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Kidney injury molecule-1 (KIM-1) mediates renal epithelial cell repair via ERK MAPK signaling pathway

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Abstract

The expression of kidney injury molecule-1 (KIM-1), a very promising sensitive and specific urinary biomarker for acute renal injury, is markedly upregulated in injured and regenerating renal proximal tubular epithelial cells following ischemic or toxic insults, suggesting a possible role for this molecule in renal repair process. In the present study we report that expression of KIM-1 facilitates renal tubular epithelial cell repair by promoting cell migration and proliferation. KIM-1 expression also enhances ERK MAPK activation, and the modulatory effect of KIM-1 on cellular repair process is likely mediated via ERK MAPK signaling pathway.

Keywords

Kidney injury molecule-1 (KIM-1); cellular repair; cell migration; cell proliferation; ERK MAPK

INTRODUCTION

Acute kidney injury is a common medical disease associated with very high overall mortality [1]. It is unlikely that this high mortality and associated cost will be reduced until we have a better understanding of the cellular and molecular mechanisms for renal cell injury and repair. Epithelial cell injury is fundamental to the pathophysiology of acute kidney injury, and the S3 segment of the proximal tubule is particularly susceptible to ischemic and toxic injury [2]. Unlike the brain and heart, where ischemically injured tissue cannot recover after injury, the kidney with its complex architecture has the ability to recover both morphologically as well as functionally. However, the underlying mechanism regulated renal epithelial cell repair processes remains largely unknown.

Kidney injury molecule-1 (KIM-1), also known as HAVCR (hepatitis A virus cellular receptor) [3] and TIM-1 (T-cell immunoglobulin and mucin protein) [4], is a type 1 transmembrane glycoprotein markedly upregulated in the injured and regenerating renal proximal tubule epithelial cells of the human and rodent kidney following ischemic [5] and

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toxic [6] injury to the kidney. KIM-1 protein is localized primarily to the apical membrane of the cells and the ectodomain of KIM-1 undergoes membrane-proximal cleavage via a highly regulated process, which leads to the release of soluble KIM-1 into the urine [7, 8]. Urinary KIM-1 has been shown to be a very promising novel biomarker in human and rodents and outperforms traditional biomarker of kidney injury in preclinical biomarker qualification studies [9–12].

Recently KIM-1 has been shown to be a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells [13]. The expression of this molecule in renal tubules mediates epithelial cell phagocytosis of apoptotic cells, which protects the kidney after acute injury by downregulating innate immunity and inflammation [14]. KIM-1 also physically interacts with and inhibits cellular Ga12 activation after inflammatory stimuli by blocking GTP binding to Ga12, which protects mice against renal ischemia-reperfusion injury [15]. On the other hand, renal tubule repair process is generally characterized by migration and proliferation of the intrinsic dedifferentiated epithelia across the denuded tubule basement membrane [16], but the mechanisms that regulate these processes remain poorly understood. Since renal epithelial cells that express KIM-1 are typically dedifferentiated, characterized by a loss of the brush border membrane and expression of mesenchymal proteins such as the intermediate filament protein vimentin [5], the present study was therefore designed to examine the role of KIM-1 in renal tubular epithelial cell repair focusing on the migration and proliferation processes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium/Han's F12 (DMEM/F12), hygromycin, and G418 were obtained from Mediatech (VA, USA). Fetal bovine serum (FBS), doxycycline, MTT (Methylthiazolyldiphenyl-tetrazolium bromide), and U0126 were obtained from Sigma (MO, USA). Epidermal growth factor (EGF) and matrigel were purchased from BD Biosciences (NY, USA). Antibodies against human KIM-1 were generated as described [7] and have been used in our previous study [8].

Cell culture and transfections

LLC-PK₁ cells were grown in DMEM supplemented with 10% FBS and maintained at 37°C in 5% CO₂. These cells were transfected with eukaryotic expression vector pcDNA3-neo containing the cDNAs encoding for wild type human KIM-1 or pcDNA3-neo alone using Superfect reagent (Qiagen, CA, USA). Stable clones were selected and maintained in DMEM containing G418 (400 µg/ml). KIM1-tet-off MDCKII cells (referred as MDCK-KIM-1 here) were generous gift from Dr. Benjamin Humphreys (Harvard medical school). They were generated as described in previous study [13] and cultured in DMEM/F-12 supplemented with 10% FBS, 250 µg/ml hygromycin, and 100 µg/ml of G418. Cells were routinely maintained in 100 ng/ml doxycycline to prevent KIM-1 transcription until induction was initiated by its withdrawal.

