Published in final edited form as:

Am J Physiol Renal Physiol. 2008 April ; 294(4): F739–F747. doi:10.1152/ajprenal.00508.2007.

Netrin-1 and kidney injury. I. Netrin-1 protects against ischemia-reperfusion injury of the kidney

Weiwei Wang, W. Brian Reeves, and Ganesan Ramesh

Division of Nephrology, The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania

Abstract

Endogenous mechanisms exist to limit inflammation. One such molecule is netrin. This study examined the impact of ischemia-reperfusion (I/R) on netrin expression and the role of netrin in preventing renal inflammation and injury. All three isoforms of netrin (1, 3, and 4) are expressed in normal kidney. I/R significantly downregulated netrin-1 and -4 mRNA expression, whereas expression of netrin-3 was moderately upregulated at 24 h of reperfusion. The netrin receptor UNC5B mRNA increased at 3 h and but decreased at later time points. Expression of a second netrin receptor, DCC, was not altered significantly. I/R was associated with dramatic changes in netrin-1 protein abundance and localization. Netrin-1 protein levels increased between 3 and 24 h after reperfusion. Immunolocalization showed an interstitial distribution of netrin-1 in sham-operated kidneys which colocalized with Von Willebrand Factor suggesting the presence of netrin-1 in peritubular capillaries. After I/R, interstitial netrin-1 expression decreased and netrin-1 appeared in tubular epithelial cells. By 72 h after reperfusion, netrin-1 reappeared in the interstitium while tubular epithelial staining decreased significantly. Downregulation of netrin-1 in the interstitium corresponded with increased MCP-1 and IL-6 expression and infiltration of leukocytes into the reperfused kidney. Administration of recombinant netrin-1 significantly improved kidney function (blood urea nitrogen: 161 ± 7 vs. 104 ± 24 mg/dl, creatinine: 1.3 ± 0.07 vs. 0.75 ± 0.16 mg/dl, $P < 0.05$ at 24 h) and reduced tubular damage and leukocyte infiltration in the outer medulla. These results suggest that downregulation of netrin-1 in vascular endothelial cells may promote endothelial cell activation and infiltration of leukocytes into the kidney thereby enhancing tubular injury.

Keywords

netrin-4; inflammation; endothelial cell; chemokine

Acute kidney injury (AKI) is an unfortunately common and serious complication of hospitalized patients. Ischemia-reperfusion (I/R) injury is the leading cause of AKI in native or transplanted kidneys (26). Despite recent advances in supportive care, the incidence of AKI continues to increase (29). The pathophysiology of AKI due to I/R includes a complex interplay between vascular endothelial cell dysfunction, subsequent inflammation, and tubular cell damage (16). Inflammation is known to contribute to I/R-induced tissue damage through release of mediators such as reactive oxygen species, proteases, cytokines, and chemokines (i.e., TNF- α , IL-1, MCP), downregulation of anti-inflammatory molecules (IL-10), and upregulation of adhesion molecules (ICAM-1, E-selectin, and VCAM-1) (4, 5,

7, 13, 21, 27). The netrins were discovered about a decade ago as neuronal guidance cues (23). Netrins are laminin-like molecules with a distinctive domain organization and belong to the laminin-related family of axon-guidance molecules (1). Four netrins have been identified so far in different species. Mice express netrin-1, -3, and -4 which are encoded by distinct genes. Mouse netrin-1 shares 52% amino acid identity with mouse netrin-3, and 98 and 87% aa identity with human and chicken netrin-1, respectively (1, 23). Netrins mediate their effects through two known receptors, Deleted in Colon Cancer (DCC) and UNC5 (1). The DCC receptors, which include DCC and neogenin in vertebrates, function as homodimers to induce growth cone attraction, whereas the four UNC5 receptors (UNC5A-D in mouse and UNC5H1-4 in human) mediate netrins' repulsive effect, either by themselves or by forming heterodimers with DCC receptors (1). Recently, it was shown that some effects of netrin are not mediated through DCC and UNC5 receptors (30), suggesting the presence of unidentified receptors for netrins. Recent studies indicate various other roles of netrins beyond axonal guidance including development of mammary gland, lung, pancreas, and blood vessels, inhibition of leukocyte migration during sepsis, mitogenesis, and chemoattraction of endothelial cells (15, 30). Netrin-1 is a potent inhibitor of leukocyte chemoattraction in vivo and prevented leukocyte migration in a peritonitis model (15). Although the kidney has one of the highest levels of netrin expression (15), the roles of netrin in kidney physiology and pathophysiology are unknown. The current studies explored the hypothesis that renal netrin expression may play a protective role against the inflammatory response seen in I/R injury.

