Netrin-1 increases proliferation and migration of renal proximal tubular epithelial cells via the UNC5B receptor

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Wang W, Reeves WB, Ramesh G. Netrin-1 increases proliferation and migration of renal proximal tubular epithelial cells via the UNC5B receptor. Am J Physiol Renal Physiol 296: F723–F729, 2009. First published February 11, 2009; doi:10.1152/ajprenal.90686.2008.—The cellular hallmark of kidney repair is a rapid proliferation of renal tubular epithelial cells ultimately leading to the restoration of nephron structure and function. Netrin-1 was discovered as a neural guidance cue and found to be expressed outside the nervous system, including in kidney. Previous work showed that netrin-1 is upregulated in response to ischemic injury and ameliorates ischemic injury. The objectives of this study were to determine the role of netrin-1 in renal tubular epithelial cell proliferation and migration in vitro. Real-time RT-PCR analysis showed that netrin-1 and its receptors UNC5B and neogenin are highly expressed in cultured mouse renal epithelial cells (TKPTS), whereas the expression of the Deleted in Colon Cancer (DCC), UNC5A, UNC5C, and UNC5D receptors is negligible or undetectable. Netrin-1 protein was induced in the edges of mechanical wounds in vitro. Netrin-1 increased TKPTS cell proliferation in a dose-dependent manner. The netrin-1-induced increase in TKPTS cell proliferation was completely prevented by small interfering RNA (siRNA) inhibition of UNC5B receptor but not UNC5C receptor expression. Netrin-1 also increased TKPTS cell migration in vitro, and this was also mediated through the UNC5B receptor. Netrin-1 increased the phosphorylation of Akt and ERK. Inhibition of phosphatidylinositol 3-kinase and MEK1/2 completely inhibited netrin-1-induced cell proliferation but not migration. These results indicate that netrin-1 increases renal tubular epithelial cell proliferation and migration through the UNC5B receptor. Moreover, the increase in cell proliferation, but not migration, was mediated via activation of Akt and ERK pathways.

MATERIALS AND METHODS

Cell culture. Immortalized mouse kidney proximal tubular epithelial cells (TKPTS) were cultured as described previously (25). To determine the effect of netrin-1 on cell proliferation, cells were plated in 96-well plates. Twenty-four hours after plating, medium was replaced with serum-free, low-glucose medium containing different concentrations of netrin-1 or vehicle (0.1% BSA). Forty-eight hours after netrin-1 addition, the cell proliferation was determined with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-3-tetrazolium bromide (MTT) assay. To determine the role of MAPK pathways in netrin-1-induced cell proliferation, cells were treated with 10 μM U0126 (ERK inhibitor) or 10 μM LY-294002 [phosphatidylinositol 3-kinase (PI3-kinase) inhibitor]. To determine the pathways activated by netrin-1, TKPTS cells were treated with 100 ng/ml netrin-1 and harvested at 5, 10, 20, 40, and 60 min after netrin-1 addition.

Western blot analysis. Protein was extracted by solubilizing cells in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was quantitated with the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, IL), and 50 μg of total protein was loaded onto 4–12% polyacrylamide gels, separated, and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was probed with rabbit anti-phospho ERK, PKC, Akt, focal adhesion kinase (FAK), and PKA antibodies (Cell Signaling Technologies) or goat anti-UNC5B, UNC5C, and neogenin antibodies (R&D Systems). Proteins were detected with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech). Protein loading was normalized by probing the membrane with anti-actin antibodies.

