

Kidney Injury Molecule-1 Is Up-Regulated in Renal Epithelial Cells in Response to Oxalate *In Vitro* and in Renal Tissues in Response to Hyperoxaluria *In Vivo*

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Abstract

Oxalate is a metabolic end product excreted by the kidney. Mild increases in urinary oxalate are most commonly associated with Nephrolithiasis. Chronically high levels of urinary oxalate, as seen in patients with primary hyperoxaluria, are driving factor for recurrent renal stones, and ultimately lead to renal failure, calcification of soft tissue and premature death. In previous studies others and we have demonstrated that high levels of oxalate promote injury of renal epithelial cells. However, methods to monitor oxalate induced renal injury are limited. In the present study we evaluated changes in expression of Kidney Injury Molecule-1 (KIM-1) in response to oxalate in human renal cells (HK2 cells) in culture and in renal tissue and urine samples in hyperoxaluric animals which mimic *in vitro* and *in vivo* models of hyper-oxaluria. Results presented, herein demonstrate that oxalate exposure resulted in increased expression of KIM-1 mRNA as well as protein in HK2 cells. These effects were rapid and concentration dependent. Using *in vivo* models of hyperoxaluria we observed elevated expression of KIM-1 in renal tissues of hyperoxaluric rats as compared to normal controls. The increase in KIM-1 was both at protein and mRNA level, suggesting transcriptional activation of KIM-1 in response to oxalate exposure. Interestingly, in addition to increased KIM-1 expression, we observed increased levels of the ectodomain of KIM-1 in urine collected from hyperoxaluric rats. To the best of our knowledge our studies are the first direct demonstration of regulation of KIM-1 in response to oxalate exposure in renal epithelial cells *in vitro* and *in vivo*. Our results suggest that detection of KIM-1 over-expression and measurement of the ectodomain of KIM-1 in urine may hold promise as a marker to monitor oxalate nephrotoxicity in hyperoxaluria.

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Introduction

Hyper-oxaluria, either as a result of inherited metabolic disorders or many other intrinsic and extrinsic factors, is one of the major risk factors for developing kidney stones [1,2]. Studies with animal models as well as tissue culture model systems, have demonstrated injury to the epithelial cells of the kidney in presence of oxalate and/or calcium oxalate crystals. Persistently high concentrations of Oxalate in the body can lead to end stage renal failure, severe systemic oxalosis and can finally result in premature death of most of the patients [3]. Current diagnostic methods depend on the estimation of concentrations of Oxalate and Glycolate in the urine, however, the accuracy of utilizing these measures is misleadingly low, especially in end stage renal failure [4]. Monitoring tubular injury in these conditions may offer a more reliable measure of severity of the disease.

In 1994, we were the first group to note that oxalate renal cell interactions involved alterations in gene expression [5]. Over the past two decades, studies [5–12] have demonstrated that oxalate interactions with renal epithelial cells result in a program of events,

including changes in gene expression and cell dysfunction, consistent with cellular stress.

Oxalate a metabolic end product is freely filtered at glomerulous and undergoes bi-directional transport in the renal tubules. We demonstrated for the first time that oxalate exposure to the renal cells results in plethora of changes, including changes in gene expression, re-initiation of DNA synthesis, and cell death [5]. Over the years others and we have shown that exposure to Oxalate results in activation of many different pathways, gene expression changes and initiation of DNA synthesis in the epithelial cells [6–16]. Studies in our laboratory have focused on changes in signal transduction pathways mediated by JNK and p38 MAPK upon Oxalate exposure [7,17]. Even though extensive studies have been attempted to understand cellular changes occurring as a result of hyperoxaluria, early detection of Oxalate exposure still remains a challenge. The need for a reliable and easily detectable early marker for tubular injury as a result of hyper-oxaluria has not been answered yet.

Several recent studies have suggested the usefulness of Kidney Injury Molecule -1 (KIM-1) as an early indicator for renal injury

resulting from chemical toxicity or ischemic injury [18,19]. KIM-1 was first identified as a putative cell adhesion molecule that can recognize and induce phagocytosis of dead cells in the tubular lumen of the kidney [20]. Expression of KIM-1 is up-regulated during tubular injury, but most importantly, the stable ectodomain is cleaved from the membrane anchor and can be detected in the urine. This phenomenon has been observed in rodent and human models of nephrotoxicity including in Renal Cell Carcinoma [21]. KIM-1 has been suggested to be an ideal biomarker in many chemical and pathological nephrotoxicity models due to its robust and marked expression in the injured state. Moreover, there is a total lack of its expression in healthy kidneys [22].