We determined the changes in netrin expression in response to I/R of the kidney and whether netrin plays a role in preventing acute renal inflammation and injury of the kidney. We found that netrins (1, 3, and 4) and their receptors (DCC and UNC5b) are expressed in the kidney. Immunolocalization revealed that netrin-4 was localized in basement membrane, whereas netrin-1 prominently localized in the interstitium in normal kidney consistent with expression in peritubular capillaries. Within 3 h after reperfusion, netrin-1 protein expression was highly induced in tubular epithelial cells with a decrease in interstitial expression. By 24 h of I/R, netrin-1 staining was seen only in tubular epithelial cells. Administration of recombinant mouse netrin-1 prevented I/R of the kidney, reduced chemokine expression, and reduced leukocyte infiltration. Together, these data suggest that downregulation of netrin-1 in the peritubular capillaries may promote infiltration of leukocytes, whereas netrin-1 normally may counter this process. We propose that modulation of netrin-1 expression may be effective in preventing ischemic-reperfusion injury of kidney.

MATERIALS AND METHODS

Renal I/R

Protocol number 2007-003, entitled "Netrin-1 in ischemic reperfusion injury of kidney," was approved by the IACUC on April 6, 2007. C57BL/6J mice (8–9 wk of age, The Jackson Laboratory, Bar Harbor, ME) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and were placed on a heating pad to maintain core temperature at 37°C. Both renal pedicles were identified through dorsal incisions and clamped for 26 min. Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline (ip) and kept in a warm incubator until they regained consciousness.

Drug administration

Animals were administered recombinant mouse netrin-1 (R&D Systems) or netrin-4 (R&D Systems) at a dose of either 1 or 5 μ g/animal or vehicle (0.1% BSA) intravenously through the tail vein in a volume of 0.3 ml 2 h before the surgery.

Renal function

Renal function was assessed by measurements of blood urea nitrogen (VITROS DT60II Chemistry slides, Ortho-clinical Diagnostics) and serum creatinine (cat. no.: DZ072B, Diazyme Labs).

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). Total RNA (1.5 μ g) was reverse transcribed in a reaction volume of 20 μ l using Omniscript RT kit and random primers. The product was diluted to a volume of 150 μ l and 6- μ l aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were as follows: mouse TNF- α (forward: GCATGATCCGCGACGTGGAA; reverse: AGATCCATGCCGTTG GCCAG), MCP-1 (forward: ATGCAGGTCCCTGTCATG; reverse: GCTTGAGGTGGTTGTGGA), IFN- γ (forward: TCAGCAACAGCAAGGCGAAAAAG; reverse: ACCCCGAATCAGCAGCGACTC), IL-6 (forward: GATGCTACCAAACTGGATATAATC; reverse: GGTCCCTTAGCCACTCCTTCTGTG), ICAM-1 (forward: AGATCACATTCACGGTGCTG; reverse: CTTGAGAGGCAGGAAACAGG), VCAM-1 (forward: ATTTTCTGGGGCAGGAAGTT; reverse: ACGTCAGAACAACCGAATCC), E-selectin (forward: AGCTACCCATGGAACACGAC; reverse: ACGCAAGTTCTCCAGCTGTT), netrin-1 (forward: AAGCCTATCACCCACCGGAAG; reverse: GCGCCACAGGAATCTTGATGC), netrin-4 (forward: AACAGGGGCTCCTAACGAAT; reverse: GTCTTCTGAGGTCGCGGTAG), UNC5A (forward: ATCCCTAACACAGGAATCAGC; reverse: CTAACGATAGGACTCAGCAGG), UNC-5B (forward: TGGATCTTTTCACTCAAGACCCAG; reverse: AAGATGGCCAGCTGGAGCCG), UNC5C (forward: GATGAAACCTCTGGTCTAATTGTG; reverse: CCTTCCGACTCTTCGTAGTG), UNC5D (forward: GTGAACATCTTCGTATCCGT; reverse: TTCTCAATGCCTCTCCTACTC), DCC (forward: CTCTTCACAGGATTGGAGAAAGGC; reverse: GAGGAGGTGTCCAACATCATGATG), and netrin-3 (forward: TGGCTGGTTGACTTACAGCGG; reverse: TACAAGAGCGAGGCTCCCTCG). The amount of DNA was normalized to the β -actin signal amplified in a separate reaction (forward primer: AGAGGGAAATCGTGCCTGAC; reverse: CAATAGTGATGACCTGGCCGT).

Cell culture

Immortalized temperature-sensitive mouse renal endothelial cells kindly provided by Prof. I. J. Fidler (The University of Texas, M.D., Anderson Cancer Center) were cultured in DMEM supplemented with glutamine, sodium pyruvate, nonessential amino acid, vitamins, 10% FBS, and antibiotics and grown at 33°C. Cells were transferred to 37°C 3 days before experimental analysis. Immortalized mouse kidney proximal tubular epithelial cells (TKPTS) were cultured as described before (19). For in vitro simulated I/R, cells were serum starved for 24 h and then the medium was replaced by washing with HBSS followed by overlaying mineral oil (Sigma, cat. no. M-5904) to induce hypoxia for 30 or 60 min. At end of the hypoxic time, the mineral oil and HBSS were aspirated, washed, and replaced

with serum-containing medium. Twenty-four hours after reoxygenation, cells were harvested and proteins were extracted for Western blot and RT-PCR analysis.