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Small interfering RNA transfection. Small interfering RNA (siRNA) pools against UNC5B and UNC5C were purchased from Dharmacon Thermo Scientific. siRNA was dissolved in siRNA buffer (Thermo Scientific) at a concentration of 100 μM. TKPTS cells were harvested by trypsinization, counted, and resuspended in serum-free medium. Two million cells were aliquoted in separate tubes and centrifuged. The cell pellets were resuspended in 100 μl of Nucleofector-T solution. Cells were electroporated with 20 nM siRNA in an Amaxa electroporation unit using the T-20 program. Cells were plated in 96-well plates. Twenty-four hours after transfection, cells were treated with vehicle (0.1% BSA) or netrin-1 (100 ng/ml). Forty-eight hours after netrin-1 addition, cell proliferation was quantitated with the MTT assay. To confirm the receptor mRNA knockdown, cells were harvested at 48 h after transfection and RNA was isolated. Receptor expression was determined by real-time RT-PCR.

Creation of stable TKPTS cell lines expressing short hairpin RNA for netrin-1, UNC5B, and neogenin. TKPTS cells stably expressing short hairpin (shRNA) for UNC5B, neogenin, or netrin-1 were created by transfecting lentiviral vectors containing shRNAs and the puromycin resistance gene. All clones selected were shown to have normal expression of the target genes. All clones selected were shown to have normal expression of the target genes. Seventy-two clones were screened. Five clones for each gene demonstrated >80% knockdown were selected and frozen in liquid nitrogen. All clones selected were shown to have normal expression of the other receptors.

Quantitation of mRNA by real-time RT-PCR. Real-time RT-PCR was performed in an Applied Biosystems 7700 Sequence Detection System (Foster City, CA). cDNA was obtained by reverse transcribing 1 μg of total RNA in a reaction volume of 20 μl with Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl, and 6 μl aliquots were used as templates for amplification with the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were mouse netrin-1 (forward: AAGCCTATCACCCACCCGAAG; reverse: GGCCCAACAGATCTTGAAGTC), UNC5A (forward: ATCCCTAACAAGATTCTGATG), UNC5B (forward: TGGATCTTTCAGCTCACAGCC; reverse: AAGGAGGCGCA-GCTGGGAGCC), UNC5C (forward: GATGAAATCTTCTGGTCATGTGGTTG; reverse: CTCCTCGACCTTCTCTGATGA), UNC5D (forward: GTGAACATCTTCGTATCCGT; reverse: TTCTCAATGCCCTTCTTACTC), DCC (forward: CTCTTACAGGATTTGGAGAAAGG; reverse: GAGGAGGTGTCACACTGATG), and neogenin (forward: CGCTACCTGATTTGAAGTG; reverse: GATGATGTAACCTGTAATCTGGC). The amount of DNA was normalized to the β-actin signal amplified in a separate reaction (forward primer: AAGGGAATCTGCGGCGT; reverse: CAAATGTGATGACCTGGCCG).

Scratch wound assay for netrin-1 expression. TKPTS cells were grown to confluence in chamber slides, and a single scratch wound was created in each well with a 1-ml pipette tip. Twenty-four hours after creation of the wound, cells were fixed in cold methanol followed by 4% paraformaldehyde for 10 min, and then immunocytochemical localization of netrin-1 was carried out. Briefly, cells were permeabilized with PBS-0.3% Triton X-100, and nonspecific sites were blocked with 5% normal goat serum-1% BSA in PBS and incubated with chicken anti-netrin-1 antibody (Neuromics) overnight at 4°C. After washing, cells were incubated with FITC-conjugated goat anti-chicken antibody for 1 h. Slides were washed and mounted with aqueous mounting medium containing DAPI. Staining was visualized and photographed with a Leica TCS SP2 AOBs (Exton, PA) confocal microscope at the Penn State Hershey College of Medicine Imaging Core.