In the present study, we determined the effect of hyper-oxaluria on the expression status and shedding of KIM-1. We used both *in vitro* and *in vivo* methods that are well documented as models for experimental hyperoxaluria for these studies. For *in vitro* studies, treating cells in tissue culture with different concentrations of Sodium Oxalate or COM-Crystals has been established as an efficient model system to test the effects of hyper-oxaluria [6–16]. In this study, HK-2 cells [23], a line of immortalized human proximal tubular epithelial cells, were exposed to oxalate for different time points. Administration of Ethylene Glycol either alone or in combination with Ammonium Chloride or Vitamin D as an efficient model in generating experimental hyper-oxaluria in rats [24–26]. Boeve *et al* [25], identified nephrotoxic effects of Ammonium Chloride independent of Ethylene Glycol and though higher doses of ethylene Glycol may be toxic to rodents, 0.75% Ethylene Glycol has been successfully used for experimental hyper-oxaluria studies without deleterious effects [26]. In this study, Male Wistar rats, with an average weight about 150 gm, were supplied drinking water with 0.75% Ethylene Glycol over 4 week duration to induce hyper-oxaluria.

Results from both the *in vivo* and *in vitro* studies indicate that hyperoxaluria increases the expression of KIM-1 mRNA and protein in renal cells and tissues. Moreover, the ectodomain of KIM-1 was also identified in the urine collected from hyper-oxaluric rats by Western blot analysis. This study, thus, suggests that KIM-1 may be a potential urinary biomarker for oxalate mediated renal injury.

Results

KIM-1 mRNA and protein levels are increased in HK-2 cells upon Oxalate exposure

Oxalate exposure has been shown to induce cellular injury in epithelial cells and one of the markers for tubular injury is the over-expression and shedding of Kidney Injury Molecule (KIM-1) [20]. To study the effect of Oxalate exposure on KIM-1 expression, we incubated HK-2 cells in the presence of oxalate for various time points and looked at the mRNA and protein levels of KIM-1. Results presented in Figure 1a suggest that there is a significant increase in the mRNA levels of KIM-1 in as little as 30 min after HK-2 cells are exposed to Sodium Oxalate. This increase in the mRNA levels is sustained over a period of more than 4 hours of Oxalate exposure. Western blot analysis indicated that the KIM-1 protein levels are also elevated 2 hours post Oxalate exposure (Figure 1b). The increase is highly significant as the control cells do not have any detectable KIM-1 protein. These results suggest that oxalate exposure triggers transcriptional up-regulation of KIM-1 gene expression in renal epithelial cells and this increase occurs very early in response to oxalate exposure.

Hyperoxaluria increased Kidney Injury Molecule (KIM-1) expression in rats

Since we observed increased KIM-1 expression upon Oxalate exposure *in vitro* using proximal tubular epithelial cells, we extended the study to an *in vivo* model for hyper-oxaluria. Wistar rats were given 0.75% Ethylene Glycol in drinking water to develop hyper-oxaluria. Immuno-histochemical staining using the antibody that binds to the C-terminal region of KIM-1 suggested that the expression of the protein is significantly higher in hyper-oxaluric rat kidney in as little as 1 week (Figure 2). Regardless of the duration of hyper-oxaluric exposure, the levels of KIM-1 protein are higher compared to the control rats.

RT-PCR analysis to determine the mRNA levels of KIM-1 also revealed significantly higher mRNA levels in rats exposed to Ethylene Glycol for as little as 1 week compared to control (Figure 3a). Densitometry analysis also suggested that the relative amounts of KIM-1 mRNA remain elevated in the treatment group at least up to three weeks, suggesting that there is sustained damage to the kidneys. To confirm that the increased mRNA levels lead to increased protein amounts, we performed western blot analysis using kidney tissue collected from control and hyper-oxaluric rats. Results presented in Figure 3b show increased levels of KIM-1 total protein in kidney tissue collected from hyper-oxaluric rats. Moreover, KIM-1 protein levels are consistently higher in hyper-oxaluric rats compared to control as long as 3 weeks beginning from the first week of Ethylene Glycol treatment. Taken together, these results suggest increased expression of KIM-1 mRNA and protein in renal tissues of hyper-oxaluric rats.

