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Protective role for netrin-1 during diabetic nephropathy

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Abstract

Recent studies implicate neuronal guidance molecules in the orchestration of inflammatory events. For example, previous studies demonstrate a functional role for netrin-1 in attenuating acute kidney injury. Here, we hypothesized a kidney-protective role for netrin-1 during chronic kidney disease, such as occurs during diabetic nephropathy. To study the role of netrin-1 during diabetic nephropathy, we induced diabetes in mice at age of 8 weeks by streptozotocin (STZ) treatment. Sixteen weeks after STZ treatment, we examined the kidneys. Initial studies in wild-type mice demonstrated robust induction of renal, urinary and plasma netrin-1 protein levels during diabetic nephropathy. Subsequent genetic studies in mice with partial netrin-1 deficiency (*Ntn1*^{+/-} mice) revealed a more severe degree of diabetic nephropathy, including more severe loss of kidney function (albuminuria, glomerular filtration rate, histology). We subsequently performed pharmacologic studies with recombinant netrin-1 treatment given continuously via osmotic pump. Indeed, netrin-1 treatment was associated with attenuated albuminuria and improved histologic scores for diabetic nephropathy compared to controls. Consistent with previous studies implicating purinergic signaling in netrin-1-elicited tissue-protection, mice deficient in the *Adora2b* adenosine receptor were not protected. Taken together, these studies demonstrate a functional role for endogenous netrin-1 in attenuating diabetic kidney disease.

Keywords

Diabetes; nephropathy; netrin-1; inflammation; vascular injury; hypoxia

Introduction

While neuronal guidance molecules have been originally characterized with regard to their functions within the developing brain, many of these molecules were later also found to have an impact on outcomes during inflammatory diseases, thereby implicating neuronal guidance molecules in the orchestration of inflammatory events. For example, the netrin

family of secreted proteins functions to provide migrational cues in the central nervous system during brain development. Indeed, gene-targeted mice for netrin-1 die shortly after birth due to very severe defects in brain development [1]. In contrast, mice with partial netrin-1 deficiency are viable and have previously examined in inflammatory disease models [2–5]. Indeed, previous studies demonstrated that netrin-1 is transcriptionally induced during conditions of limited oxygen availability [6–8], and in that context, netrin-1 signaling dampens hypoxia-induced inflammation of different organs, including the lungs, the intestine and the kidneys [5]. Other studies have implicated netrin-1 signaling during vascular inflammation and atherosclerosis [9]. Again other studies demonstrated that during intestinal inflammation – such as occurs in the setting of inflammatory bowel disease – netrin-1 signaling dampens intestinal inflammation via signaling events involving extracellular adenosine receptors [3,10]. Consistent with these findings, again other studies revealed that netrin-1 signaling through the Adora2b adenosine receptor is protective during inflammatory peritonitis [11] or acute lung injury [12,13].

Several previous studies had shown a functional role for netrin-1 in acute renal disease models. Indeed, netrin-1 is highly expressed in the kidneys, and functions to attenuate ischemic AKI [4]. Mice with partial netrin-1 deficiency experience a more severe degree of ischemic kidney injury, while treatment with recombinant netrin-1 is kidney protective [4]. Moreover, a very recent study found that transgenic mice with tubule-specific netrin-1 overexpression are protected during diabetic nephropathy [14]. While diabetic control mice experienced increased infiltration of neutrophils and macrophages, chemokine expression, and tubular epithelial cell apoptosis in kidney, these changes were attenuated in kidney of netrin-1 transgenic mice. To further expand on the functional role of netrin-1 during diabetic nephropathy, we decided to pursue studies in mice with genetic netrin-1-deficiency. Due to the fact that *Ntn-1*^{-/-} mice die shortly after birth with severe brain defects [1], we used mice with partial netrin-1 deficiency (*Ntn-1*^{+/-} mice). These mice are viable and can be used to address the functional role of netrin-1 during inflammatory disease models [3–5]. In addition, we performed pharmacologic studies with netrin-1 treatment, and genetic studies to address the role of netrin-1 receptors important in kidney protection. Together, our findings implicate endogenous netrin-1 signaling in attenuating diabetic nephropathy via enhancing purinergic signaling events.

