Fibrinogen β-derived Bj15-42 peptide protects against kidney ischemia/reperfusion injury

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Short Title: Fibrinogen in Kidney Damage

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ABSTRACT

Ischemia/reperfusion (I/R) injury in the kidney accounts for the majority of acute kidney injury (AKI) and is associated with significantly high mortality. To identify genes that modulate kidney injury and repair we conducted genome-wide expression analysis in the rat kidneys following I/R and found that the mRNA levels of Fgα, Fgβ and Fgγ chains significantly increase in the kidney and remain elevated throughout the regeneration process. Cellular characterization of Fgα and Fgγ chain reactive proteins shows a predominant expression in renal tubular cells and the localization of immunoreactive Fgβ chain protein is primarily in the renal interstitium in healthy and regenerating kidney. We also show that urinary excretion of Fg is massively increased after kidney damage and is capable of distinguishing human patients with acute or chronic kidney injury (n=25) from healthy volunteers (n=25) with high sensitivity and specificity (ROC of 0.98). Furthermore, we demonstrate that Fgβ-derived Bβ15-42 peptide administration protects mice from I/R induced kidney injury by aiding in epithelial cell proliferation and tissue repair. Given that kidney regeneration is a major determinant of outcome for patients with kidney damage, these results provide new opportunities for the use of Fg in diagnosis, prevention, and therapeutic interventions in kidney disease.

Keywords: Biomarker, Therapeutic, Urine, BBeta, Inflammation, Acute Kidney Injury
INTRODUCTION

Kidney disease is a major public health concern receiving increased global attention owing to the significantly increased prevalence and high mortality rates\(^1,2\). Renal ischemia/reperfusion (I/R) accounts for the majority of acute kidney injury (AKI) in humans. Studies suggest that damage to the renal microvascular architecture and deterioration of the angiogenic response constitutes the early steps in the complex multiple pathways involved in both early and chronic ischemic renal injury\(^3\). Restoration of blood flow to the site of injured tissue is crucial for developing a successful repair response that involves the surviving dedifferentiated cells spreading over the denuded basement membrane, undergoing mitogenesis and ultimately re-differentiating to re-establish and restore functional integrity of the nephron\(^4,5\). While these processes are well described at the pathological level, very little is known about the cellular and molecular mechanisms of action of blood proteins within the kidney and their contribution to pathogenesis of renal disease.

Fibrinogen (Fg), a 340kDa dimeric blood protein, is made up of two sets of three different polypeptide chains, Fg\(\alpha\), Fg\(\beta\) and Fg\(\gamma\) that span a length of 50kb on chromosome 4 in region q28\(^6\). Although the primary site for Fg synthesis is shown to be liver, extrahepatic synthesis of Fg by epithelial cells of intestine\(^7\), cervix\(^8\), and lung\(^9,10\) has been reported suggesting that it may function in other structural and functional capacities. Furthermore, endogenous expression of Fg\(\alpha\) and Fg\(\beta\) chain mRNA has been shown in the normal rat kidneys, levels of which, significantly increase at 2 h following the onset of brain death injury\(^11,12\) potentially restoring hemostasis by supporting extracellular matrix and wound repair processes following injury\(^12\). In addition
to its major role in blood clotting and circulation via interaction with platelets\textsuperscript{13}, Fg has also been recognized as an important regulator of inflammation\textsuperscript{14}, wound healing\textsuperscript{15}, angiogenesis\textsuperscript{16}, and neoplasia\textsuperscript{17} via its action on a wide range of cellular receptors such as integrins, intracellular adhesion molecule-1, and vascular endothelial cadherin\textsuperscript{18}.

In this study we identify Fg\textsubscript{α}, Fg\textsubscript{β}, and Fg\textsubscript{γ} chain mRNA transcripts to be significantly up regulated in the kidney following bilateral renal I/R injury. We provide evidence suggesting the ability of urinary Fg to serve as a translational, non-invasive, and sensitive biomarker for early detection of AKI. We also demonstrate a therapeutic potential of Fg derived B\textsubscript{β}_{15-42} peptide that elicits $\sim$ 50% protection from bilateral I/R injury by increasing renal tissue repair and decreasing apoptosis. These results provide novel insights into the role of Fg as a diagnostic biomarker and therapeutic candidate in kidney disease and suggest that the presence of Fg in the kidney could serve as a protective mechanism against ischemic injury to facilitate tubular epithelial cell proliferation and tissue repair.
MATERIALS AND METHODS

Peptides and Proteins.

Endotoxin free Bβ15-42 (GHRPLDKKREEAPSLRPAPPPISGGGYR) and random peptide (DRGAPAHRPPRGPIGRSTPEKEKLPG) was custom synthesized (Invitrogen, Carlsbad, CA) with 95% modification and N-terminal amine group addition and free acid modification.

Animals.

Male Wistar rats (280-320 g) and male C56Bl/6 mice (22-25 g) were purchased from Harlan Laboratories (Indianapolis, IN) and Charles River Laboratories (Wilmington, MA) respectively. The animals were maintained in central animal facility over wood chips free of any known chemical contaminants under conditions of 21 ± 1°C and 50-80% relative humidity at all times in an alternating 12 h light-dark cycle. Animals were fed with commercial rodent chow (Teklad rodent diet # 7012), given water ad lib, and were acclimated for 1-week prior to use. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health and were approved by the Harvard Medical School Animal Care and Use Committees (IACUC).