Increased KIM-1 expression is associated with deposition of COM-Crystals in the renal tubules of hyper-oxaluric rats

Kidneys were dissected out of control and hyper-oxaluric rats after each week and processed for histology, to determine tubular injury. Hematoxylin and Eosin staining showed increased luminal volume suggesting injury leading to tubular dilation. These changes were observed in as little as 2 weeks after the rats were given 0.75% EG in their drinking water (data not shown). Kidney tissue dissected out of rats fed EG for 3 weeks showed evidence of crystal deposition in the lumens of the tubules. Bi-refringent crystals were observed under polarized light (Figure 4a) and there were no significant differences in crystal deposition in the cortical and papillary region. Moreover, the tubular epithelium in the vicinity of the deposited crystals showed evidence of structural damage.

To determine the nature of the chemical composition of the crystals, tissue sections were stained with Von Kossa's staining for Calcium. Tissue sections collected from rats after 3 week of EG diet, showed positive von Kossa's staining, suggesting that the observed crystal deposits were primarily composed of Calcium Oxalate (Figure 4b). Tissue collected from control rats did not show any staining further suggesting that the observed staining in the tissue from hyper-oxaluric rats was from Calcium oxalate crystal deposition.

Hyper-oxaluria results in shedding of the ectodomain of KIM-1 in rats

Tubular injury in rodents has been shown to cause shedding of the ectodomain of KIM-1 protein, which can be detected in the urine. Urine was collected both from control and hyper-oxaluric rats for a period of 24 hours and western blot analysis was used to identify KIM-1 in the urine. Hyper-oxaluric rat urine showed shed KIM-1 protein in the urine, where as the control rats did not have

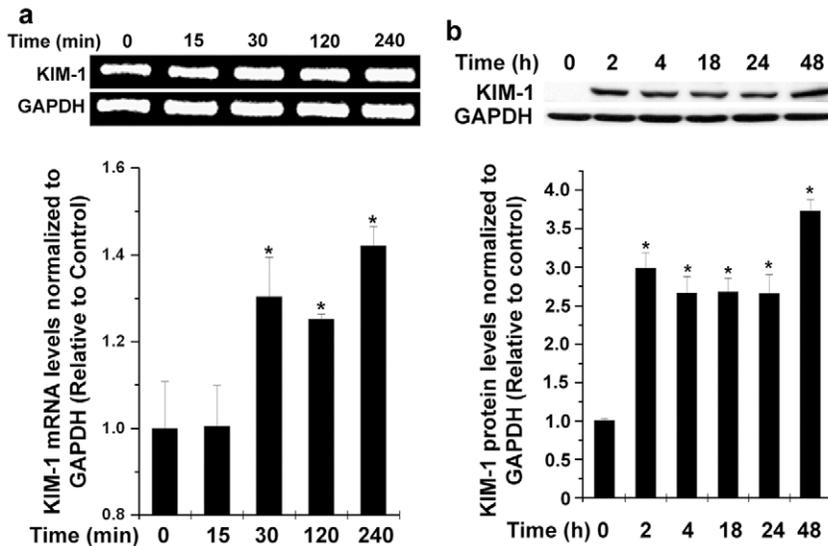


Figure 1. Human Kidney Epithelial Cells (HK-2) in culture, exposed to Sodium Oxalate showed increased KIM-1 mRNA and protein levels. (a) Oxalate was added to serum starved HK-2 cells and incubated for various time points. KIM-1 mRNA levels were estimated by Relative quantitative PCR and (Top Panel) representative gel image is shown. (Lower Panel) Quantification of mRNA levels was performed by band density analysis and represented as a relative intensity compared to control. Each data point represents mean \pm S.D. of two independent PCRs (* indicates $p < 0.05$ compared to control, $n = 2$). (b) Total protein was extracted as in materials and methods. Western blot analysis was used to detect KIM-1 protein levels in the cells. Representative blots from two individual experiments are shown (Top Panel). Densitometric analysis was used to quantify the KIM-1 protein levels. Each data point represents mean \pm S.D. of two independent gels (*indicates $p < 0.05$ compared to control, $n = 2$). doi:10.1371/journal.pone.0044174.g001

any protein in the urine (Figure 5a). This suggests that hyperoxaluria not only increases the expression of KIM-1 but also causes protein cleavage and shedding in the urine. Shedding of the ectodomain of KIM-1 protein was observed in conjunction with increased urinary Oxalate excretion (Figure 5b). This increase in Oxalate excretion was significantly higher in as little as 1 week after drinking water with added Ethylene Glycol.