Cell migration assay. Epithelial cell migration assays were carried out according to the manufacturer’s instructions (Cell Biolabs). Briefly, TKPTS cells grown in T-25 flasks were serum starved for 24 h. Cells were harvested by trypsinization and counted. A cell suspension containing 1 million cells/ml was prepared in serum-free medium. Aliquots (300 μl) of the cell suspension were added to the upper well of the migration chamber. The lower chambers contained 500 μl of medium containing 10% fetal bovine serum with or without varying concentrations of recombinant mouse netrin-1. Inhibitors of MEK1/2 (U0126) and PI3-kinase (LY-294002) were added to both the upper and lower chambers at a concentration of 10 μM, as needed. Cells were incubated for 24 h in a CO2 incubator. At the end of the incubation, the medium from the upper chamber was aspirated and the filter insert was transferred into a clean well containing 225 μl of cell detachment solution. The insert was incubated at 37°C for 30 min to allow detachment of cells from the bottom surface of the filter. Cells recovered from bottom of the insert into the detachment solution were quantitated with CyQuant GR dye. CyQuant GR dye was diluted in 4X lysis buffer, 75 μl of 4X lysis buffer was added to each well and incubated for 20 min at room temperature, and fluorescence was read at 480 nm (excitation) and 530 nm (emission).

RESULTS

Netrin-1 and its receptors are expressed in mouse proximal tubular epithelial cells. Netrin-1 and its receptors UNC5B and UNC5C are abundantly expressed in mouse kidney. However, the site of receptor expression is unknown. To determine the physiological roles of netrin-1 and its receptors in kidney epithelial cells, we first determined the expression of netrin-1 and its receptors in mouse proximal tubular epithelial cells (TKPTS). Netrin-1 is abundantly expressed, at levels similar to those in brain (Fig. 1A). UNC5B and neogenin were expressed at levels ~30% and 54%, respectively, of those in brain. However, the netrin-1 receptors DCC, UNC5A, UNC5C, and UNC5D were either undetectable or showed very low levels of expression. Western blot analysis of netrin-1, UNC5B, UNC5C, and neogenin was also performed. Both UNC5B and neogenin protein expression were detectable in confluent and subconfluent TKPTS cells (Fig. 1B). However, there was no detectable UNC5C protein expression. As shown in Fig. 1C, basal netrin-1 expression in TKPTS cells was 4 ng/50 μg of total protein or per million cells.

Netrin-1 increases proximal tubular epithelial cell proliferation. Although netrin-1 is abundantly expressed in renal proximal tubular epithelial cells in vitro and highly induced after ischemia-reperfusion injury in vivo (30), the physiological actions of netrin-1 in the kidney are unknown. To determine whether netrin-1 affects tubular epithelial cell proliferation, TKPTS cells were treated with increasing concentrations of mouse recombinant netrin-1. Netrin-1 increased cell proliferation in a biphasic manner (Fig. 2). The maximum effect was seen at 100 ng/ml. Beyond 100 ng/ml, netrin-1 suppressed cell proliferation.

ERK MAPK kinase is rapidly activated by netrin-1 and mediates proliferative effect of netrin-1. Several pathways are activated in response to netrin-1 binding to its receptors in various cells and tissues (2, 9). However, the pathways that are activated by netrin-1 and their role in mediating the proliferation of renal epithelial cells are unknown. To determine the pathways that are activated by netrin-1, TKPTS cells were treated with 100 ng/ml of netrin-1 and harvested at different times after netrin-1 addition. As shown in Fig. 3A, netrin-1 rapidly activates ERK and Akt as reflected by increased phosphorylation of these kinases. However, there was no activation of PKA, PKC, and FAK-1, which are known to be activated in...
neuronal cells by netrin-1. Increases in phosphorylation of ERK and Akt were seen as early 10 min after netrin-1 treatment and reached a maximum at 60 min (Fig. 3B). Addition of the MEK1/2 inhibitor U0126 suppressed the proliferative effect of netrin-1 in TKPTS cells. Unlike U0126, a PI3-kinase inhibitor (LY-294002) inhibited both basal and netrin-1-induced proliferation of tubular epithelial cells (Fig. 4).