Epithelium wound healing assay

Cellular repair was assessed by an *in vitro* model of epithelium wound healing. Confluent cell monolayers grown in glass coverslips were wounded with a sterile surgical blade and remained in culture for various times. At the end of experiment, the monolayers were washed with ice-cold PBS, fixed with 100% methanol, and stained with 0.1% crystal violet, and photographed. In some experiments, the “wounded area” (cell free region) was assessed using Image J program (NIH). The “wound-healing” at different time points were calculated and expressed as a percentage of the wounded area at baseline (0 h).

Cell migration

To assess the complex migration processes of epithelial monolayers in cell culture without available time-lapse video microscopy, we have developed a test system that reproducibly measures the ability of cells to cover a cell culture surface coated with selected components of extracellular matrix. For this assay, a small glass cover slip covered with monolayer of confluent cells was placed upside-down on cell culture plate coated with or without matrigel (2.5 µg/ml) and cultured in complete media for various times. Cell migration from the edge of cover slip was then observed under a phase contrast microscope and photographed. To distinguish cell migration from proliferation, mitomycin C (Sigma, MO, USA) at 0.01 mg/ml was added to culture media to inhibit cell proliferation.

Cell proliferation

After overnight serum deprivation, cells were harvested with trypsin/EDTA (inactivated by trypsin inhibitor, not serum containing media), seeded in 96-well cell culture plate, and cultured in the presence or absence of 20% FBS for various times. Cell proliferation was then assessed by MTT staining and expressed as percentage of cell proliferation according to the following formula: Cell survival (%) = OD (optical density) at various times/OD at baseline (0 h) × 100.

Activation of ERK MAPK

Confluent cells were serum deprived overnight and then incubated with various concentrations of FBS or EGF for various times. Activation of ERK MAPK was then assessed by western blot analysis using phospho-specific antibody against ERK MAPK.

Western blot analysis

The cell monolayers were lysed on ice in lysis buffer supplemented with protease inhibitors. Aliquots of cell lysates were boiled in SDS-PAGE sample buffer, fractionated on 10% SDS-PAGE gel, and transferred to PVDF membrane. After blocking with 5% nonfat dry milk in TBS/0.05% Tween, the blots were then incubated with the respective primary antibodies overnight at 4°C, followed by peroxidase-conjugated secondary antibody and ECL detection.

Statistical Analyses

Continuous variables were reported as mean $\pm$ standard deviation. The differences between groups were analyzed by the Student *t* test or one-way analysis of variance (ANOVA). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Heterologous expression of KIM-1 facilitates renal tubular epithelial cell repair

LLC-PK₁ cells, a porcine renal tubular epithelial cell line, were chosen for the current study because they do not express detectable endogenous KIM-1. Overexpression of human KIM-1 in these cells was achieved via stable transfection. As shown in Figure 1A, the expression pattern of KIM-1 in KIM-1-LLC cells was similar to that of 769-P cells, a human renal cell adenocarcinoma cell line expressing high levels of endogenous KIM-1 [7, 8]. The 100 kDa band is the actual cell surface glycosylated protein (marked with*) and the 60 kDa band most likely corresponds to KIM-1 transiting through the Golgi [7]. By contrast, there is no KIM-1 expression at all in control (pcDNA3-LLC) cells. In the current study, we tested the effect of KIM-1 overexpression in an *in vitro* model of epithelium wound healing. As shown in Figure 1B, a mechanical wound was completely healed in less than 16 hours in KIM-1 expressing LLC-PK₁ cells; while in control pcDNA3-LLC cells, it remained unhealed even after 24 hours, indicating that upregulation of KIM-1 facilitates renal tubular epithelial cell repair.

KIM-1 expression promotes migration and proliferation of renal tubular epithelial cells

Cell migration and proliferation are two most important cellular events during the repair processes. We next examined whether the cellular repair conferred by KIM-1 overexpression are related to these cellular functions. Since a time-lapse video microscopy is not easily accessible, we have developed a novel assay to assess the migration of cell monolayer under physiologic condition. As shown in Figure 2A, cell migration over a surface coated with matrigel, an extract of the EHS sarcoma that is enriched in basement membrane matrix, is significantly faster in KIM-1 expressing LLC-PK₁ cells compared to their controls (pcDNA3-LLC), indicating KIM-1 overexpression promotes renal epithelial cell migration. On the other hand, migration over an uncoated surface is slow in both cells without notable differences between them (Figure 2B), suggesting that the enhanced cell migration conferred by KIM-1 expression is not just a general feature but is specific to interaction with basement membrane components, similar to a wound healing process.