Material and Methods

Induction of diabetes using STZ

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver, and is in accordance with the National Institutes of Health guidelines for use of live animals. Previously described mice with partial netrin-1 deficiency (*Ntn-1*^{+/-} mice) and *Adora2b*^{-/-} or littermate controls matched in age, gender and weight, were used [5,15]. At 8 wk of age, mice received daily streptozotocin injections intraperitoneally (50 mg/kg, made fresh in 0.1 M citrate buffer, pH 4.5) for 5 consecutive days. Vehicle-injected mice served as controls. The fasting level of blood glucose was examined weekly with a B-Glucose Analyzer. The mice were considered diabetic if the blood glucose concentration (BGC) was above 400 mg/dl. After 16 weeks following induction of diabetes, mice were placed in metabolic cages and 24-h urine samples were collected. For continuous application of recombinant netrin-1 Alzet pumps (2006, 0.15µl/hr for 6 weeks, 2µg/mouse/per day) were subcutaneously implanted under isoflurane anesthesia.

Determination of glomerular filtration rate

Inulin clearance was measured as previously described [15].

Determination of albuminuria

A mouse albumin ELISA was used by following the manufacturer's instructions (Exocell).

Determination of netrin-1

We used a netrin-1 ELISA (Hoelzel-Biotech) and followed the manufacturer's instructions.

Renal histology

Kidneys were excised and harvested as previously described [9]. Extracellular matrix deposition in glomeruli was assessed by periodic acid-Schiff (PAS) staining. An investigator scored sections in a blinded fashion, according to an established scoring system (range 0–4: 0, no extracellular matrix deposition; 4, extracellular matrix deposition in all sections of the glomeruli) [4].

Glomerular size was determined using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>) analysis software. The largest area on sections was measured in 3–4 different mice per group with at least 10 glomeruli of each mouse.

Statistics

Data are presented as mean $\pm$ SEM from three to six animals per condition. We performed statistical analysis using the Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Renal and urinary netrin-1 protein levels are induced during diabetic nephropathy

To study the role of netrin-1 during diabetic nephropathy, we induced diabetes in mice at age of 8 weeks by streptozotocin (STZ) treatment. For this purpose, we treated mice with 5 single injections of 50 mg/kg, freshly dissolved in 0.1 M citrate buffer, pH 4.5, on five successive days. As previously described, this treatment is associated with the destruction of insulin-producing beta-cells in the pancreas, hyperglycemia, and the development of diabetic nephropathy. Sixteen weeks after STZ treatment, we performed studies to address the role of netrin-1 during diabetic nephropathy. Initial studies in BL6 mice showed that renal netrin-1 protein levels – as assessed by ELISA from renal tissues – were significantly elevated 16 weeks following STZ treatment in comparison to vehicle-treated controls matched in age, gender and weight (Fig. 1A). Due to the fact that netrin-1 is secreted into the urine [4], we went on to examine urinary netrin-1 levels in vehicle-treated control mice or mice with diabetic nephropathy. Indeed, we observed very robust increases of netrin-1 protein levels in the urine obtained from diabetic mice (Fig. 1B). In addition serum netrin-1 levels in control mice were in the same range as previously published [16] and showed a significant increase in diabetic mice (Fig. 1C). Taken together, these studies indicate that renal netrin-1 levels are induced during STZ-induced diabetic nephropathy, and allude to the fact that urinary netrin-1 levels could potentially be examined in further studies as a biomarker for diabetic nephropathy.

Mice with partial netrin-1 deficiency (*Ntn-1*^{+/-} mice) experience a more severe degree of kidney disease during diabetes mellitus

To demonstrate a functional role for netrin-1 during diabetes-associated kidney disease, we took a genetic approach. Previous studies had examined gene-targeted mice for netrin-1 during brain development [1]. Indeed, *Ntn-1*^{-/-} mice die shortly after birth due to severe neurologic problems in the context of incomplete brain development. However, several subsequent studies have utilized mice with partial netrin-1 deficiency (*Ntn-1*^{+/-} mice) to