Selection of participants.

Critically ill patients in the intensive care unit with elevated SCr > 1.5 mg/dL were recruited. Causes of acute kidney injury (AKI) or chronic kidney disease (CKD) were obtained by detailed chart review including the treating nephrologist's consultation note.
and evaluation of laboratory data by a co-author not involved in the patients’ care (SSW). Healthy volunteers were recruited from the staff at BWH. Healthy volunteers were excluded if they reported a recent hospitalization, diagnosis of chronic kidney disease, or treatment with nephrotoxic medications (non-steroidal anti-inflammatory drugs were allowed). All participants were patients or employees (healthy volunteers) of Brigham and Women’s Hospital, a tertiary care teaching hospital. The Institutional Review Board approved the protocols for recruitment and sample collection.

**Human Kidney Biopsy Sections.**

Human kidney biopsy sections were obtained through Brigham and Women’s Hospital’s pathology service core, classified as patient without evidence of acute tubular injury (ATI): 58 year old female diagnosed with renal oncocytoa and patient with ATI: 78 year old male diagnosed with active glomerulonephritis with diffuse proliferative and crescentic pattern of injury, active interstitial nephritis, active tubular injury involving focal tubular atrophy and interstitial fibrosis (30 %).

**Experimental design.**

*Rat studies:* For whole genome expression profiling studies, nine male Wistar rats underwent ischemia reperfusion (I/R) surgery and three rats underwent sham surgery simulating I/R. In order to perform I/R surgery, the rats were anesthetized using pentobarbital sodium (30 mg/kg, ip) and renal ischemia was induced by nontraumatic vascular clamps over the pedicles for 20 min as described before

[19,20]. Upon release of the clamps, the incision was closed in two layers with 2-0 sutures. The sham rats
underwent anesthesia and a laparotomy only and were sacrificed after 24 h. The rats in I/R group were further divided in subgroups of three rats each and sacrificed after 6, 24, and 120 h of reperfusion. To confirm the results of gene expression analysis twenty male Wistar rats underwent 20 minutes bilateral I/R surgery and five rats underwent sham surgery as described above and were sacrificed at 6, 24, 72 and 120 h following reperfusion (n=5/timepoint).

Mouse studies: Forty male C57Bl6 wild type mice were anesthetized using pentobarbital sodium (30 mg/kg, ip) and subjected to 27 min of bilateral renal I/R surgery by the retroperitoneal approach. Sham surgery was performed with exposure of both kidneys but without induction of ischemia. Immediately upon the start of reperfusion, 3.6mg/kg of Bβ<sub>15-42</sub> or random peptide were administered intravenously to the mice via tail vein. One ml of warm saline (37°C) was injected ip three hours after surgery for volume supplementation. Mice (n=5-10/group) in the respective groups (sham or I/R administered Bβ<sub>15-42</sub> or random peptide) were sacrificed at 24 and 48 h following reperfusion using overdose of pentobarbital (180 mg/kg, ip).

Blood and Urine Analysis.

At sacrifice, blood was collected from dorsal aorta in heparinized tubes. Serum creatinine (SCr) concentrations were measured using a Beckman Creatinine Analyzer II. Blood urea nitrogen (BUN) was measured spectrophotometrically at 340 nm using a commercially available kit (Thermo Scientific, Rockford, IL) as described before<sup>21</sup>. Urines were collected by placing animals in individual metabolic cages and one ml of RNALater (Ambion, Austin, TX) was added to the tubes to preserve RNA. Urinary Kidney
Injury Molecule-1 (Kim-1 in rats and KIM-1 in humans) was measured using previously established luminex-based assays\textsuperscript{20,22}. Urinary Kim-1 in mice was measured using a luminex-based assay in Dr. Bonventre’s laboratory. Urinary N-acetyl-\(\beta\)-D-galactosaminidase (NAG) was measured spectrophotometrically according to the manufacturers’ protocols (Roche diagnostics, Basel Switzerland). Urinary creatinine concentration was used to normalize biomarker measurements in order to account for the influence of urinary dilution on biomarker concentrations. Fibrinogen in mouse, rat and human urines was measured using commercially available species-specific luminex assay based kit from Millipore (Billerica, MA).

**Whole Genome Expression profiling and hierarchical clustering.**

For genome-wide expression analysis, RatRef-12 bead array (Illumina, San Diego, CA) was used which contains about 22,523 50-mer oligonucleotide probes primarily based on NCBI RefSeq database (Release 16). Gene expression and hybridization array dataset has been submitted to the NCBI Gene expression omnibus. Accession:GSE27274:


Total RNA was extracted from 30 mg of frozen tissue samples using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Integrity of the isolated total RNA was determined by 1 % agarose gel electrophoresis and the RNA concentration was measured by ultraviolet light absorbance at 260 nm using the Nanodrop 2000C spectrophotometer (Thermo Scientific, Rockford, IL). Aliquots of RNA were converted into ds-cRNA and biotinylated using the Illumina TotalPrep RNA
Amplification Kit (Ambion, Austin, TX, USA). The cRNA samples were then labeled with streptavidin-Cy3 and hybridized onto RatRef-12 Expression Beadchip. The image was scanned using the Illumina BeadArray Reader and the data was analyzed by Illumina Beadstudio software (version 3.3.7). For non-redundant 22,523 symbols, the intensity profiles were quantile normalized. We used median absolute deviation (MAD) to select highly variable genes (MAD > 0.4; n = 1571) for subsequent analysis. Hierarchical clustering was performed using 1 – Pearson correlation coefficient as distance with average linkage option.