Western blot analysis

Protein was extracted by solubilizing cells or kidney in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was quantitated using the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL) and 50 μ g of total protein were loaded onto 4–12% polyacrylamide gels, separated, and then transferred onto a PVDF membrane. The membrane was probed with rabbit anti-netrin-1 antibody (Calbiochem cat. no. PC344). Proteins were detected using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Immunohistochemical localization of netrin-1 and -4

Immunohistochemical localization of netrin-1 and -4 was performed as described before (15) with modification. Briefly, the kidneys were perfused with PBS and fixed overnight in 4% paraformaldehyde and then transferred to 30% sucrose. Kidney was placed in a cryomold and frozen. Six-micrometer-thick sections were placed on glass slides. Sections were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS, washed, and blocked with PBS containing 5% donkey serum and 1% BSA. Primary antibodies included a chicken anti-netrin-1 polyclonal antibody (Neuromics cat. no. CH23002) and goat anti-netrin-4 polyclonal antibody (R&D Systems, cat. no. AF1132). Primary antibodies were detected using secondary antibodies conjugated with FITC (Abcam). Slides were mounted in aqueous mounting medium (Santa Cruz Biotechnology) and viewed using a Leica confocal microscope. To colocalize netrin-1 and Von Willebrand Factor (VWF) or Tie-2 GFP, we used an FITC-conjugated sheep anti-VWF antibody (abcam, cat. no. 8822) and chicken anti-netrin-1 polyclonal antibody followed by a goat anti-chicken cy5 conjugate (Abcam).

Histology

Kidney tissue was fixed in buffered 10% formalin for 12 h and then embedded in paraffin wax. For assessment of injury, 5- μ m sections were stained with periodic acid Schiff (PAS). To quantitate leukocyte infiltration, sections were stained with naphthol AS-D choroacetate esterase (Sigma kit no. 91A) which identifies neutrophils and monocytes. Twenty-five $\times$ 40 fields of esterase-stained sections were examined for quantitation of leukocytes.

Statistical methods

All assays were performed in duplicate. The data are reported as means $\pm$ SE. Statistical significance was assessed by an unpaired, two-tailed Student's *t*-test for single comparison or ANOVA for multiple comparisons.

RESULTS

Netrin mRNA expression is downregulated in response to I/R

Earlier studies showed that netrins are expressed in tissues outside the nervous system and highest expression was found in the kidney (6, 14, 15). The expression of netrin was downregulated during sepsis-induced inflammation of the lung (15). We hypothesized that netrin-1 was downregulated in the kidney after I/R and that this downregulation contributes to leukocyte infiltration and tissue injury. All three netrins (1, 3, and 4) and the netrin receptors UNC5B and UNC5C were highly expressed in normal kidney (Fig. 1, *A* and *B*). DCC and UNC5A expression were low but detectable while UNC5D mRNA expression was undetectable (Fig. 1*A*). The expression of netrin-1 was significantly downregulated at 6 and

24 h after reperfusion and recovered to baseline levels by 48 h, whereas netrin-4 expression was chronically downregulated after 24 h (Fig. 1B). Netrin-3 expression was not altered significantly (not shown). Two of the prominent netrin receptors were analyzed by real-time PCR. DCC and UNC5B receptor expression showed an early increase and then decrease after 48 h of reperfusion (Fig. 1B).

Netrin-1 protein is induced in tubular epithelial cells and reduced in endothelium after I/R

To determine whether mRNA expression correlated with protein expression, netrin-1 and -4 were analyzed by Western blot. In contrast to mRNA, netrin-1 protein increased gradually starting from 3 h and reached peak levels at 24 h after reperfusion (3-fold; Fig. 2) and returned to baseline levels by 72 h. This result suggests that netrin-1 may be regulated at the translational level. Netrin-4 protein abundance was not altered significantly (not shown). We next examined the cellular distribution of netrin-1 and -4. Netrin-4 was localized in peritubular basement membranes in sham-operated animals and its localization was not altered significantly after I/R except at later time points when netrin-4 staining decreased and appeared in the luminal surface or mixed with cell debris (Fig. 3). Netrin-1 protein was primarily localized in the interstitium with very little staining in tubular epithelial cells in normal kidney (Fig. 3). Netrin-1 expression was colocalized with VWF and with endothelial-targeted GFP (Fig. 4) suggesting expression in the peritubular capillaries. However, within 3 h after reperfusion, netrin-1 appeared in tubules and decreased in the interstitium. Twenty-four hours after reperfusion, most of the staining was in tubular epithelial cells and very little or no staining was seen in the interstitium. By 72 h after reperfusion, tubular epithelial staining decreased and interstitial staining reappeared (Fig. 3).