study the functional role of netrin-1 during inflammatory diseases [3–5]. In contrast to *Ntn-1^{-/-}* mice who due to severe brain defects early after birth [1], *Ntn-1^{+/-}* mice are viable, do not exhibit any obvious immunologic alteration, and are undistinguishable from their littermate control counterparts [5]. Again, we induced diabetes mellitus by STZ treatment at the age of 8 weeks, and performed measurements 16 weeks after the induction of diabetes (Fig. 2A). For the purpose of these studies, we systematically examined the phenotype of *Ntn-1^{+/-}* mice at 16 weeks following diabetes induction. In control studies, we utilized littermate control mice matched in age, gender and weight (*Ntn-1^{+/+}* mice). Indeed, *Ntn-1^{+/-}* mice had significantly lower levels of Ntn-1 protein expression, compared to their matched littermate controls (Fig. 2B). From a functional perspective, they experienced a more severe loss of body weight and increase in kidney weight in the disease course (Fig. 2C,D), in conjunction with elevated drinking and urine volumes (Fig. 2E,F), thereby indicating a more severe degree of kidney injury. Glomerular filtration rate was significantly higher in *Ntn-1^{-/-}* mice compared to wild-type mice following induction of diabetes (Fig. 2G). Moreover, they exhibited a more severe degree of albuminuria (Fig. 2H). To assess the histologic degree of renal tissue injury, we also performed histologic staining studies, which were examined by an examiner blinded to the treatment group with significant experience in scoring histologic kidney injury in mice. Indeed, these studies revealed a more severe degree of histologic tissue injury in *Ntn-1^{+/-}* mice as compared to their control counterparts (Fig. 3 A–C). Taken together, these genetic studies indicate a protective role for endogenous netrin-1 during diabetic nephropathy.

Pharmacologic studies utilizing treatment with recombinant netrin-1 show protection from diabetic nephropathy

As proof of principle for the assertion that netrin-1 plays an important role in protecting the kidneys from diabetic nephropathy, and to rule out biologic compensation in gene-targeted mice for netrin-1, we subsequently performed pharmacologic studies. For this purpose, and as we have done in previous studies [2–5], we treated wild-type mice with recombinant netrin-1 after we induced diabetes mellitus by STZ treatment. Indeed, we believe that netrin-1 induction during diabetic nephropathy represents an endogenous protective pathway resulting in attenuated diabetic nephropathy. Netrin-1 treatment – particularly at early stages of the disease – could enhance this adaptive pathway and thereby provide potent kidney protection during diabetic kidney disease. Such findings would be consistent with many other studies of acute or chronic forms of kidney disease, where an endogenous adaptive response is pharmacologically enhanced, and thereby can provide kidney protection [4,17–19]. For this purpose, we implanted osmotic pumps for continuous subcutaneous recombinant netrin-1 treatment (Alzet 2006, 0.15 μ l/hr for 6 weeks, 2 μ g/mouse/per day) at week three and ten following the induction of diabetes (50mg/kg STZ on five successive days) (Fig. 4A), and examined kidney function after 16 weeks of hyperglycemia. Indeed, treatment with soluble recombinant netrin-1 was associated with statistically significant improvements of body weight, kidney weight, drinking volume, urine volume and albuminuria (Fig. 4B–F). Based on histologic scoring performed by a blinded examiner, continuous treatment with recombinant netrin-1 also dramatically improved the histologic degree of diabetic nephropathy (Fig. 5A–C). Taken together, these pharmacologic studies confirm our findings in *Ntn-1^{+/-}* mice and implicate netrin-1 treatment in kidney protection during diabetic nephropathy.

Protection by recombinant netrin-1 was abolished in *Adora2b^{-/-}* mice

Previous studies had implicated purinergic signaling pathways through the adenosine receptor *Adora2b* in netrin-1-dependent attenuation of inflammation [5]. To investigate if the *Adora2b* plays a role in mediating the protective effect of netrin-1 during diabetic nephropathy we treated *Adora2b* gene-targeted mice with exogenous netrin-1. As described

before we implanted osmotic pumps for continuous subcutaneous recombinant netrin-1 treatment (Alzet 2006, 0.15 μ l/hr for 6 weeks, 2 μ g/mouse/per day) at week three and ten following the induction of diabetes (50mg/kg STZ on five successive days) (Fig. 6A), and examined kidney function after 16 weeks of hyperglycemia. Consistent with a functional role for Adora2b signaling in netrin-1-elicited kidney protection, treatment with recombinant netrin-1 failed to improve body weight, kidney weight, drinking volume, urine volume and albuminuria in *Adora2b*^{-/-} mice compared to wild type treated mice (Fig. 6B–F). Taken together, these pharmacologic studies in *Adora2b*^{-/-} mice suggest that the kidney protective effect of netrin-1 during diabetic nephropathy is - at least in part - mediated by Adora2b signaling events.