Our observations also did not suggest any changes in the water or feed intake of the treatment group compared to the control rats. Results presented in Figure 5c, showed that there were no appreciable differences in the volume of urine or in the creatinine clearance by the EG fed rats compared to the control group. In

contrast, there was a significant reduction in Calcium excretion (Figure 5d). Moreover, there was a gradual reduction in the amount of total calcium excretion up to 3 weeks. Taken together, these results suggest that hyper-oxaluria increased the expression and shedding of the ectodomain of KIM-1 protein in rats.

Discussion

Hyper-oxaluria either as a result of increased Oxalate intake or metabolic disorders afflicts different age groups and is a primary source of discomfort. Persistent and prolonged exposure of the kidneys and urinary tract to higher Oxalate loads leads to

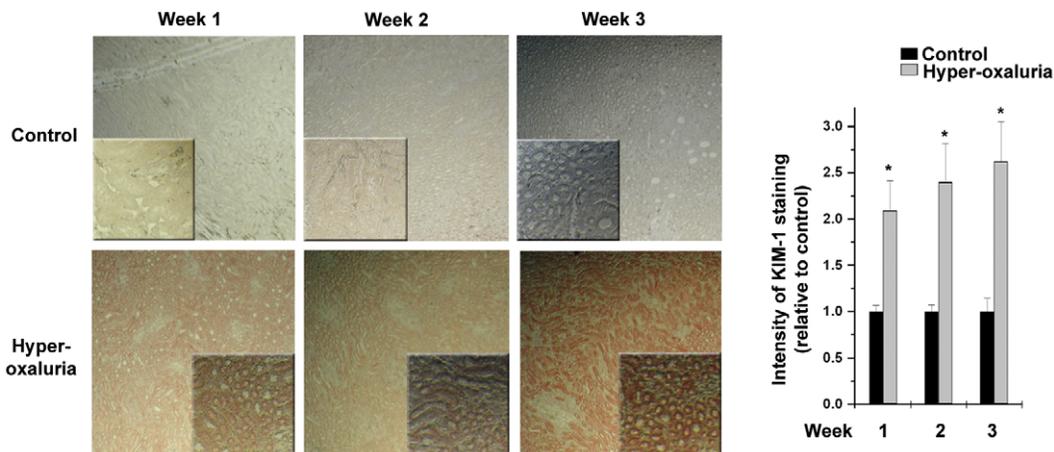


Figure 2. Immunohistochemical analysis of KIM-1 expression in kidney tissue of hyper-oxaluric rats. (Left Panel) KIM-1 protein expression in kidney tissue was detected using immunohistochemistry. (Right Panel) protein expression was quantified based on color density from five different 1 cm^2 regions of the section. Each data point represents mean \pm S.D. of two independent sections (* indicates $p < 0.05$ compared to control, $n = 2$). doi:10.1371/journal.pone.0044174.g002