In vitro hypoxia alters netrin-1 expression in cultured endothelial and renal tubular epithelial cells

Consistent with its interstitial localization in vivo, netrin-1 is expressed in renal microvascular endothelial cells in vitro (Fig. 5). Netrin-1 mRNA expression in endothelial cells was upregulated significantly 24 h after hypoxia-reoxygenation (Fig. 5C); however, the protein expression was not changed (0.9 ± 0.02 -fold vs. control, $P > 0.05$ at 30 min; Fig. 5A). In contrast, mouse proximal tubular epithelial cells (TKPTS) expressed low levels of netrin-1 which were highly induced after 30 min of hypoxia-reoxygenation (1.7 ± 0.02 -fold vs. control, $P < 0.001$; Fig. 5B) and which was associated with downregulation of netrin-1 mRNA (Fig. 5C).

Recombinant netrin-1 improves kidney function and reduces leukocyte infiltration

Netrin-1 is highly expressed in endothelial cells where it is downregulated in response to ischemia in vitro. Earlier studies showed that netrins act on leukocytes to inhibit migration across the endothelium (15). We hypothesized that the observed downregulation of netrin-1 in the interstitium may promote endothelial cell activation, leukocyte infiltration, and subsequent renal injury. Administration of recombinant netrin-1 ($1 \mu\text{g}/\text{animal}$) 2 h before I/R improved renal function moderately [blood urea nitrogen (BUN): 148 ± 10 vs. 180 ± 4 mg/dl] at 24 h while administration of $5 \mu\text{g}/\text{animals}$ improved renal function significantly at 24 h (BUN: 104 ± 24 vs. 162 ± 7 mg/dl; creatinine: 1.2 ± 0.07 vs. 0.7 ± 0.1 mg/dl at 24 h, $P > 0.05$, $n = 4-6$) and 48 h after reperfusion (Fig. 6). Consistent with the improvement in renal function, neutrophil infiltration was also reduced significantly in netrin-1-treated kidneys compared with vehicle-treated animals (Fig. 7). In addition, histological assessment indicated that vehicle-treated kidneys contained severe injury in the outer stripe of outer medulla including cast formation, sloughing of tubular epithelial cells, and loss of brush-border membrane. These changes were minimal in netrin-1-treated animals. Of interest, administration of a similar dose ($5 \mu\text{g}/\text{animals}$) of recombinant netrin-4 did not improve renal function (BUN: 178 ± 20 vs. 151 ± 7 at 24 h, $P > 0.05$, $n = 5-7$; Fig. 8).

I/R injury is associated with upregulation of adhesion molecules and inflammatory cytokines. Inhibition of the expression or function of many of these molecules provides protection against ischemic renal injury and leukocyte infiltration (8, 11, 12, 18). Therefore, we examined the effect of netrin-1 administration on MCP-1, IL-6, ICAM-1, VCAM-1, and E-selectin expression. As shown in Fig. 9, I/R increased expression of MCP-1, IL-6, and ICAM-1 several fold as early as 3 h and persisted even after 24 h. Administration of recombinant netrin-1 significantly downregulated MCP-1 mRNA at 24 h. There was no change in IL-6 expression. Although ICAM-1, VCAM-1, and E-selectin showed a 50% reduction in expression in netrin-1-treated animals, this did not reach statistical significance.

DISCUSSION

I/R injury of the kidney results from a sequence of events which includes endothelial cell activation and swelling, increase in vascular permeability, infiltration of leukocytes, loss of polarity and cytoskeletal integrity of the tubules, necrosis and apoptosis, production of proinflammatory cytokines, and interstitial inflammation (2–4, 7, 8, 16, 24). Barriers are present in normal tissues to prevent the influx of inflammatory cells, endothelial cell activation, and inflammation. These molecules are produced by kidney epithelial, endothelial, vascular, and interstitial cells and include growth factors (e.g., VEGF, IGF-1), anti-inflammatory cytokines (IL-10), nitric oxide, and heme oxygenase (10, 22, 25, 26, 28). We propose, based on the current results, that endothelial netrin-1 represents yet another homeostatic protein whose dysregulation after ischemia contributes to the development of organ failure.