**Real Time-Polymerase Chain Reaction (RT-PCR).**

The isolated RNA was treated with Quantitect Reverse Transcription kit (Qiagen Sciences, Germantown, MD). Real Time PCR of the tissue samples was performed with Quantifast SYBR Green (Qiagen Sciences, Germantown, MD) using CFX96 RT-PCR instrument (Biorad, Hercules, CA)\(^1\). Primers were designed to amplify 120-150 base pair fragment with the following cycle conditions: 95 °C for 3 min, the following steps were repeated 40 times: 95 °C for 30 sec, 55 °C for 30 sec. Forward and reverse primer sequences for rat and mouse specific genes were designed using MacVector software (MacVector Inc., Cary, NC) and are listed in table 2.

**Immunofluorescence staining.**

Kidney tissues were perfused with cold PBS before harvesting and then fixed in formalin for 16 h and embedded in paraffin. The sections incubated overnight at 4 °C in rabbit monoclonal anti-Fibrinogen alpha (Epitomics, Burlingame, CA), rabbit polyclonal
anti-Fibrinogen beta (ProteinTech Group, Chicago, IL), rabbit polyclonal anti-Fibrinogen gamma (ProteinTech Group, Chicago, IL), anti rat fibrinogen (Nordic Immunological Laboratories, Tilburg, The Netherlands), anti human fibrinogen (Sigma-Aldrich, St. Louis, MO) and rabbit monoclonal anti-Ki67 (Vector Laboratories, Burlingame, CA). The primary antibody was detected using goat anti-rabbit Cy3 labeled and donkey anti goat Cy3 labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). DAPI (Sigma Aldrich, St. Louis, MO) was used for nuclear staining. The tissue sections were mounted using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). The images were captured at 100X for rats, 60X for mice and humans using NIKON Eclipse 90i fluorescence microscope.

**TUNEL assay.**

Apoptosis was measured in kidney tissues by TUNEL assay using the In Situ Cell Death detection kit (Roche Applied Science, Indianapolis, IN) according to manufacturer’s instructions21.

**In vitro experimental design.**

The renal tubular epithelial cell line, LLC-PK1, established from pig renal cortex was obtained from ATCC (Manassas, VA) and maintained in DMEM containing 10% FBS. Two thousand five hundred LLC-PK1 cells were plated in 96 well plate for 24 h in DMEM 10 % fetal bovine serum (FBS) at which time they formed a 50 % confluent monolayer in the well. They were pretreated with 6 μM of β15-42 or random peptide for 6 h and were immersed in 100 μl of mineral oil on top of DMEM medium without any
serum for 6 h. This oil immersion simulates in vivo ischemic conditions by restricting cellular exposure to oxygen and nutrients as well as limiting metabolite washout\textsuperscript{23}. After 6 h, the mineral oil was removed and cells were incubated with 6 \( \mu \text{M} \) of \( \text{B}\beta_{15-42} \) or random peptide for 48 h in serum free conditions. Bromodeoxyuridine (5-bromo-2-deoxyuridine (BrdU)) was measured as an index of cell proliferation by incubating cells with BrdU for 2 h before harvesting and the absorbance was quantified using a spectrophotometer at 450 nm wavelength as per manufacturer’s instructions (Millipore, Bellerica, MA). Absorbance obtained from untreated cells was taken as 100\% \((n=6 \text{ wells/group})\) and the experiment was repeated twice.

**Statistics.**

Data are expressed as average + standard error. Statistical difference \((p<0.05)\) as calculated by one way ANOVA or student’s t-test. \(P<0.05\) was considered significant and represented by ‘\(*\)’ where applicable. All graphs were generated by GraphPad Prism (GraphPad, Inc., La Jolla, CA). Diagnostic performance (i.e., the ability of a urinary biomarker to identify kidney injury) was assessed by evaluating sensitivity and specificity using the receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) and 95\% confidence interval (CI) were calculated using the non-parametric method\textsuperscript{22}. The AUC for a diagnostic test ranges from 0.5 (no better than chance alone) to 1.0 (perfect test, equivalent to the gold standard). Statistical analyses were performed with MedCalc for Windows, version 11.5 (Mariakerke, Belgium).
RESULTS

mRNA expression of α, β and γ chains of fibrinogen (Fg) increases in the kidney following ischemia reperfusion injury.