We have also evaluated the effect of KIM-1 overexpression on cell proliferation. As shown in Figure 2C&D, KIM-1 expressing LLC-PK₁ cells grew significantly faster than the control pcDNA3-LLC cells in the absence or presence of serum. More interestingly, in response to serum-derived growth factor, KIM-1 expressing cells grew exponentially, as compared to a nearly linear pattern of growth in controls cells (Figure 2D), suggesting KIM-1 expression may enhance cellular response to growth signaling and promote cell proliferation.

Regulated expression of KIM-1 enhances renal tubular epithelial cell repair

Stable transfection may alter the phenotypic characteristics of cells over time and results from those experiments may not reflect the situation of endogenous KIM-1 expression under physiologic conditions. We have therefore established a clone of MDCK cells (MDCK-KIM-1) bearing a doxycycline sensitive promoter, which has the ability to control when and how much of KIM-1 is expressed. MDCK is a canine renal tubular epithelial cell line expressing only modest amount of endogenous KIM-1. As shown in Figure 3A, withdrawal of doxycycline in the culture media resulted in a time-dependent expression of KIM-1 in MDCK-KIM-1 cells as early as less than 12 hours, achieving a significant level of expression in these cells after 48 hours of doxycycline withdrawal. Thus, to allow sufficient KIM-1 expression, doxycycline was withdrawn from culture media for at least 48 hours prior to experiment. In a similar model of epithelium wound healing, the wounded area was significantly smaller in cells expressing KIM-1 (with doxycycline withdrawn) compared with control cells that were cultured in the presence of doxycycline to prevent KIM-1 transcription (Figure 3B&C). These results were consistent with that performed with stable transfection in different cell line, indicating our findings were common features in renal epithelial cells.

KIM-1 expression enhances ERK MAPK activation

Activation of ERK MAPK plays important role in epithelial wound healing process [17]. To evaluate whether overexpression of KIM-1 influences ERK MAPK activities, serum-deprived MDCK-KIM-1 cells were exposed to various concentration of FBS and ERK activation was monitored. In the presence of doxycycline, lack of KIM-1 expression in these cells resulted in a weak activation of ERK MAPK in response to high concentration of FBS (20%, Figure 4A, upper panel). On the other hand, induction of KIM-1 expression in the absence of doxycycline led to a very strong response to FBS even at a concentration as low as 5% (Figure 4A, upper panel). Time course study also indicated that the activation of ERK MAPK was more sustained in KIM-1 expressing cells (Figure 4A, lower panel). Similarly, KIM-1 expression induced a more potent and sustained ERK MAPK activation in response to EGF (Figure 4B), one of the most important growth factors involved in epithelial wound healing process [18].

The modulatory effect of KIM-1 on cellular repair is mediated via ERK MAPK signaling pathway

Since KIM-1 expression enhances ERK MAPK activation, the modulatory effect of KIM-1 on renal epithelial cell repair could be mediated by ERK MAPK signaling pathway. To test this hypothesis, we examined whether the enhanced wound healing process modulated via overexpression of KIM-1 will be altered by the inhibition of ERK MAPK activities. As shown in Figure 4C&D, induction of KIM-1 expression in the absence of doxycycline resulted in a significant decrease in the wounded area ($10.06 \pm 2.29\%$ vs $45.68 \pm 10.14\%$, $p < 0.01$). Incubation with vehicle DMSO did not affect the wound healing process (wounded area $10.62 \pm 1.30\%$ vs $10.06 \pm 2.29\%$). By contrast, incubation with U0126, a specific inhibitor of ERK MAPK, resulted in a significantly dose-dependent inhibition of wound healing (Figure 4C&D), which is associated with its ability to inhibit ERK MAPK activation

(Figure 4D, top panel). The enhanced wound healing conferred by KIM-1 expression was affected in the presence of U0126 even at a concentration as low as 1 μ M (wound area $16.81 \pm 2.47\%$ vs $10.06 \pm 2.29\%$, $p < 0.01$, Figure 4D), and was further compromised in the presence of 5 μ M of U0126 ($45.34 \pm 6.83\%$ vs $10.06 \pm 2.29\%$, $p < 0.01$, Figure 4D) that is similar to baseline without KIM-1 expression ($45.68 \pm 10.14\%$ in the presence of doxycycline, Figure 4D), suggesting the modulatory effect of KIM-1 on cell repair is likely mediated via ERK MAPK signaling pathway.