Netrins were discovered as prototypical axonal attractants, initially identified as extracellular factors secreted from the floor plate that attract spinal commissural axons toward the midline (23). The netrin family in mouse includes netrin-1, -3, -4 and the netrin-related molecules netrin-G1 and netrin-G2. Netrins mediate their action through deleted in colorectal cancer (DCC and neogenin) and the UNC5 family of receptors (a–d), particularly UNC5B. Recent studies indicated a role for netrins beyond the nervous system including the development of mammary gland, lung, pancreas, and blood vessels (6, 30). More recently, it was shown that netrin-1 promotes blood perfusion after skeletal muscle ischemia, promotes revascularization, improves nerve conduction velocity in diabetic mice (30), and inhibits leukocyte migration in sepsis (15). Netrin-1 is expressed in many tissues including brain, lung, heart, liver, intestine, and kidney (15). Kidney is among the highest netrin-1-expressing organs studied. Despite its high expression, the role netrins play in kidney physiology and pathophysiology is unknown. The current study sheds some novel insights regarding the roles of netrins in I/R injury of kidney.

We found that all three netrins (1, 3, and 4) and their receptors, DCC and UNC5A–C, were expressed in normal kidney. I/R injury was associated with dramatic changes in the abundance and localization of netrin-1. Expression of netrin-1 and -4 mRNA was downregulated 6 h after reperfusion. Although netrin-1 returned to normal by 48 h after reperfusion, netrin-4 mRNA was persistently downregulated. In contrast to the mRNA levels, netrin-1 protein abundance in the kidney increased approximately threefold after ischemia. Moreover, the distribution of netrin-1 changed from being primarily endothelial to being primarily epithelial.

The changes in endothelial and epithelial netrin-1 expression could be recapitulated in vitro using cultured renal microvascular endothelial cells and proximal tubule cells. The functional significance of these changes was assessed by administering recombinant netrins to mice before I/R injury. Netrin-1 exerted a dose-dependent protection against renal dysfunction. Netrin-4 had no effect. Since exogenous netrin-1 was protective, we speculate

that the observed loss of netrin-1 in endothelial cells, rather than increase in epithelial netrin-1, contributes to I/R injury. Netrin-1 was colocalized with VWF suggesting vascular expression in sham-operated animals. Studies showed that VWF is produced in cells other than endothelial cells such as megakaryocytes (present in α -granules of platelets) and subendothelial connective tissues. Apart from plasma, VWF is present in the extracellular matrix of the subendothelium (20). The VWF and netrin-1 staining pattern suggests that netrin-1 in normal kidney might be localized in the extracellular matrix of the subendothelium where it may regulate the function of the infiltrating leukocytes.

Netrin-1 is potent inhibitor of leukocyte migration across the endothelium (15). Netrin receptor DCC and UNC5b showed to be expressed on the leukocytes. We show that exogenous netrin-1 improved renal function and decreased the expression of MCP-1, adhesion molecules, and the infiltration of leukocytes into the kidney. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates monocyte migration into the intima of arterial walls and organs (21). MCP-1 is produced by both macrophages and renal parenchymal cells (9, 19). Using CCR2-deficient mice, Furuichi et al. (9) established a role for MCP-1 in ischemic kidney injury. Likewise, adhesion molecules, such as ICAM-1, play important roles in ischemic renal injury (12). We hypothesize that netrin-1 produced by vascular endothelial cells may inhibit MCP-1 and ICAM production and leukocyte infiltration or may act directly on leukocytes to inhibit migration across endothelial cells. Downregulation of netrin-1 in the endothelium during ischemia may relieve this inhibitory effect on leukocytes and lead to increased inflammation. Netrin-1 is also known to increase blood perfusion after an ischemic insult in skeletal muscle (30). Whether a similar mechanism may exist in I/R of the kidney is not clear. The signal for the downregulation of endothelial netrin-1 was not investigated here. Earlier studies showed that netrin-1 mRNA expression is downregulated by TNF- α and INF- γ in cultured endothelial cells (15). Since TNF- α mRNA and protein are increased in renal I/R (7), TNF- α may be responsible for the changes in netrin-1 expression. Further in vitro studies using cultured renal microvascular endothelial cells and in vivo studies in TNF- α -deficient mice could address this possibility.

The protective effect of recombinant netrin-1 against I/R injury was significantly better than netrin-4, although they belong to same family of proteins. It is interesting to note that the localization pattern and regulation of expression of netrin-1 and -4 were completely different. Earlier studies showed that although netrin-1 and -4 were equally effective in inducing endothelial proliferation and tube formation in vitro, when administered in vivo to ischemic skeletal muscle, netrin-1 induced more vessel formation and VEGF than netrin-4 (30). Moreover, netrin-1 and -4 do not bind the same receptors to mediate their actions. The localization of netrin-4 in basement membrane suggests it may participate in adhesion and migration of epithelial cells and may function in maintaining the polarity of tubular epithelium. The cells which produce netrin-4 in adult mouse kidney are not known. It was shown to be expressed by tubular epithelial cells during development (31).