UNC5B mediates proliferative effects of netrin-1 on renal epithelial cells. To examine the receptor subtype that mediates proliferation in response to netrin-1, TKPTS cells were transfected with UNC5B or UNC5C siRNA. As shown in Fig. 5A, netrin-1 increased cell proliferation in mock-transfected and UNC5C siRNA-transfected epithelial cells. The proliferative effect of netrin-1 was completely abolished in UNC5B siRNA-transfected cells. As shown in Fig. 5B, siRNA transfection in TKPTS cells reduced UNC5B mRNA levels by 80%, whereas UNC5C mRNA levels were undetectable before and after UNC5C siRNA transfection.

Fig. 2. Dose-dependent regulation of tubular epithelial cell proliferation by netrin-1 in vitro. Tubular epithelial cells were treated with increasing concentrations of netrin-1. Forty-eight hours after netrin-1 addition, cell proliferation was quantitated by MTT assay. *P < 0.005 vs. vehicle-treated group. n = 4. OD, optical density.
Netrin-1 is highly induced in edges of wounds in vitro. Netrin-1 is known to mediate cell proliferation and migration during development (3, 10, 29, 33). Recovery from acute renal failure requires the replacement of injured cells with new cells that reline and restore tubule epithelial integrity (23). The growth of new cells from the edges of the injured tubules may involve the expression of migratory cues, as occurs in skin regeneration (19). To determine whether netrin-1 is induced after injury in vitro, we performed wound assays. Netrin-1 expression was minimal in confluent cells (Fig. 6B) but highly induced at the edges of wounds (Fig. 6, C and D). High levels of netrin-1 were also seen in the tubular epithelial cells located at the edges of islands of proliferating cells (Fig. 6F). A second antibody control did not show any staining (Fig. 6, A and E).

Netrin-1 increases renal tubular epithelial cell migration via UNC5B in vitro. Cell migration plays an important role in tissue recovery after injury. To determine the effects of netrin-1 on renal tubular epithelial cell migration, we quantified the migration of cells through filter inserts into chambers filled with medium containing netrin-1. Netrin-1 increased the migration of TKPTS cells in a dose-dependent manner, with a maximum effect at a concentration of 100 ng/ml (Fig. 7). There was no further increase at 200 and 400 ng/ml concentrations. The migration was enhanced when endogenous netrin-1 expression was knocked down with siRNA (Fig. 7D). To determine which receptor mediates netrin-1-induced migration, the migration assay was repeated with cells in which the expression of UNC5B and neogenin were knocked down. UNC5B knockdown completely abolished the effect of netrin-1 on epithelial cell migration (Fig. 7B), suggesting that UNC5B mediates netrin-1 effects on cell migration. In contrast, cells with neogenin knockdown showed enhanced migration (Fig. 7C), suggesting that neogenin activation may inhibit cell migration.

ERK and PI3-kinase pathways inhibit netrin-1-induced renal tubular epithelial cell migration. To determine whether the ERK and Akt pathways activated by netrin-1 (Fig. 3) mediate tubular epithelial cell migration, specific pathway inhibitors were added during the migration assay. Netrin-1-induced cell migration was enhanced in the presence of U0126 or LY-294002 (Fig. 8). The inhibitors alone also increased the basal migration. These data suggest that ERK and Akt pathways may antagonize netrin-1-induced migration.

DISCUSSION

In recent work (30), we determined that netrin-1 is highly upregulated in renal epithelial cells in response to ischemia-reperfusion injury. We also determined that exogenous netrin-1 administration ameliorates ischemia-reperfusion injury. However, the cellular mechanisms whereby netrin-1 reduces renal injury were not determined. The present studies provide several insights into this issue. First, we show for the first time that netrin-1, a neuronal attractant, has a previously unidentified role in stimulating the proliferation and migration of renal tubular epithelial cells. Second, using stable cell lines in which
the expression of netrin-1 and netrin-1 receptors UNC5B, UNC5C, and neogenin were reduced by shRNA, we show that netrin-1-induced cell proliferation and migration are mediated via the UNC5B receptor. Third, we show that the proliferative effect is mediated by the ERK MAPK pathway, whereas both ERK and Akt pathways inhibit migration. Together our results suggest that netrin-1 regulates renal tubular epithelial cell proliferation and migration in response to injury.