DISCUSSION

In the present study, we found that KIM-1 expression facilitates renal tubular epithelial cell repair process by promoting cell migration and proliferation, which is likely mediated via ERK MAPK signaling pathway. The complexity of renal tubular structures is lined with epithelial cells that are frequently exposed to various ischemic and toxic insults. Epithelial cell injury is therefore fundamental to the pathophysiology of acute kidney injury [2]. To restore epithelial integrity and function following injury, several cellular events are engaged sequentially, including cell migration, proliferation, and differentiation [19]. Cell migration plays a central role in a wide variety of biological phenomena [20]. Movement of the regenerating cells to cover the denuded basement membranes is one of the first mechanisms of epithelial repair. Indeed, migration of renal epithelial cells specific on basement membrane matrix matrigel (not observed on plastic surface) is significantly enhanced by overexpression of KIM-1 in our study, suggesting a potential role for KIM-1 in regulating this event to initiate the renal tubular epithelial cell repair process.

Proliferation of epithelial cells forms the cornerstone of wound healing. There had been a debate about the origin of cells that repair renal tubular epithelia after injury. Recent studies have challenged the traditional belief that those cells are come from endogenous surviving tubules [21]. They suggested that bone marrow-derived cells, including hematopoietic stem cells (HSCs) and mesenchymal stem cells, could possibly replenish the epithelial cells that have been lost [22, 23]. Additional study [24], however, indicated that bone marrow-derived cells do not directly replace the tubule epithelial cells that are lost with injury, but exert paracrine effects that facilitate repair potentially by reducing inflammation, which may be mediated by microvesicles that transfer membrane receptors, proteins, mRNAs, microRNAs, and organelles [25–28]. The most definite evidence came from genetic fate-mapping techniques employed in transgenic mice, and the results demonstrated that intratubular progenitor cells proliferate and this accounts for replenishment of the tubular epithelium after ischemia [16]. Since we observed that proliferation of renal tubular epithelial cells, either in the presence or absence of growth factors is significantly increased via upregulation of KIM-1 expression, a possible role for KIM-1 in propagating the renal tubular epithelial cell repair process by stimulation of endogenous progenitor cell proliferation could also be speculated.

In response to injury, renal epithelial cells initiate several signaling pathways to act as intracellular communication lines that regulate the tubular structural and functional restoration. Mitogen-activated protein kinases (MAPKs) are among the most commonly studied signaling pathways involved in these processes [29]. Extracellular signal-regulated

kinases (ERKs; ERK1 and ERK2), the classic and best characterized MAPKs identified in the context of growth factor-related signaling, have been implicated in a variety of kidney diseases [30, 31]. Our study indicated that overexpression of KIM-1 induces not only more robust but also more sustained activation of ERK MAPK in response to serum derived growth factors or more specifically, EGF. The activation of ERK MAPK in response to growth factor is typically biphasic, a rapid phase appearing at 5–10 min followed by a late and sustained phase lasting for hours [32]. The second phase is usually more important since it is correlated well with the mitogenic potential and is an obligatory event for growth factor-induced cellular functions [32]. The booster effect of KIM-1 on the sustained phase of ERK MAPK activation is likely critical, since the modulatory effect of KIM-1 on renal epithelial cell repair disappears after inhibition of ERK MAPK pathway.

In summary, we demonstrate that KIM-1 enhances cell repair, likely by promoting cell migration and proliferation in renal tubular epithelial cells. KIM-1 also induces a more robust and sustained activation of ERK MAPK signaling pathway, which could mediate its cellular functions. Modulation of KIM-1 expression in renal tubular epithelial cells could represent a novel therapeutic approach to facilitate renal recovery after acute kidney injury.

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A.

B.