In the quest to identify early genes modulating kidney injury and repair process we conducted gene expression profiling in the cortex and medulla of rat kidney following 20 minutes bilateral renal ischemia/reperfusion (I/R). This reversible model of kidney injury results in elevated kidney dysfunction [measured by serum creatinine (SCr) and blood urea nitrogen (BUN)] (supplementary Fig. 1A) and proximal tubular injury [measured by kidney injury molecule-1 (Kim-1) mRNA levels and histopathological findings characterized by proximal tubular necrosis and apoptosis] (supplementary Fig. 1B and 1C) at 24 h of reperfusion followed by recovery at 120 h. We selected highly variable genes (median absolute deviation > 0.4; n = 1571) and performed hierarchical clustering to investigate their co-expression pattern during kidney regeneration after ischemic injury (Fig. 1A). The selected genes include the previously identified candidate genes lipocalin-2 (LCN2) \(^24\), clusterin (CLU) \(^25\), tissue inhibitor of metalloproteinase-1 (TIMP1) \(^26\) and kidney injury molecule-1 (Kim-1) \(^20\). While the up regulation of LCN2 and CLU were more dominant in renal medulla compared to cortex, TIMP1 and Kim-1 were up regulated both in cortex and medulla at 24 hr after ischemic injury. We observed a local cluster of genes that includes Fgβ and Fgγ chains (arrow in Fig. 1A) whose expression pattern is similar to Kim-1, i.e., clear up regulation after 24hr of ischemic injury both in cortex and medulla. The probe for Fgα chain is absent in the RatRef-12 chip from Illumina. Therefore, we further evaluated the expression profile of Fgα, Fgβ and Fgγ chains by real-time PCR (RT-PCR) in kidney (cortex and medulla) (Fig. 1B),
liver (Fig. 1C), lung, spleen and heart (supplementary Fig. 2) over time and we found that there was a modest elevation (3-fold) of \( Fg\alpha \) and \( Fg\gamma \) chains in the liver at early time points (Fig. 1C), whereas, there was no significant change in the mRNA levels of any of the chains in lung, spleen and heart (supplementary Fig. 2). The medulla showed significantly higher expression of all three chains as compared to cortex with the highest up regulation after 72 h of reperfusion (\( Fg\alpha \) chain-14 fold, \( Fg\beta \) chain-50 fold and \( Fg\gamma \) chain-10 fold) (Fig. 1B).

**Immunoreactivity of \( Fg\alpha \), \( Fg\beta \) and \( Fg\gamma \) chains in the kidney.**

Immunostaining, to evaluate the cellular expression profile of Fg whole protein and \( Fg\alpha \), \( Fg\beta \) and \( Fg\gamma \) chains, revealed immunoreactive protein for all three chains and Fg whole molecule in renal tubular cells. Positive staining was observed using the anti-\( Fg\beta \) chain and anti-Fg whole molecule antibodies in the interstitial spaces, indicative of extracellular Fg. In sham kidneys (Fig. 2Aa), the \( Fg\alpha \) chain is expressed with fine granular cytoplasmic reactivity and more pronounced expression at the peak of injury by 24 h (Fig. 2Ab). \( Fg\alpha \) chain immunoreactive protein, as detected by the monoclonal antibody, continued to be expressed in the proximal tubular epithelial cells and in the glomeruli throughout the time course of injury (Fig. 2Ac). \( Fg\beta \) chain immunoreactive molecules in the uninjured kidneys, assessed by a polyclonal antibody against \( Fg\beta \) chain, showed focal reactivity in the renal interstitium (Fig. 2Ad). At the peak of injury by 24 h, \( Fg\beta \) immunoreactivity distinctly outlined the peritubular capillaries (Fig. 2Ae) and a small proportion of tubular epithelial cells expressed the \( Fg\beta \) chain immunoreactive component in their cytoplasm. By 72 h, intense, irregular and coarse distal tubular staining (Figs. 2Af) and a distinct luminal outline along proximal tubules featured. The
Fgγ chain staining, assessed by a polyclonal antibody against Fgγ chain, was primarily located in the distal tubules and collecting ducts with a diffuse cytoplasmic distribution that gravitated along the basolateral side in uninjured kidneys (Fig. 2Ag). At the peak of injury by 24 h, the Fgγ chain immunoreactive protein stained in a coarse granular pattern, distributed centrally in the cytoplasm in the distal tubules and collecting ducts (Fig. 2Ah). By 24 h, the Fgγ chain in the cortex was confined towards the apical side of the proximal tubules while in the medulla, the cellular debris of injured S3 segments non-specifically stained for the Fgγ chain as well. By 72 h, Fgγ chain immunoreactive proteins showed a mixed pattern that resembled sham and 24 h injured kidneys in the staining and distribution patterns (Fig. 2Ai). The expression of fibrinogen whole molecule was identified in a linear pattern along the apical surface of epithelial cells (Figs. 2Aj to 2Al) as well as along the glomerular basement.

A consistent pattern of increased expression of Fg and its chains was observed in human kidney biopsy sections obtained from patients pathologically diagnosed with acute tubular injury (ATI) as compared to patient without evidence of ATI (Fig. 2Ba to 2Bh). All three chains along with Fg were present in the interstitium in both ATI and non-ATI patients in addition to which, Fgγ chain (Fig. 2Bf) and Fg (Fig. 2Bh) was predominantly expressed on the apical side of the tubules in the ATI patient.