Netrin-1 protein expression was found in normal kidney localized mostly in the endothelium consistent with in vitro finding that netrin-1 is highly expressed in renal microvascular endothelial cells. Within 3 h after reperfusion, the endothelial staining decreased and netrin-1 began to appear in tubular epithelial cells. The signal for netrin-1 upregulation in the tubular epithelial cell is not known. Since netrin-1 mRNA did not increase in concert with protein abundance, netrin-1 production by epithelial cells may be regulated at the translational level. We previously showed that production of TNF- α by renal epithelial cells is also under translational regulation (19). However, the physiological role of epithelial netrin-1 in renal injury is unclear. Studies using tissue-specific deletion of netrin-1 may be needed to address this issue.

In summary, all three netrins and two receptors (DCC and UNC5b) were expressed in the normal mouse kidney. Netrin-1 was localized in the interstitium consistent with vascular endothelial expression, whereas netrin-4 was localized in the tubular basement membrane. In response to I/R, netrin-1 expression was downregulated in the subendothelial matrix but upregulated in tubular epithelial cells. Administration of recombinant netrin-1 improved ischemia-induced renal dysfunction, perhaps by reducing chemokine expression and leukocyte infiltration. Since previous studies suggest a role for netrin-1 in maintaining vascular endothelial function (17), netrin may have some utility as therapeutic agent in ischemic renal injury and other kidney diseases involving microvascular dysfunction, such as diabetic nephropathy. In addition, the role of urinary netrin-1 measurement for the early diagnosis of AKI is addressed in a companion paper to this report (20a).

Acknowledgments

GRANTS

This work was supported in part by an Alyce Spector Research Grant from the Kidney Foundation of Central Pennsylvania to G. Ramesh and National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-063120 to W. Brian Reeves.

References

1. Barallobre MJ, Pascual M, Del Rio JA, Soriano E. The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Res Brain Res Rev.* 2005; 49:22–47. [PubMed: 15960985]
2. Bijuklic K, Jennings P, Kountchev J, Hasslacher J, Aydin S, Sturn D, Pfaller W, Patsch JR, Joannidis M. Migration of leukocytes across an endothelium-epithelium bilayer as a model of renal interstitial inflammation. *Am J Physiol Cell Physiol.* 2007; 293:C486–C492. [PubMed: 17428840]
3. Bonventre JV. Mechanisms of ischemic acute renal failure. *Kidney Int.* 1993; 43:1160–1178. [PubMed: 8510397]
4. Bonventre JV, Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol.* 2003; 14:2199–2210. [PubMed: 12874476]
5. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int.* 2004; 66:480–485. [PubMed: 15253693]
6. Dalvin S, Anselmo MA, Prodhan P, Komatsuzaki K, Schnitzer JJ, Kinane TB. Expression of Netrin-1 and its two receptors DCC and UNC5H2 in the developing mouse lung. *Gene Expr Patterns.* 2003; 3:279–283. [PubMed: 12799072]
7. Donnahoo KK, Meng X, Ayala A, Cain MP, Harken AH, Meldrum DR. Early kidney TNF- α expression mediates neutrophil infiltration and injury after renal ischemia-reperfusion. *Am J Physiol Regul Integr Comp Physiol.* 1999; 277:R922–R929.
8. Friedewald JJ, Rabb H. Inflammatory cells in ischemic acute renal failure. *Kidney Int.* 2004; 66:486–491. [PubMed: 15253694]
9. Furuichi K, Wada T, Iwata Y, Kitagawa K, Kobayashi K, Hashimoto H, Ishiwata Y, Asano M, Wang H, Matsushima K, Takeya M, Kuziel WA, Mukaida N, Yokoyama H. CCR2 signaling contributes to ischemia-reperfusion injury in kidney. *J Am Soc Nephrol.* 2003; 14:2503–2515. [PubMed: 14514728]
10. Gobe G, Willgoss D, Hogg N, Schoch E, Endre Z. Cell survival or death in renal tubular epithelium after ischemia-reperfusion injury. *Kidney Int.* 1999; 56:1299–1304. [PubMed: 10504480]
11. Jo SK, Sung SA, Cho WY, Go KJ, Kim HK. Macrophages contribute to the initiation of ischaemic acute renal failure in rats. *Nephrol Dial Transplant.* 2006; 21:1231–1239. [PubMed: 16410269]
12. Kelly KJ, Williams WW Jr, Colvin RB, Bonventre JV. Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. *Proc Natl Acad Sci USA.* 1994; 91:812–816. [PubMed: 7904759]

13. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der PT, Weening JJ, Florquin S. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest*. 2005; 115:2894–2903. [PubMed: 16167081]
14. Liu Y, Stein E, Oliver T, Li Y, Brunken WJ, Koch M, Tessier-Lavigne M, Hogan BLM. Novel role for netrins in regulating epithelial behavior during lung branching morphogenesis. *Curr Biol*. 2004; 14:897–905. [PubMed: 15186747]
15. Ly NP, Komatsuzaki K, Fraser IP, Tseng AA, Prodhan P, Moore KJ, Kinane TB. Netrin-1 inhibits leukocyte migration in vitro and in vivo. *Proc Natl Acad Sci USA*. 2005; 102:14729–14734. [PubMed: 16203981]
16. Molitoris BA, Sutton TA. Endothelial injury and dysfunction: role in the extension phase of acute renal failure. *Kidney Int*. 2004; 66:496–499. [PubMed: 15253696]
17. Nguyen A, Cai H. Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism. *Proc Natl Acad Sci USA*. 2006; 103:6530–6535. [PubMed: 16611730]
18. Rabb H, Daniels F, O'Donnell M, Haq M, Saba SR, Keane W, Tang WW. Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am J Physiol Renal Physiol*. 2000; 279:F525–F531. [PubMed: 10966932]
19. Ramesh G, Kimball SR, Jefferson LS, Reeves WB. Endotoxin and cisplatin synergistically stimulate TNF- α production by renal epithelial cells. *Am J Physiol Renal Physiol*. 2007; 292:F812–F819. [PubMed: 17032936]
20. Rand JH, Glanville RW, Wu XX, Ross JM, Zangari M, Gordon RE, Schwartz E, Potter BJ. The significance of subendothelial von Willebrand factor. *Thromb Haemost*. 1997; 78:445–450. [PubMed: 9198194]
21. Reeves WB, Kwon O, Ramesh G. Netrin-1 and kidney injury. II. Netrin-1 is an early biomarker of acute kidney injury. *Am J Physiol Renal Physiol*. In press.
22. Rollins BJ, Yoshimura T, Leonard EJ, Pober JS. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am J Pathol*. 1990; 136:1229–1233. [PubMed: 2113354]
23. Scalia R, Booth G, Lefer DJ. Vascular endothelial growth factor attenuates leukocyte-endothelium interaction during acute endothelial dysfunction: essential role of endothelium-derived nitric oxide. *FASEB J*. 1999; 13:1039–1046. [PubMed: 10336886]
24. Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell*. 1994; 78:409–424. [PubMed: 8062384]
25. Sheridan AM, Bonventre JV. Pathophysiology of ischemic acute renal failure. *Contrib Nephrol*. 2001; 132:7–21. [PubMed: 11395913]
26. Sinuani I, Averbukh Z, Gitelman I, Rapoport MJ, Sandbank J, Albeck M, Sredni B, Weissgarten J. Mesangial cells initiate compensatory renal tubular hypertrophy via IL-10-induced TGF- β secretion: effect of the immunomodulator AS101 on this process. *Am J Physiol Renal Physiol*. 2006; 291:F384–F394. [PubMed: 16571592]
27. Star RA. Treatment of acute renal failure. *Kidney Int*. 1998; 54:1817–1831. [PubMed: 9853246]
28. Takada M, Nadeau KC, Shaw GD, Marquette KA, Tilney NL. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest*. 1997; 99:2682–2690. [PubMed: 9169498]
29. Wagner M, Cadetg P, Ruf R, Mazzucchelli L, Ferrari P, Redaelli CA. Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts. *Kidney Int*. 2003; 63:1564–1573. [PubMed: 12631374]
30. Waikar SS, Curhan GC, Wald R, McCarthy EP, Chertow GM. Declining mortality in patients with acute renal failure, 1988 to 2002. *J Am Soc Nephrol*. 2006; 17:1143–1150. [PubMed: 16495376]
31. Wilson BD, Li M, Park KW, Suli A, Sorensen LK, Larrieu-Lahargue F, Urness LD, Suh W, Asai J, Kock GA, Thorne T, Silver M, Thomas KR, Chien CB, Losordo DW, Li DY. Netrins promote developmental and therapeutic angiogenesis. *Science*. 2006; 313:640–644. [PubMed: 16809490]
32. Yin Y, Sanes JR, Miner JH. Identification and expression of mouse netrin-4. *Mech Dev*. 2000; 96:115–119. [PubMed: 10940631]

Fig. 1.

A: expression of netrin-1 and netrin receptors in the normal mouse (C57BL/6) kidney. mRNA abundance was analyzed by real-time RT-PCR as described in MATERIALS AND METHODS. Values are means $\pm$ SE from 3 animals. *B:* expression of netrins and the netrin receptors DCC and UNC5b mRNA in the kidney at various times after reperfusion in C57BL/6 mice. The mRNA was analyzed by real-time RT-PCR. *Left:* netrin-1 (■) and netrin-4 (●) mRNA expression. *Right:* DCC (■) and UNC5B (●) mRNA expression. The mRNA levels were normalized to β -actin mRNA and expressed as relative to levels in sham-operated mouse kidneys. Values are means $\pm$ SE for 4 wild-type (WT), 6 sham-operated mice. * $P < 0.05$ and + $P < 0.001$ vs. sham-operated.

Fig. 2.