Discussion

In the present studies we combined genetic and pharmacologic approaches to investigate the functional role of the neuronal guidance molecule netrin-1 during diabetic nephropathy. Initial findings suggested that renal and urinary netrin-1 protein levels are dramatically induced during diabetic nephropathy. Moreover, functional studies utilizing mice with partial netrin-1 deficiency (*Ntn-1*^{+/-} mice) experienced a far more severe course of kidney disease during STZ-induced diabetes mellitus as compared to diabetic littermate control mice. Finally, treatment studies with recombinant netrin-1 - given continuously via an osmotic pump - revealed robust treatment effects, as shown by an attenuated disease severity in all parameters that we assessed. However, that treatment effect was absent in *Adora2b* gene-targeted mice. Taken together, these studies indicate for the first time a protective role of netrin-1 during diabetic nephropathy.

Consistent with the present study, a very recent study utilized a different approach to study netrin-1 functions during diabetic nephropathy. They found that transgenic mice with tubule-specific netrin-1 overexpression are protected during diabetic nephropathy [14]. While diabetic control mice experienced increased infiltration of neutrophils and macrophages, chemokine expression, and tubular epithelial cell apoptosis in kidney, these changes were attenuated in kidney of netrin-1 transgenic mice. These findings are consistent with the present study in knockout mice showing that partial netrin-1 deletion aggravates diabetic nephropathy. However, the present study provides the first evidence that netrin-1 can be used to treat diabetic nephropathy. Indeed, continuous treatment with recombinant netrin-1 given via an osmotic pump was effective in attenuating diabetic kidney injury. Moreover, and beyond the studies of Mohamed et al. the present studies define a signaling mechanism that is responsible for netrin-1-dependent kidney protection. Indeed, knockout mice for purinergic *Adora2b* receptor are not protected by netrin-1 treatment. These findings are consistent with previous reports implicating the Adora2b in mediating netrin-1-dependent attenuation of inflammation [5,20].

The results from the present study on netrin-1 expression during diabetic nephropathy are consistent with previous findings showing increased expression of netrin-1 during disease conditions such as hypoxia, ischemia, or inflammation [21]. For example, previous studies examined the transcriptional regulation of netrin-1 during conditions of hypoxia [5]. In this study, the authors exposed intestinal epithelial cells to conditions of ambient hypoxia and found very robust increases of netrin-1 transcript and protein levels. In order to address the mechanisms of hypoxia-elicited induction of netrin-1, the authors generated netrin-1 promoter constructs, and examined them under conditions of ambient hypoxia. They found a functional role for a previously unrecognized site for binding of the transcription factor hypoxia-induced factor (HIF)-1 in netrin-1 induction during ambient hypoxia. Moreover, studies with site directed mutagenesis of promoter constructs, transcription factor binding assays and combinations of in vivo and in vitro loss-and-gain of HIF1 function confirmed

their initial results, and provide strong evidence that hypoxia-dependent induction of netrin-1 is mediated by HIF. It is important to point that inflammatory disease conditions such as diabetic nephropathy are typically characterized by tissue hypoxia [2,8,22–26]. Indeed, it is tempting to speculate that potentially the renal and urinary induction of netrin-1 during diabetic nephropathy could also be mediated by HIF-1.

The present studies implicate purinergic signaling events as a mechanism by which netrin-1 provides kidney protection during diabetic nephropathy. Previous studies had shown different netrin-1 receptors in the regulation of inflammatory endpoints. For example, a previous study examined the functional role of netrin-1 signaling during leukocyte-endothelial interactions [27]. In this study, the authors demonstrate that netrin-1 is expressed on vascular endothelia, where it is regulated by infection and inflammatory cytokines. Moreover, they went on to show that the classic netrin-1 receptor UNC5b is strongly expressed by leukocytes, upon which netrin-1 acts as a potent inhibitor of migration to different chemotactic stimuli both in vivo and in vitro. Together, these studies indicate that a crosstalk between endothelial-released netrin-1 and the classic netrin-1 receptor UNC5b is involved in attenuating vascular inflammation [27]. Consistent with our findings, other studies have implicated purinergic signaling events in netrin-1 dependent alteration of inflammatory endpoints – particularly through the adenosine Adora2b receptor. Indeed, many previous studies have shown very robust effects of adenosine signaling in attenuating inflammation, particularly in the context of tissue hypoxia [28–46]. Consistent with the idea that netrin-1 signaling could alter inflammatory endpoints by interacting with the Adora2b [5], previous studies showed that this receptor is expressed on vascular endothelia [17,18,45,47], inflammatory cells [48,49], cardiac tissues [40,50–52], or epithelial cells, and provides potent protection from inflammation [6]. Indeed, several studies had shown that an interaction between netrin-1 and the Adora2b is critical in netrin-1-mediated attenuation of inflammation, such as experimental colitis, or hypoxia-induced inflammation [5,6]. At present, the nature of netrin-1-Adora2b interaction remains unclear. For example, it is conceivable that this interaction involves indirect effects of netrin-1-dependent enhancement of Adora2b signaling.