**Increased urinary levels of Fg in rats and humans serve as a potential biomarker for acute kidney injury.**

We hypothesized that if Fg was secreted into the urine upon injury, then urinary Fg may serve as a biomarker for kidney injury. Following 20 min bilateral renal I/R injury in
rats, we observed ~100-fold increase in urinary Fg concentration as early as 6 h (Fig. 3Aa) that remained higher than baseline till day 5 (~ 4 fold) following reperfusion, correlating with proximal tubular necrosis as assessed by histopathologic injury (supplementary Fig. 1C) and elevated urinary N-acetyl-β-D-glucosaminidase (NAG) (Fig. 3Ab) as well as urinary kidney injury molecule-1 (Kim-1) (Fig. 3Ac). There was no increase in plasma Fg (supplementary Fig. 3) levels after sham or kidney I/R injury as compared to rats that did not undergo sham or I/R surgery. To evaluate the performance of urinary Fg in distinguishing healthy volunteers against patients with chronic kidney disease (CKD) and/or acute kidney injury (AKI), urinary Fg was measured in 25 patients admitted to the intensive care unit with abnormal serum creatinine (> 1.5 mg/dL) with established kidney damage from a variety of causes and 25 healthy volunteers. We also compared urinary Fg against two other well-studied AKI or CKD biomarkers, NAG and KIM-1. Demographic and clinical data are shown in table 1. Median urinary concentration of Fg was significantly higher in patients with AKI and CKD than in healthy volunteers (p < 0.001) (Fig. 3B) and corresponded with the increased levels of urinary NAG and KIM-1 (supplementary Fig. 4). The diagnostic ability of urinary Fg to distinguish between patients with AKI or CKD versus patients without kidney injury was 0.98 as calculated by area under the receiver operating characteristic curve (ROC) (Fig. 3C).

**Fibrinogen Bβ15-42 protects the kidney against ischemia-reperfusion (I/R) injury.**

Given that in our model Fgβ chain was the highest up regulated gene following kidney injury amongst the three chains (Fig. 1B) and the fact that exogenous Bβ15-42
peptide administration has been shown to protect against myocardial I/R injury\textsuperscript{27-29} and lung injury\textsuperscript{30}, we next evaluated the therapeutic potential of Bβ\textsubscript{15-42} peptide in renal I/R injury. Bβ\textsubscript{15-42} or random peptide (3.6 mg/kg) was administered intravenously (iv) 1 min after reperfusion following 27 min bilateral renal I/R injury in C57BL/6 mice (n=5 to 10/group). A significant reduction in the infarct size and vascular congestion (outlined by white dots) (Fig. 4A) was observed. Approximately 50 % reduction in kidney dysfunction [measured by serum creatinine (SCr), blood urea nitrogen (BUN)] and kidney proximal tubular injury [measured by urinary levels of kidney injury molecule-1 and Fg] (Fig. 4B), and a significant decrease in proximal tubular damage in the outer stripe of outer medulla [histopathological evaluation of H & E stained kidney sections] (Fig. 4C) was recorded at 24 h after I/R injury in the mice administered Bβ\textsubscript{15-42} peptide as compared to random peptide. The kidney injury and dysfunction parameters appeared to decrease by 48 h suggesting the onset of a complete structural and functional recovery in both groups.

**Decreased apoptosis and increased tissue repair in the kidneys of Bβ\textsubscript{15-42} treated mice as compared to mice treated with random peptide following ischemia-reperfusion (I/R) injury.**

To elucidate the mechanism of Bβ\textsubscript{15-42}-induced protection in I/R mice, we measured candidate markers of inflammation, leukocyte infiltration, apoptosis and proliferation in kidney tissues over time. There was no difference in mRNA levels of inflammatory cytokines (IL-1β, IL-6, IL-10, TNFα, ICAM), or macrophage marker (CD68) between the Bβ\textsubscript{15-42} and random peptide treatment groups (supplementary Fig. 5). Similarly
leukocyte infiltration (measured by myeloperoxidase staining) also appeared to be similar between the two groups (supplementary Fig. 6). The number of TUNEL positive apoptotic cells in the renal medulla was similar at 24 h (Fig. 5a & c). However, at 48 h there was a significant decrease in apoptosis (p<0.05) in the mice administered Bβ15-42 as compared to random peptide (Fig. 5b & d). Interestingly, a significant number of cells appeared to be in a proliferative state (Ki67 positive) in the renal medulla at 48 h following administration of the Bβ15-42 peptide as compared to random peptide administration (Fig. 5g). *In vitro* experiments using proximal tubular epithelial cells (LLC-PK1) mimicked the *in vivo* findings in demonstrating a protective effect of Bβ15-42 from hypoxic injury by stimulating renal epithelial cell proliferation (supplementary Fig. 7) suggesting that the Bβ15-42 peptide promotes an efficient resolution of ischemic injury by inducing rapid tissue regeneration response, thereby decreasing the necrosis and apoptosis in the kidney.
DISCUSSION

Although it has been recognized that progressive kidney disease is characterized by gradual deterioration of the renal endothelium, which correlates with the development of tubulointerstitial injury, fibrosis and glomerulosclerosis \(^3\) there has been little effort to characterize the regulatory role of blood proteins in pathophysiology of AKI. Here we show that: i) in whole genome expression profiling studies Fgβ and Fgγ chains are amongst the highly up regulated genes after 24hr of ischemic injury both in kidney cortex and medulla; ii) Fg could serve as an effective safety and efficacy biomarker for kidney injury not only because of the marked increase in urinary Fg following kidney damage (Fig. 3), but also due to their reduced levels upon Bβ\(_{15-42}\) peptide mediated protection from kidney injury (Fig. 5) demonstrating responsiveness to both injury and recovery; iii) Fg derived Bβ\(_{15-42}\) administration protects mice from I/R induced kidney injury by aiding kidney tissue repair thus demonstrating for the first time its therapeutic potential in AKI. These findings not only highlight the important role of Fg in renal tissue injury and repair, but also offer a therapeutic strategy to enhance kidney regeneration as opposed to simply preventing further injury or deleterious inflammation in the damaged tissue.