Regulation of netrin-1 expression during ischemia-reperfusion (I/R) of the kidney. C57BL/6 mice were subjected to sham surgery or I/R and kidney tissue was collected at various time points. The content of netrin-1 was determined by Western blot analysis. *Top*: densitometric quantitation of netrin-1 levels in fold compared with sham operation. *Bottom*: Western blot analysis of netrin-1 in kidney homogenate. * $P < 0.05$ vs. sham-operated kidney.

Fig. 3.

Immunohistochemical localization of netrin-1 (*left*) and -4 (*right*) in kidney after I/R and sham operation in C57BL/6 mice. Paraformaldehyde-fixed frozen tissue sections were stained with anti-netrin-1 and anti-netrin-4 antibody (green stain) as described in MATERIALS AND METHODS. Nuclei were stained blue with DAPI. No netrin-1 and -4 staining was seen in the primary antibody negative controls (*top*). In sham-operated kidney, netrin-1 staining was mainly in the interstitium. Three hours after reperfusion, staining appeared in tubules and intense tubular staining was seen at 24 h after reperfusion. At the same time, interstitial staining disappeared. Seventy-two hours after reperfusion, interstitial staining reappeared and tubular staining decreased. Netrin-4 staining was mostly seen around tubules in the basement membrane in sham-operated animals. There was not much change in staining at 3 and 24 h after reperfusion. Seventy-two hours after reperfusion, staining was present within tubules and inside the lumens.

Fig. 4.

Immunohistochemical colocalization of netrin-1 and endothelial markers.

Paraformaldehyde-fixed frozen tissue sections were stained with anti-netrin-1 antibody (red stain) and anti-Von Wille-brand Factor (VWF) antibody (green stain) as described in MATERIALS AND METHODS. Nuclei were stained blue with DAPI. In sham-operated kidney (*B–D*), netrin-1 staining was mainly in the interstitium and colocalized with VWF. Similarly, netrin-1 and VWF colocalized in the kidney section from 72 h of reperfusion (*E* and *F*). No staining was seen in the primary antibody negative control (*A*). Colocalization of netrin-1 (red) and GFP (green) in kidney from Tie2-GFP mice was also observed under normal conditions (*G* and *H*).

Fig. 5.

In vitro hypoxia alters netrin-1 expression in cultured renal tubular epithelial cells (TKPTS) and renal microvascular endothelial cells. Cells were made hypoxic for 30 and 60 min followed by 24 h of reoxygenation (H/R) and then harvested. *A*: Western blot analysis of netrin-1 showed no change in netrin-1 protein in endothelial cells. *B*: netrin-1 protein expression was increased after hypoxia-reoxygenation (H/R) in TKPTS cells. *C*: netrin-1 mRNA expression increased significantly ($*P < 0.001$ vs. control) in endothelial cells but decreased by 50% ($+P < 0.001$ vs. control) in TKPTS cells; $n = 3-4$.

Fig. 6. Effect of recombinant netrin-1 on I/R-induced kidney dysfunction. Blood urea nitrogen (BUN; *left*) and serum creatinine (*right*) levels following renal I/R in mice treated with vehicle (■) and recombinant netrin-1 (●). Male C57BL/6J mice, 8 to 10 wk old, were subjected to 26 min of warm renal ischemia followed by up to 96 h of reperfusion. * $P < 0.05$ vs. vehicle-treated animals. Values are averages $\pm$ SE; $n = 4-5$ for each group.

Fig. 7. Immunohistochemical localization of neutrophils following renal ischemia and 24-h reperfusion in sham, I/R with vehicle, or netrin-1-treated mice. Sections were fixed and stained for neutrophils as described in MATERIALS AND METHODS. Sham-operated mice showed few neutrophils. I/R with vehicle showed increased neutrophil staining. *Bottom:* quantitative summary data of neutrophil infiltration (+ $P < 0.001$ vs. sham-operated. * $P < 0.05$ vs. vehicle-treated animals. $n = 4-6$ in each group).

Fig. 8.

Effect of recombinant netrin-4 on I/R-induced kidney dysfunction. BUN levels following renal I/R in mice treated with vehicle (■) and recombinant netrin-4 (●). Male C57BL/6J mice, 8 to 10 wk old, were subjected to 26 min of warm renal ischemia followed by up to 96 h of reperfusion. Values are averages $\pm$ SE; $n = 5-8$ for each group.

Fig. 9.

Effect of recombinant netrin-1 on cytokine and adhesion molecule gene expression. Effect of netrin-1 on gene expression was measured at 24 h after sham operation (gray bar), I/R treated with vehicle (black bar), and I/R treated with netrin-1 (hatched bar). Values are means $\pm$ SE for 6 vehicle-treated, 4 sham surgery, and 3 netrin-1-treated mice. * $P < 0.05$ vs. vehicle-treated I/R. + $P < 0.01$ vs. sham surgery.