In conclusion, the present study on the role of netrin-1 signaling during diabetic nephropathy demonstrate a protective role of endogenous netrin-1 in attenuating disease severity. Several challenges remain to further elucidate this pathway, and to characterize the biological role of renal netrin-1 in kidney protection, before these findings can be translated into the treatment of human disease. For example, additional studies will be necessary to more clearly define how the netrin-1-Adora2b-axis functions to dampen kidney inflammation, or to alter vascular responses during diabetic nephropathy.

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Figure 1. Netrin-1 is induced by diabetic nephropathy

Age-, sex-, and weight-matched mice were subjected to streptocotozin (STZ)-induced diabetes for 16 weeks until organs were removed for analysis. Wild-type mice show (a) increased renal netrin-1 concentrations, (b) increased urine netrin-1 excretion related to creatinine and (c) no significant increase in netrin-1 serum concentrations following 16 weeks of diabetes compared to wild-type controls without diabetes measured by ELISA (n=3–4).

Figure 2. Diabetic nephropathy is increased in *Ntn-1*^{+/-} mice. (a) *Ntn-1*^{+/-} mice and age-, weight- and gender-matched wildtype mice were subjected to streptozotocin (STZ)-induced diabetes for 16 weeks until measurement of (b) renal netrin-1 content, (c) body weight, (d) kidney weight, (e) drink volume, (f) urine volume, (g) glomerular filtration rate and (h) albumin excretion, (n=4–6 in each group).

Figure 3. Severe histologic injury in *Ntn1*^{+/-} mice with diabetic nephropathy
 (a) Extracellular matrix deposition as determined by PAS staining. PAS staining was increased in diabetic wild-type mice and in particular in *Ntn1*^{+/-} mice (arrows). (b) histological scores of PAS staining sections. (c) Glomerular size in all groups. Panels b and c show result of at least 4–10 glomeruli in sections of two to four different mice.

Figure 4. Treatment with recombinant netrin-1 attenuates diabetic nephropathy in wild-type mice

Wild-type mice were subjected to streptozotocin (STZ)-induced diabetes for 16 weeks. (a) Two weeks following STZ injection and confirmation of diabetes (glucose levels above 400 mg/dl) mice were treated with netrin-1 or vehicle via Alzet pump (2 μ g/mouse/per day). Delivery time of the Alzet pumps was six weeks thus they were replaced at week 10. After 16 weeks of diabetes with or without netrin-1 treatment (b) body weight, (c) kidney weight, (d) drink volume, (e) urine volume and (f) albumin excretion (n=4–6 in each group) were determined.

Figure 5. Treatment with recombinant netrin-1 attenuates histologic injury in wild-type mice with diabetic nephropathy

Wild-type mice were subjected to streptozotocin (STZ)-induced diabetes for 16 weeks. Two weeks following STZ injection and confirmation of diabetes (glucose levels above 400 mg/dl) mice were treated with netrin-1 or vehicle via Alzet pump (2 μ g/mouse/per day). Delivery time of the Alzet pumps was six weeks thus they were replaced at week 10. (a) Extracellular matrix deposition as determined by PAS staining. PAS staining was attenuated in diabetic wild-type mice with netrin-1 treatment compared to wild-type mice without treatment (arrows). (b) histological scores of PAS staining sections. (c) Glomerular size in all groups. Panels b and c show result of at least 4–10 glomeruli in sections of two to four different mice.

Figure 6. The protective effect of treatment with recombinant netrin-1 is absent in *Adora2b^{-/-}* mice

Adora2b^{-/-} mice were subjected to streptozotocin (STZ)-induced diabetes for 16 weeks. (a) Two weeks following STZ injection and confirmation of diabetes (glucose levels above 400 mg/dl) mice were treated with netrin-1 or vehicle via Alzet pump (2 μ g/mouse/per day). Delivery time of the Alzet pumps was six weeks thus they were replaced at week 10. After 16 weeks of diabetes with or without netrin-1 treatment (b) body weight, (c) kidney weight, (d) drink volume, (e) urine volume and (f) albumin excretion (n=4–6 in each group) were determined.