further demonstrated that the rmGas6 treatment can prevent the I/R-induced degradation of I κ B- α , subsequently resulting in translocation of NF- κ B from cytosol to nucleus for the activation. In consistent, it has been proposed that activation of TAMs, the receptors of Gas6, leads to the suppression of NF- κ B signaling for the immunomodulation [34]. At the same token, we also observed that the expression of proinflammatory chemokines, KC and MIP-2, is down-regulated by rmGas6 treatment. KC and MIP-2 are major factors of attracting neutrophils infiltrating to the damaged organs [35]. Although neutrophil

infiltration is needed to eliminate the invaded pathogens and clear up the cell debris, excessive neutrophils retained in the organs can cause severe tissue damage [36].

The generation of nitrosative stress is another characteristic of I/R pathophysiology and contributes to tissue damage, which is reflected on the induction of iNOS [37]. We have demonstrated that rmGas6 treatment can effectively reduce the elevation of iNOS expression in the kidneys after I/R. The inhibition of iNOS by Gas6 has also been observed in LPS-stimulated microglia [38]. The strategy of attenuating iNOS expression and activity has been shown to have renoprotective effects in an animal model of renal I/R injury [39]. We have also detected that COX-2 expression is inhibited by rmGas6 treatment. COX-2 is the main enzyme to produce prostaglandins, which can trigger a broad spectrum of inflammation cascade [40]. A recent study has also reported that COX-2 expression can be regulated by NF- κ B signaling [41]. In addition, it has been proposed that blockage of COX-2 can attenuate the renal tissue damage induced by I/R [42].

Finally, we have demonstrated that rmGas6 treatment can promote renal cell progression after I/R-induced injury, using the protein levels of cyclin D1 as an indicator [30]. Several studies indicate that the Gas6/Axl system via the activation of the RAS/RAF/MAPK and PI3K/Akt signaling pathways to promote growth, survival and proliferation of various cell type [18]. Correspondingly, Llacuna *et al* demonstrate the involvement of Gas6 in regulating the phosphorylation of Akt to execute its protective role in the liver against I/R injury [31].

5. Conclusions

Gas6 is a multiple-functional protein involved in regulating phagocytosis, inflammation and cell survival. With the administration of rmGas6, the tissue damage, apoptosis and inflammatory responses in the kidneys underwent I/R are significantly decreased, as well as the renal function and renal cell proliferation are significantly improved. These results clearly indicate that rmGas6 provides renoprotective activity in an animal model of renal I/R injury. Therefore, rmGas6 can be considered as a potential therapeutic candidate in an effort to treat patients with acute renal failure.

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Fig. 1.

Effect of rmGas6 on renal injury markers after I/R. Serum samples were harvested at 23 h after I/R for measuring (A) creatinine and (B) BUN. Data presented as means $\pm$ SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * P < 0.05 versus sham; # P < 0.05 versus vehicle.

Fig. 2.

Effect of rmGas6 on renal tissue damage and apoptosis after I/R. Kidneys were harvested at 23 h after I/R and subjected to immunohistochemical evaluation. (A) Representative images of renal tissues stained with hematoxylin and eosin (H&E). Original magnification 100×. (B) Representative images of renal tissues stained with TUNEL (green fluorescence). Original magnification 200×. (C) The extent damage of renal tissues based on H&E staining was graded with a modified schema as described in Materials and Methods. (D) A graphical indication of the number of TUNEL-positive staining cells averaged over 10 microscopic fields/animal. Data presented as means ± SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * $P < 0.05$ versus sham; # $P < 0.05$ versus vehicle.

Fig. 3.

Effect of rmGas6 on cytokine expression in the kidneys after I/R. Kidneys were harvested at 23 h after I/R for total RNA isolation. The mRNA levels of (A) IL-1 β , (B) IL-6 and (C) TNF- α were determined by qPCR. Results are normalized by β -actin as an internal control and are expressed as fold induction in comparison to the sham group. Data presented as means $\pm$ SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * P < 0.05 versus sham; # P < 0.05 versus vehicle.

Fig. 4.

Effect of rmGas6 on chemokine expression in the kidneys after I/R. Kidneys were harvested at 23 h after I/R for total RNA isolation. The mRNA levels of (A) KC and (B) MIP-2 were determined by qPCR. Results are normalized by β -actin as an internal control and are expressed as fold induction in comparison to the sham group. Data presented as means $\pm$ SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * P < 0.05 versus sham; # P < 0.05 versus vehicle.

Fig. 5.

Effect of rmGas6 on the expression of inflammatory mediators in the kidneys after I/R. Kidneys were harvested at 23 h after I/R for total RNA isolation. The mRNA levels of (A) iNOS and (B) COX-2 were determined by qPCR. Results are normalized by β -actin as an internal control and are expressed as fold induction in comparison to the sham group. Data presented as means $\pm$ SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * P < 0.05 versus sham; # P < 0.05 versus vehicle.

Fig. 6.

Effect of rmGas6 on IkB-α expression in the kidneys after I/R. Kidneys were harvested at 23 h after I/R for total cell lysate preparation. The protein levels of IkB-α were determined by Western blotting. Band intensity of IkB-α was normalized to its corresponding band intensity of β-actin. The ratio of the sham group is designated as 1 for comparison. Data presented as means ± SE (n=4–6/group) and compared by one-way ANOVA and SNK method; **P* < 0.05 versus sham; #*P* < 0.05 versus vehicle.

Fig. 7.

Effect of rmGas6 on cyclin D1 expression in the kidneys after I/R. Kidneys were harvested at 23 h after I/R for total cell lysate preparation. The protein levels of cyclin D1 were determined by Western blotting. Band intensity of cyclin D1 was normalized to its corresponding band intensity of β -actin. The ratio of the sham group is designated as 1 for comparison. Data presented as means $\pm$ SE (n=4–6/group) and compared by one-way ANOVA and SNK method; * P < 0.05 versus sham; # P < 0.05 versus vehicle.

Table 1

A list of primer sequences used in this study

Name	Gene Bank	Forward	Reverse
IL-1 β	NM_008361	CAGGATGAGGACATGAGCACC	CTCTGCAGACTCAAACCTCCAC
IL-6	NM_031168	CCGGAGAGGAGACTTCACAG	CAGAATTGCCATTGCACAAC
TNF- α	X_02611	AGACCCTCACACTCAGATCATCTTC	TTGCTACGACGTGGGCTACA
KC	NM_008176	GCTGGGATTACCTCAAGAA	ACAGGTGCCATCAGAGCAGT
MIP-2	NM_009140	CCCTGGTTCAGAAAATCATCCA	GCTCCTCCTTTCCAGGTCAGT
iNOS	NM_010927	GCAGGTCGAGGACTATTTCTTTCA	GAGCACGCTGAGTACCTCATTG
COX-2	NM_011198	CTCAGCCAGGCAGCAAATC	ACATTCCCCACGGTTTTGAC
β -actin	NM_007393	CGTGAAAAGATGACCCAGATCA	TGGTACGACCAGAGGCATACAG