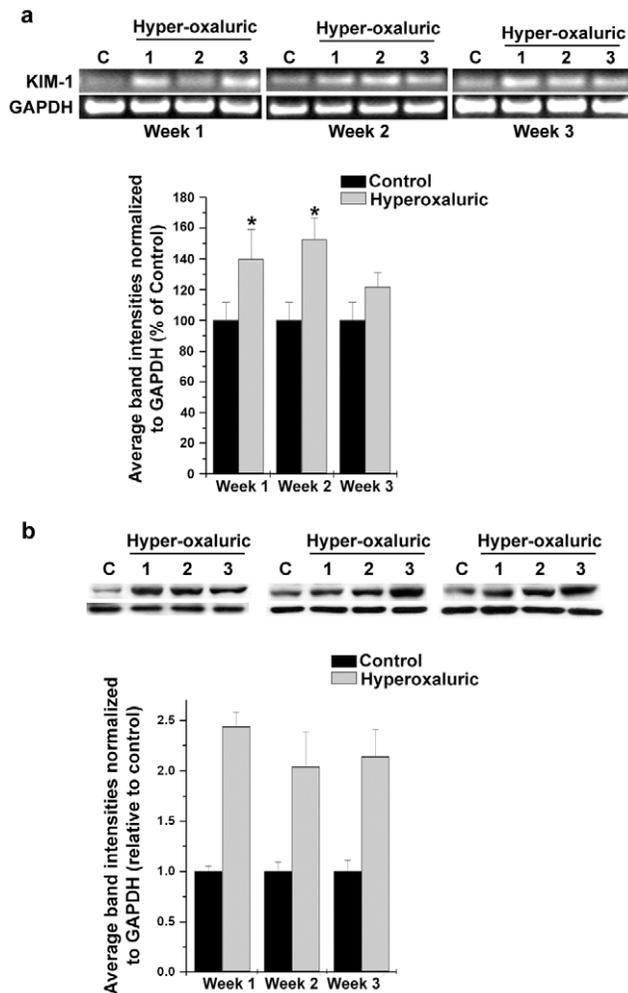


Figure 3. Hyper-oxaluric rats have increased mRNA and protein levels of KIM-1 in renal tissue. (a) KIM-1 mRNA levels were detected by relative quantitative RT-PCR in kidney tissue of control and three individual hyper-oxaluric rats. Representative gel images from three individual PCRs are shown (**Top Panel**). Quantitative representation of relative KIM-1 mRNA expression across the hyper-oxaluric group compared to control is shown in the **Bottom panel**. Each data point represents mean \pm S.D. of three experiments (*indicates $p < 0.05$ compared to control, $n = 3$). (b) Representative gel images from three individual western blots are shown (**Upper Panel**). Quantitative representation of the relative amounts of KIM-1 protein in the kidney tissue is shown in the **lower panel**. Each data point represents mean \pm S.D. of three blots (*indicates $p < 0.05$ compared to control, $n = 3$). doi:10.1371/journal.pone.0044174.g003

increased renal injury and is usually considered to be an initiator for developing kidney stones [12]. Instances of uncontrolled hyper-oxaluria leading to multi organ oxalosis and renal failure are well documented [23] Recent studies have identified Kidney Injury Molecule-1 (KIM-1) over-expression and shedding upon nephrotoxicity. This molecule is known to be highly up-regulated in both human and rodent kidney during nephrotoxicity [18]. The present study was designed to determine the status of KIM-1 upon hyper-oxaluric exposure using both *in vivo* and *in vitro* models of hyper-oxaluria.

In the present study, exposure of HK-2 cells to increased Oxalate concentrations over-expresses KIM-1 mRNA and protein. KIM-1 mRNA levels are significantly increased in as little as

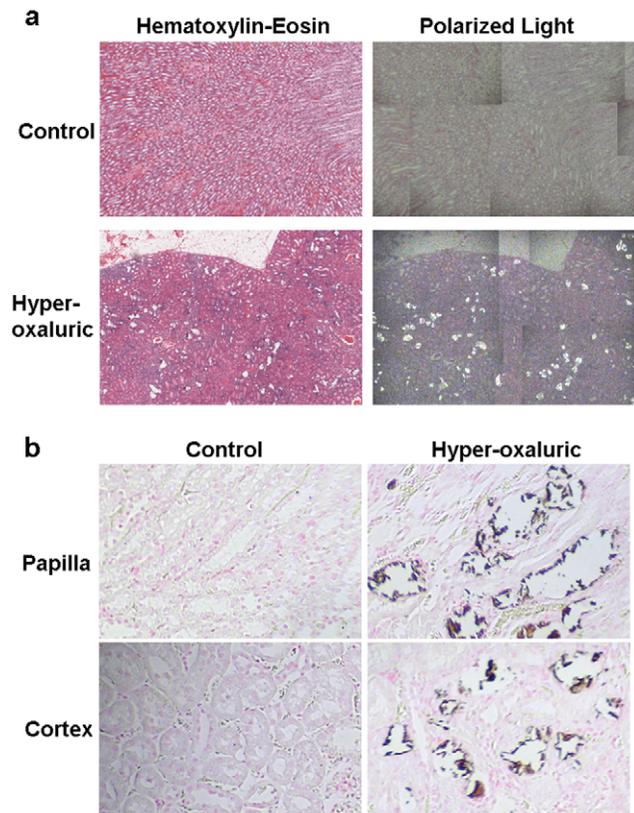


Figure 4. Hyper-oxaluric rats showed crystal deposits of Calcium Oxalate in renal tubules after 3 weeks. (a) Hematoxylin and Eosin stained paraffin embedded 5 μ m sections were photographed under polarized light to detect crystal deposits in the tubules. Representative images captured using a 10 \times objective magnification are shown. (b) Von Kossa's staining method was used to identify Calcium deposition in tubules of hyper-oxaluric rat kidneys. Digital photo-micrographs were taken with a 10 \times magnification. Representative images are shown. doi:10.1371/journal.pone.0044174.g004

30 min after exposure to oxalate (Figure 1a). Control HK-2 cells do not have KIM-1 protein but increased protein levels are observed in HK-2 cell lysates after exposure to oxalate (Figure 1b). This suggests that KIM-1 mRNA is expressed even in normal epithelial cells but protein expression is found only upon Oxalate exposure. Moreover, stable protein expression is detected in a very short time upon Oxalate exposure.