The presence of Fgα, Fgβ, and Fgγ chain transcripts in the kidney at baseline \(^1^1\) as well as its up regulation following nephrotoxicity \(^3^1\) or brain death induced vascular endothelial activation in kidneys \(^1^2,3^2\) has been observed before. Here we characterized the cellular expression patterns of Fg and its individual chains in the renal tubule following injury. Because we used polyclonal antibodies for immunostaining of the Fgβ and Fgγ chain respectively, there is no way to distinguish between the intact fibrinogen
versus intermediates such as alpha/gamma, beta/gamma, or the half-molecule of one each alpha/beta/gamma polypeptide chains. However, we found distinct expression patterns of Fgα, Fgβ, and Fgγ chains in the renal tubular epithelial cells, glomeruli and interstitium at baseline and during the regeneration in the injured kidney. The increased Fg expression following injury can potentially be a consequence of plasma leakage due to organ damage, as seen after spinal cord injury\textsuperscript{33}, but the observation of detectable transcript levels of Fgα, Fgβ, and Fgγ chains (Fig. 1) and corresponding immunoreactivity of all three chains as well as whole Fg molecule in sham rats and in patient without any evidence of tubular injury (Fig. 2) suggests that the protein could be potentially synthesized and assembled in the kidney.

Plasma Fg has been associated with vascular disease in numerous epidemiological studies\textsuperscript{34}. Although we did not find any increase in plasma Fg levels following I/R injury (SFig. 3), urinary Fg levels increased massively as early as 6 hours following I/R injury that decreased over time, but remained modestly elevated during the resolution phase of injury (Fig. 3). In 1974, Naish et al\textsuperscript{35} reported higher levels of urinary fibrin degradation products (FDP) in patients with glomerulonephritis. Subsequently, urinary FDP were shown to be able to make a diagnosis of 25 out of 26 acute rejection episodes at least 24 h before deterioration in renal function and the elevation of FDP preceded the rise in NAG in 9 out of 11 patients.\textsuperscript{36} Consistent with these published reports, we show in a cross sectional study of individuals with and without kidney damage that urinary Fg performed very well in differentiating between patients with and without AKI/CKD with ROC of 0.98. The urine samples were obtained well after the diagnosis of kidney injury was made, and therefore this study does not address the
issue of early diagnosis prior to a rise in SCr. However, it does suggest that the sensitivity and specificity of urinary Fg was comparable to the other more advanced biomarkers of AKI such as NAG and KIM-1\textsuperscript{19,20,22,37}, and further warrants an investigation to evaluate the comparative efficacy and temporal pattern of urinary Fg excretion with other AKI/CKD biomarkers in longitudinal studies. Urinary fragments of Fg\textbeta\textsc{b} (ROC of 0.85) and Fg\textalpha chains (ROC of 0.74) were found to distinguish patients with malignant vs. benign ovarian cancer\textsuperscript{38} and plasma levels of Fg\gamma' chain (a splice variant of Fg\gamma chain) associated with a number of traditional cardiovascular risk factors in Framingham Offspring cohort.\textsuperscript{39} The current assay is a sandwich ELISA based luminex assay using two polyclonal antibodies against Fg protein and therefore it will be interesting to use a more targeted approach like liquid chromatography–multiple reaction monitoring/mass spectrometry (LC-MRM/MS) to identify whether there is a predominant excretion of Fg\textbeta chain polypeptides in the urine following kidney injury that would correlate with the highest Fg\textbeta chain mRNA levels in the kidney.

We tested the therapeutic efficacy of Fg\textbeta chain derived peptide (B\textbeta\textsubscript{15-42}) in mice subjected to bilateral renal I/R injury and found that B\textbeta\textsubscript{15-42} substantially reduces acute tubular injury. Others have investigated the role of Fg during kidney damage using the Malayan pit viper venom, ancrod, and found that it provided remarkable protection in experimental allergic glomerulonephritis in rabbits\textsuperscript{40}. This provides an indirect evidence for protective role of B\textbeta\textsubscript{15-42} because ancrod reduces plasma levels of Fg and simultaneously increases fibrinogen degradation products such as the bioactive polypeptide (B\textbeta\textsubscript{15-42}).\textsuperscript{41} B\textbeta\textsubscript{15-42} is a naturally occurring, 28 amino acid long product, cleaved from fibrin fragments and at suprapharmacological doses, it has shown to
protect from myocardial infarction\textsuperscript{28} and acute lung injury\textsuperscript{30,42} in animal models. In a multicentered phase IIa clinical trial with Bβ\textsubscript{15-42} peptide administration, successful protective effects were seen in patients with acute myocardial infarction, whose endothelial barrier integrity had not been compromised\textsuperscript{27,29}. The peptide has also been shown to be vasculoprotective in models of vascular leak in a Fyn-dependent pathway\textsuperscript{42}. Bβ\textsubscript{15-42} has been shown to mediate platelet spreading, proliferation, capillary tube formation and Von Willebrand Factor release and has a binding site for heparin\textsuperscript{42,43}. Bβ\textsubscript{15-42} also has low affinity interactions with VE-Cadherin, efficiently disrupting the interaction between Fg with its receptors VE-Cadherin on endothelial cells, thereby stabilizing endothelial barriers, which in turn elicits beneficial anti-inflammatory properties\textsuperscript{28,42}.