Although a possible role of netrin in nerve regeneration has long been suggested in a variety of experimental models (5, 18), the molecular details are still unknown. Recent studies indicate that netrin-1 may promote nerve regeneration through Schwann cell proliferation (13). The kidney has an enormous capacity to recover from mild to moderate injury. Our earlier study (30) suggested that exogenous netrin-1 reduces ischemia-reperfusion injury of the kidney. However, the exact mechanism responsible for this effect was not determined. The recovery from tubular epithelial death involves both proliferation of surviving cells and their migration to reestablish an intact epithelium. In the present study, netrin-1 was found to stimulate renal tubular epithelial cell proliferation and also epithelial cell migration. Accordingly, netrin-1-mediated protection against ischemia-reperfusion injury may be due to enhanced rates of proliferation and migration of renal tubular epithelial cells after injury. Consistent with our findings, Lee et al. (13) and Park et al. (24) reported that netrin-1 may also increase cell proliferation of Schwann cells and vascular smooth muscle cells. In addition, that netrin-1 may also increase cell proliferation in vivo is suggested by our preliminary findings that netrin-1 transgenic mice have increased numbers of proximal tubular epithelial cells proliferating under basal conditions (unpublished data).

We report that only UNC5B and neogenin, and not DCC, UNC5A, UNC5C, or UNC5D, are expressed in proximal tubule cells. Recent studies have indicated a crucial role for UNC5B rather than DCC and neogenin in mediating the effects of netrin-1 in adult nonneuronal cells such as endothelial cells and leukocytes (15, 17). Our studies using siRNA knockdown revealed that UNC5B but not neogenin or UNC5C is required for the netrin-1-induced proliferation and migration of tubular epithelial cells.

Akt and ERK MAPK pathways have been shown to play important roles in the proliferation of various cells (26, 31) including renal epithelial cells (12, 20, 35). Our studies show
that netrin-1 rapidly activates both Akt and ERK MAPK in tubular epithelial cells. Although both pathway inhibitors inhibited the basal rate of cell proliferation, only the ERK inhibitor reduced netrin-1-induced epithelial cell proliferation. Although several other pathways, including FAK-1, PKA, and PKC, have been shown to be activated in other cells in response to netrin-1 (2), these kinases were not activated in renal tubular epithelial cells. The reason for this difference is not clear. Of note, our data reveal inhibitory roles for PI3-

kinase and ERK in netrin-1-induced epithelial cell migration. The pathways that mediate tubular epithelial cell proliferation and migration may be stimulus specific (34, 35). For example, EGF receptor (EGF-R) stimulation activates Akt, ERK, and p38 MAPK. However, EGF-R-induced cell proliferation proceeds through Akt rather than ERK MAPK, and cell migration proceeds through p38 MAPK (34, 35).

Neogenin is a multifunctional receptor regulating diverse developmental processes. The functional outcome of neogenin activation is dictated by both the nature of the ligand as well as the developmental context. A new family of neogenin ligands, the repulsive guidance molecule (RGM) family, was recently identified (4). Netrin-1-neogenin interactions are known to mediate chemoattractive axon guidance, while RGM-neogenin interactions repel axons (4). Our studies with shRNA knockdown suggest that, unlike in the nervous system, neogenin may antagonize the migration mediated by UNC5B. Whether netrin-1, as opposed to an RGM ligand, initiates the antimigration effects of neogenin on renal epithelial cells is not clear.

In conclusion, a new role for netrin-1 as a mitotic factor for renal tubular epithelial cells and as a migration factor has been identified in this study. Our results reinforce the concept that chemoattractant/repulsive cues may provide significant signals for cell proliferation and survival. The identification of these novel functions of netrin-1 has implications for the treatment of acute kidney injury and for research into renal regeneration.

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