For developing an *in vivo* model, Wistar rats were given drinking water with 0.75% Ethylene Glycol for a period of 3 weeks. Rat models of hyper-oxaluria were based on Ethylene Glycol in various concentrations ranging from 0.75% to 2.5% in drinking water [28] In addition to Ethylene Glycol; many studies have utilized Ammonium Chloride as an additive for generating hyper-oxaluria in rats. Though, these results in crystal deposition in a shorter time period [29], the nephrotoxic effects cannot be ignored. Our results indicated that rats fed with 0.75% Ethylene Glycol alone have a significant increase in the amount of urinary Oxalate and developed signs of hyper-oxaluria as early as 1 week, without any discomfort or perceptible weight or appetite loss. Our observed results are consistent with other studies wherein, only Ethylene Glycol was used to induce hyper-oxaluria [26–29].

Immuno-histochemical staining using antibodies against the C-terminal region of KIM-1 showed up-regulation of KIM-1 in tissue sections collected from hyper-oxaluric rats compared to

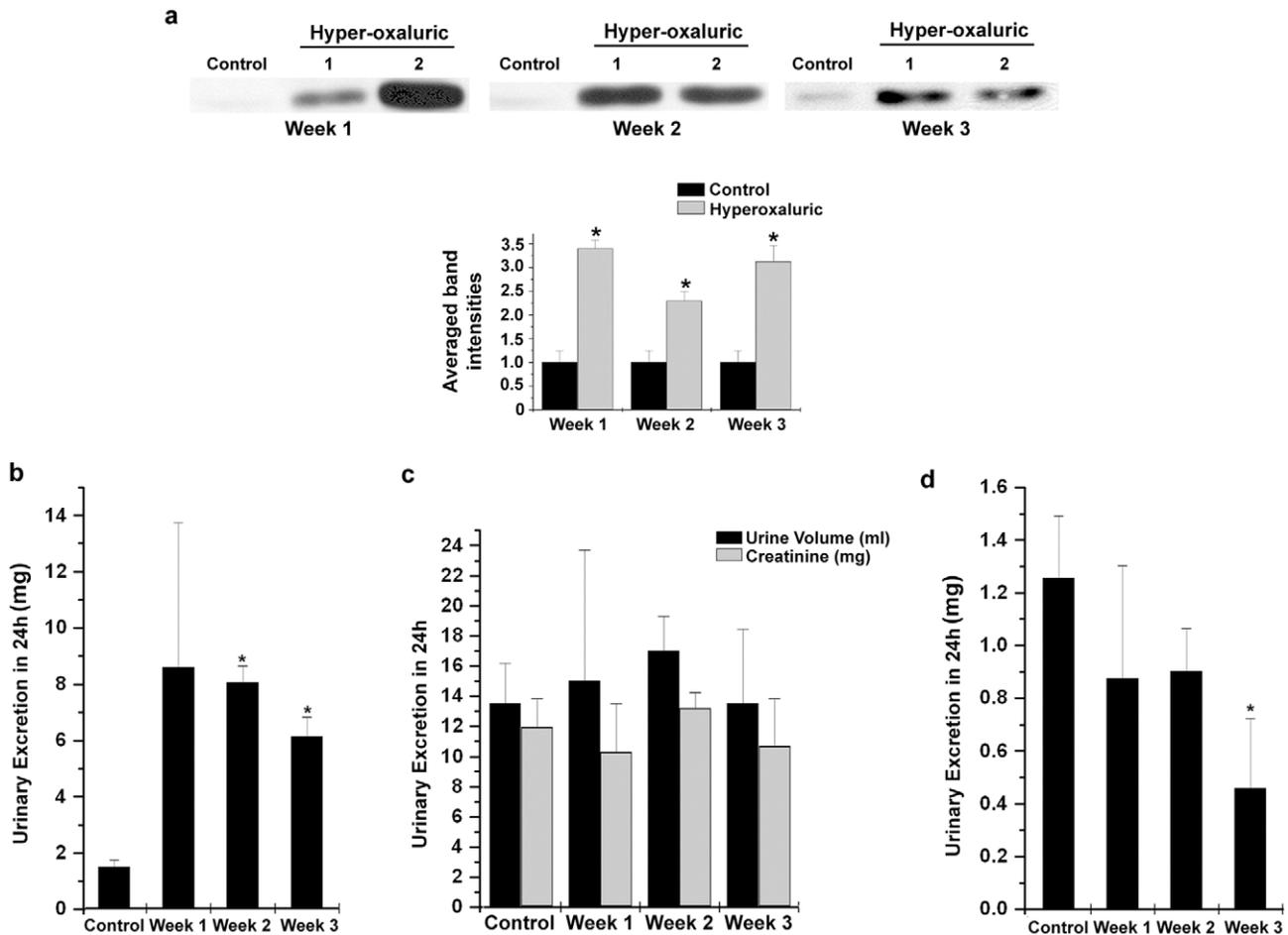


Figure 5. Hyper-oxaluria results in increased shedding of KIM-1 ectodomain in rat urine. (a) Western blot to detect KIM-1 protein shed in the urine of two individual hyper-oxaluric rats. Representative gel images from three individual western blots are shown (Upper Panel). Quantitative representation of the relative amounts of KIM-1 protein in urine, averaged across the treatment groups, is shown in the lower panel. Each data point represents mean \pm S.D. of three blots (* indicates $p < 0.05$ compared to control, $n = 3$). (b) Urine Volume (ml) and Creatinine excretion (mg) values (c) Oxalate (mg) and (d) Calcium (mg) over a period of 24 hours. Each data point represents mean \pm S.D. of individual animals in each group (* indicates $p < 0.05$ compared to control). doi:10.1371/journal.pone.0044174.g005

control (Figure 2). The increase in KIM-1 protein was observed in as little as 1 week after Ethylene Glycol treatment. Even though crystal deposition was not observed after week 1 of Ethylene Glycol diet, KIM-1 mRNA levels were higher suggesting that tubular injury occurs earlier than crystal deposition (Figure 3a). Though, the Creatinine clearance in the rats on Ethylene Glycol diet was comparable to the control animals up to 3 weeks, the up-regulation of KIM-1 mRNA and increased shedding of KIM-1 protein (Figure 5a) in the urine of hyper-oxaluric rats suggests that this may be a better marker for associating tubular injury to hyper-oxaluria. This may be particularly useful as previous studies have not been able to identify tubular damage associated with this experimental model based on creatinine clearance data.