Here we show a unique mechanism of Bβ\textsubscript{15-42} peptide mediated protection \textit{in vivo} and \textit{in vitro} that results in increased proliferation of renal tubular epithelial cells resulting in decreased necrosis and apoptosis following damage (Fig. 5). The fact that amongst the three chains of Fg, Fgβ chain transcript levels increase the highest (~ 50 fold) at 72 h (Fig. 1B) which is the peak of regeneration in this model \textsuperscript{44} further underscores the finding that Fg is up regulated in the kidney as a protective mechanism to aid in regeneration. This result would be consistent with the studies conducted thus far suggesting that Fg appears to be most important in tissue repair when cells must infiltrate and organize fluid-filled areas such as internal dead space created by injury\textsuperscript{15}. In a cutaneous wound healing model Fg deficient (Fg\textsuperscript{-/-}) mice revealed an abnormal pattern of tissue repair including misguided epithelium, delayed wound closure, reduced tensile strength and diminished ability to organize a dead space\textsuperscript{15}. Fg has been shown
to have multiple binding partners such as β3 integrin \(^4^5\) intracellular adhesion molecule-1 (ICAM) \(^4^6\), regulating tissue repair process in neurons and lung epithelial cells\(^4^7\). Given that both β3 integrin and ICAM-1 expression on renal epithelial cells increases following kidney injury\(^4^8-5^0\), it remains to be investigated whether Fg or the B\(β_{15-42}\) peptide aids in renal epithelial proliferation and extracellular remodeling via one of the known ligands and pathways. Kidney regeneration, after an episode of AKI, is a major determinant of outcome for patients with AKI and therefore the use of B\(β_{15-42}\) peptide offers a novel therapy to improve the rate or effectiveness of the tissue repair process after ischemic kidney damage.

In summary our study provides evidence that Fg may function as a key molecular link between tubulo-vascular damage and regeneration in the kidney and provides new opportunities for the use of Fg in diagnosis, prevention, and therapeutic interventions in kidney disease.
ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTIONS

A.K., A.K.A, V.S.V. designed research; A.K., A.K.A, D.H., T.K., V.R., G.C., performed research; N.A.B. and S.S.W. contributed bio specimens from rodents and humans; T.K. provided bioinformatics support and data analysis; A.K., A.K.A., D.H., S.S.W. and V.S.V. analyzed data; and A.K. and V.S.V. wrote the paper. The authors have no conflict of interest to disclose.
REFERENCES

Table 1. Demographic and clinical characteristics of human subjects

<table>
<thead>
<tr>
<th></th>
<th>Acute kidney injury (AKI) or Chronic kidney disease (CKD) (N=25)</th>
<th>Healthy volunteers** (N=25)</th>
</tr>
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<tbody>
<tr>
<td>Mean age*, years, + SD</td>
<td>64.8 + 19.5</td>
<td>35.6 + 10.7</td>
</tr>
<tr>
<td>Female†</td>
<td>64%</td>
<td>68%</td>
</tr>
<tr>
<td>Black‡</td>
<td>20%</td>
<td>16%</td>
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<tr>
<td>Cause of elevated serum creatinine (SCr)</td>
<td>AKI from shock or sepsis (72%), obstruction (4%), multifactorial (12%), pre-renal (4%), CKD (8%)</td>
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</tr>
<tr>
<td>Mean (SD) peak SCr</td>
<td>4.5 (4.7) mg/dL</td>
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</tbody>
</table>

* P < 0.001
† P = 0.73
‡ P = 0.68
** Healthy volunteers were excluded if they reported a diagnosis of chronic kidney disease; serum creatinine was not measured.
Table 2. Real Time-PCR primers used for the quantification of mRNA expression levels in the study.

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>CD68</td>
<td>F</td>
<td>TCTTTCTCCAGCTGTTCACC</td>
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<td></td>
<td>R</td>
<td>ATGATGAGAGGAGCAAGAG</td>
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<td>Gapdh</td>
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<td>ICAM1</td>
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<td>IL-1β</td>
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<td>Mouse Fgα chain</td>
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<td></td>
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<td>Mouse Kim-1</td>
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<td></td>
<td>R</td>
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FIGURE LEGENDS

Figure 1: Fibrinogen (Fgα, Fgβ and Fgγ chains) is significantly up regulated in kidney cortex and medulla of male Wistar rats following 20 minutes bilateral renal ischemia/reperfusion injury as compared to sham surgery. A) A heatmap shows the expression levels of the most variable 1571 genes, which were selected from 22,523 transcripts (See Methods). Co-expressed genes are grouped by hierarchical clustering. The samples are presented in order of time post ischemic injury (0hr, 6hr, 24hr, and 120hr; triplicate for each). B) Real time PCR analysis in kidney and C) liver tissues for Fgα, Fgβ and Fgγ chains, normalized using a housekeeping gene (Gapdh), and fold change determined over sham group. (n=5/group). * represents p<0.05 as determined by one way ANOVA in comparison with sham rats.