Figure 1. Stable expression of KIM-1 enhances renal tubular epithelial cell repair

A. Overexpression of KIM-1 in LLC-PK₁ cells. LLC-PK₁ cells were stably transfected with eukaryotic expression vector pcDNA3-neo encoding cDNA of human KIM-1 (KIM-1-LLC) or empty vector alone (pcDNA3-LLC). Levels of human KIM-1 expression were determined by western blot analysis in cell lysates (upper panel). The mature, fully glycosylated KIM-1 protein is marked with an (*). Detection of ERK-2 in the same blot was included to assess sample loading (lower panel). **B.** KIM-1 expression facilitates “epithelium wound healing”. Stably transfected LLC-PK₁ cells were grown on a glass cover slip. The cell monolayers

were then wounded via mechanical scratch, and the wounded areas were recorded at various times.

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A.

B.

C.

D.

Figure 2. Overexpression of KIM-1 enhances renal tubular epithelial cell migration and proliferation

LLC-PK₁ cells were stably transfected with human KIM-1 (KIM-1-LLC) or empty vector alone (pcDNA3-LLC). To assess cell migration, confluent cell monolayer grown on a small glass cover slip was placed upside-down on cell culture plate coated with (A) or without matrigel (B) and cultured for various times. Cell migration from the edge of cover slip was then recorded. For cell proliferation, cells were seeded in a 96-well cell culture plate in the absence (C) or presence (D) of serum (10% FBS) and incubated for various times. Proliferation of these cells was then measured by MTT staining and expressed as percentage

of growth. Results are mean $\pm$ standard deviation from 3 different experiments performed in triplicate for each treatment. ** $p < 0.01$ compared with pcDNA3-LLC.

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Figure 3. Regulated expression of KIM-1 enhances renal tubular epithelial cell repair
MDCK-KIM-1 cells, a clone of MDCK cells in which the expression of human KIM-1 can be regulated under the control of a doxycycline sensitive promoter, were routinely cultured in media containing doxycycline to suppress KIM-1 expression. The expression of KIM-1 in these cells was then induced by withdrawal of doxycycline from culture media for various times and the levels of human KIM-1 expression were determined in cell lysates by western blot analysis (A). The mature, fully glycosylated KIM-1 protein is marked with an (*), and detection of ERK-2 in the same blot was included to assess sample loading (lower panel). To

evaluate the effect of regulated expression of KIM-1 on epithelial cell repair, MDCK-KIM-1 cells were grown on a glass cover slip in the presence or absence of doxycycline for at least 48 hours to allow sufficient expression of KIM-1 prior to experiment. The cell monolayers were then wounded via mechanical scratch, and the wounded areas were recorded (**B**) and quantitated (**C**) after 6 hours. * $p < 0.01$ compared with MDCK-KIM-1 cells cultured with doxycycline.

A.

B.

Figure 4. KIM-1-modulated renal tubular epithelial cell repair is mediated by ERK MAPK signaling pathway

MDCK-KIM-1 cells were cultured in the presence or absence of doxycycline for at least 48 hours to allow sufficient expression of KIM-1 prior to experiment. **A.** Activation of ERK MAPK in response to FBS. Cells were exposed to various dilution of FBS for 1 hour (upper panel) or exposed to 20% FBS for various times (lower panel) after overnight serum deprivation, The levels of ERK MAPK activation were determined in cell lysates by western blot analysis using an antibody against phosphorylated ERK, and detection of ERK-2 in the same blot was included to assess sample loading (lower lane of each panel). **B.** Activation of ERK MAPK in response to EGF. Cells were exposed to various concentrations of EGF for 1 hour (upper panel) or exposed to 1000 ng/ml of EGF for various times (lower panel) after overnight serum deprivation. The levels of ERK MAPK activation were determined in cell

lysates by western blot analysis using an antibody against phosphorylated ERK, and detection of ERK-2 in the same blot was included to assess sample loading (lower lane of each panel). **C-D**. Effect of ERK MAPK inhibition on epithelium wound healing. The cell monolayers were wounded via mechanical scratch and cultured in the presence of various concentrations of U0126 or its vehicle DMSO. The wounded areas were then recorded (**C**) and quantitated (**D**) after 6 hours. The inhibition of ERK MAPK activation by U0126 was also confirmed in these cells (**D**). * $p < 0.01$ compared with MDCK-KIM-1 cells cultured with doxycycline, # $p < 0.01$ compared with MDCK-KIM-1 cells cultured without doxycycline.