KIM-1 has been shown to be localized to proximal tubular cells and may play an important role in the phagocytosis of apoptotic and dead cells [30]. Recent studies have also identified its potential role in removal of dead cells and therefore, contribute to tissue repair [31]. Though the functional significance of KIM-1 shedding is not known, Mitogen Activated Protein Kinases, especially ERK and p38 MAPK, have been shown to play an important role in this process [32]. Previous studies in our laboratory have identified activation of p38 MAPK in kidney epithelial cells exposed to

Oxalate and COM-Crystals [7,8,17]. Based on these observations, it is tempting to speculate that signaling by p38 MAPK pathway may also play an important role in KIM-1 overexpression and shedding during hyperoxaluria mediated tubular injury, however, additional studies are needed to support such conclusions.

We observed a reduction in the Urinary Oxalate on week 3 in the hyper-oxaluric rats compared to the amount of urinary oxalate on weeks 1 and 2. This may be a reflection of the observed deposition on birefringent crystals in the lumen of the tubules (Figure 4a). In addition to increased urinary Oxalate, there was also a large reduction in the amount of calcium excreted in the urine after 2 weeks. This further suggested that the deposited crystals in the renal tubules may be composed of Calcium Oxalate. Positive staining results obtained by Von Kossa's staining further confirmed that the observed crystalline deposits were composed of Calcium (Figure 4b).

In summary, our study shows that KIM-1 is over-expressed in human kidney epithelial cells upon exposure to higher oxalate concentrations. Since, increased KIM-1 expression is observed over a sustained period of time after hyper-oxaluric exposure, over-expression and shedding of KIM-1 may be potentially used as an early marker in hyper-oxaluria mediated tubular injury.

Materials and Methods

Chemicals and reagents

All chemicals used in this study were procured from Sigma (Sigma-Aldrich, St. Louis, MO). Microcentrifuge concentrators Microcon YM-10 for concentrating rat urine were procured from Millipore Corporation (Billerica, MA).