Figure 2: Fibrinogen (immunoreactive Fgα, Fgβ and Fgγ chains) protein is expressed in the kidney of rats and humans. A) Representative formalin fixed paraffin embedded kidney tissue sections of sham male Wistar rats and those undergone 20 min bilateral ischemia reperfusion and stained for immunoreactive proteins to Fgα (a to c), Fgβ (d to f), Fgγ (g to i) and Fg (j to l) following 24 and 72 h of reperfusion and compared with healthy (sham) kidneys. Bar represents 50μm. B) Representative formalin fixed human biopsied kidney sections of AKI and non-AKI patients, stained for immunoreactive molecules recognizing Fgα (a, b), Fgβ (c, d), Fgγ (e, f) and Fg (g, h). Bar represents 50μm. Arrowheads indicate respective immunoreactive molecules. Asterisk in Fig. 2 (g) and (i) mark tubules with similar expression pattern along the basolateral side of respective tubules.
Figure 3: A significant increase in urinary fibrinogen after kidney injury in rats and humans.

A) Urinary fibrinogen (Fg) was compared with tubular injury biomarkers N-acetyl-β-glucosaminidase (NAG) and kidney injury molecule-1 (Kim-1) in rats subjected to 20 minutes bilateral renal ischemia/reperfusion injury (n=5/group). * represents p<0.05 as determined by Student’s t test in comparison with sham rats. B) Urinary Fg was measured in a human cross-sectional study with clinically established multifactorial AKI (n=25) versus healthy volunteers (n=25). Magenta line and corresponding number marked by arrow indicates a threshold cut off value at 95% specificity. C) Receiver Operating Curve (ROC) comparing the sensitivity and specificity of urinary Fg, NAG and KIM-1 to distinguish patients with acute kidney injury (AKI) or chronic kidney disease (CKD) from healthy volunteers.

Figure 4: Bβ15-42 peptide protects mice from renal ischemia/reperfusion (I/R) injury. Male C57Bl6 mice were subjected to 27 minutes bilateral renal ischemia/reperfusion injury or sham surgery and 3.6 mg/kg of Bβ15-42 peptide or random peptide was administered intravenously 1 min following reperfusion. A) The infarct size following ischemia was significantly smaller in the Bβ15-42 peptide administered mice compared to mice administered random peptide. Outline of infarcts are traced by white dots. B) Serum creatinine (SCr), blood urea nitrogen (BUN) as indicators of renal dysfunction and urinary levels of fibrinogen (Fg) and kidney injury molecule-1 (Kim-1) as indicators of kidney injury was measured at 24 and 48 h in all the groups. (n=5/group of
sham, n=10/ group at 24 hours and n=5/ group at 48 hours).* represents p<0.05 as determined by one way ANOVA in comparison with sham mice. C) Representative histology sections comparing sham, Bβ_{15-42} and random peptide administered groups of mice at 24 and 48 h post ischemia. Bar represents 100μm.

Figure 5: Bβ_{15-42} peptide aids in the resolution of injury by decreasing necrosis/apoptosis and inducing rapid tissue regeneration.

Paraffin embedded kidneys of mice subjected to 27 min bilateral ischemia/reperfusion that were administered either Bβ_{15-42} or random peptides were compared at 24 and 48 h for A) number of apoptotic cells (green) by TUNEL assay (bar represents 100μm). Arrowheads indicate TUNEL positive nuclei. B) The number of proliferative cells determined by Ki67 positive staining cells (red) (bar represents 50μm). The numbers of positive staining TUNEL and Ki67 nuclei are represented graphically on the right of respective photomicrographs. Arrowheads indicate Ki67 positively stained nuclei. * represents p<0.05 as determined by Student’s t-test between the two groups within the same time point.
FIGURE 1

A  Hours post bilateral I/R

B  KIDNEY

C  LIVER

Fg α/Gapdh

Cortex

Medulla

Fg β/Gapdh

Fg γ/Gapdh

Hours post bilateral I/R

Hours post bilateral I/R

*
FIGURE 5

Hours post bilateral I/R

<table>
<thead>
<tr>
<th>Peptide administered</th>
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<th>48</th>
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<tr>
<td>Bβ₁₅₋₄₂</td>
<td><img src="a.png" alt="Image" /></td>
<td><img src="b.png" alt="Image" /></td>
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<td>Random</td>
<td><img src="c.png" alt="Image" /></td>
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<tr>
<td>Bβ₁₅₋₄₂</td>
<td><img src="f.png" alt="Image" /></td>
<td><img src="g.png" alt="Image" /></td>
</tr>
<tr>
<td>Random</td>
<td><img src="h.png" alt="Image" /></td>
<td><img src="i.png" alt="Image" /></td>
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</tbody>
</table>

**e**

![Graph](e.png)

**j**

![Graph](j.png)
Fibrinogen β-derived Bβ15-42 peptide protects against kidney ischemia/reperfusion injury

Aparna Krishnamoorthy, Amrendra Kumar Ajay, Dana Hoffmann, Tae-Min Kim, Victoria Ramirez, Gabriela Campanholle, Norma A. Bobadilla, Sushrut S. Waikar and Vishal S. Vaidya