Animals and Treatments

Male Wistar rats (Harlan Laboratories, Indianapolis, IN) 3 to 5 week old with an average weight of 150 gm were according to the Center for Laboratory Animal Care (CLAC) guidelines at the University of Colorado Vivarium. Randomized groups were given either control water or water with 0.75% Ethylene Glycol. For metabolic studies, the rats were housed in metabolic cages for a period of 24 hours for measuring water intake and collecting urine for chemical analysis. The rats were acclimatized for a period of 24 hours to ensure continuity. Urine was collected for a period of 24 h at the end of each week and tissue samples from sacrificed animals was aseptically collected and processed. For histological studies, part of the tissue was fixed in formalin and embedded in paraffin wax; or stored in RNAlater solution (Qiagen, Valencia, CA) for extracting RNA.

Cells and Cell Culture

Human Kidney Epithelial Cells, HK-2 were procured from ATCC and maintained in DMEM medium supplemented with 10% Fetal Bovine Serum and antibiotics. Before Oxalate treatments, cells were serum starved for 16 to 20 hours. Media components were procured from Invitrogen Corporation (Carlsbad, CA) as described previously [9].

Urine Composition analysis

Urine analysis for concentrations of different metabolites was performed with the assistance of University of Colorado Hospital Clinical laboratory using standard procedures. Oxalate concentration in the urine was estimated using a AS10 ion chromatography column [33].

Immuno-histochemical analysis

Kidney tissue dissected from rats was fixed in 10% Formaldehyde and embedded in Paraffin wax. 5 μ M thin sections were used for histochemical staining; sections were stained for assessing fine structures by Hematoxylin and Eosin [34]. Immuno-histochemical staining for expression of KIM-1 was performed as described [35]. Briefly, the tissue sections were heated in Citrate buffer (10 mM sodium citrate and 1 mM EDTA, pH 6.0) for 15 minutes in a microwave oven and blocked with 10% donkey serum. Biotin conjugated secondary antibodies were used to detect primary antibody binding and then stained with streptavidin conjugated HRP antibodies using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Antibody binding was detected using diaminobenzidine color reaction (Sigma-Aldrich, St. Louis, MO).

Detection of Calcium Oxalate crystal deposits was by VonKossa's staining and was done by the Pathology Laboratory of the University of Colorado Denver using standard procedures [36].

RNA isolation and Reverse Transcriptase PCR

RNA was isolated from tissue stored in RNAlater using the RNEasy Kit according to manufacturer's recommendations

Table 1. Sequences of primers used in RT-PCR.

Primer	Sequence
KIM-1 (Rat) forward	5'-GGT CAC CCT GTC ACA ATT CC-3'
KIM-1 (Rat) reverse	5'-CTC GGC AAC AAT ACA GAC CA-3'
KIM-1 (Human) forward	5'-CTG CAG GGA GCA ATA AGG AG-3'
KIM-1 (Human) reverse	5'-ACC CAA AAG AGC AAG AAG CA -3'
GAPDH forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
GAPDH reverse	5'- TCC ACC ACC CTG TTG CTG TA-3'

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(Qiagen, Valencia, CA). 1 μ g total RNA was used to synthesize cDNA using iScript cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA). PCR was performed with gene specific primers using Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) and separating the products on a 1% agarose gel. Primers were procured from Integrated DNA Technologies (Coralville, IA) and primer sequences for rat and human Kidney Injury Molecule (KIM-1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are described in Table 1.

Protein isolation and Western blot analysis

Kidney tissue from rats and HK-2 cells were washed with Ice cold PBS and then lysed immediately with boiling hot 2X SDS gel loading buffer with 2- Mercaptoethanol. Cellular proteins were resolved using SDS-Polyacrylamide gels and then transferred onto Polyvinylidene difluoride membrane (Millipore Corporation). Two different antibodies that represent the N- terminal region and the C-terminal region of KIM-1 protein were procured from Abcam Inc. (Cambridge, MA), while GAPDH antibody was procured from Cell Signaling Technology Inc. Quantification of the band intensities was performed using the densitometry analysis function of Quantity One 1-D gel analysis software (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Unless otherwise mentioned, two dimensional two sample equal variance student's T-test was used for statistical analysis. A $p < 0.05$ was considered significant.

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Author Contributions

Conceived and designed the experiments: HK SK LK. Performed the experiments: LK SK. Analyzed the data: HK RBM SK LK. Contributed reagents/materials/analysis tools: SK HK LK. Wrote the paper: HK LK SK. Designed experiments: HK. Conducted all experiments with animal model: LK. Conducted all experiments with cell cultures: SK. Analyzed data: HK SK LK RBM. Wrote the manuscript: HK SK LK. Supervised experiments: HK.

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