Diabetic retinopathy (DR) is a major complication of diabetes and a leading cause of blindness in the working-age population. Impaired blood-retinal barrier function leads to macular edema that is closely associated with the deterioration of central vision. We previously demonstrated that the neuronal guidance cue netrin-1 activates a program of reparative angiogenesis in microglia within the ischemic retina. Here, we provide evidence in both vitreous humor of diabetic patients and in retina of a murine model of diabetes that netrin-1 is metabolized into a bioactive fragment corresponding to domains VI and V of the full-length molecule. In contrast to the protective effects of full-length netrin-1 on retinal microvasculature, the VI-V fragment promoted vascular permeability through the uncoordinated 5B (UNC5B) receptor. The collagenase matrix metalloprotease 9 (MMP-9), which is increased in patients with diabetic macular edema, was capable of cleaving netrin-1 into the VI-V fragment. Thus, MMP-9 may release netrin-1 fragments from the extracellular matrix and facilitate diffusion. Nonspecific inhibition of collagenases or selective inhibition of MMP-9 decreased pathological vascular permeability in a murine model of diabetic retinal edema. This study reveals that netrin-1 degradation products are capable of modulating vascular permeability, suggesting that these fragments are of potential therapeutic interest for the treatment of DR.

Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes (1). DR develops in a protracted manner and can ultimately result in severe impairment of visual function. Initially, nonproliferative DR is associated with microvascular complications such as microaneurysms, intraretinal hemorrhages, and eventual capillary closure and degeneration. As the disease progresses, advanced stages may lead to proliferative diabetic retinopathy (PDR), characterized by growth of leaky, hemorrhaging pre-retinal neovessels and potential vitreal contraction and retinal detachment (2, 3).

Throughout the course of the disease, deterioration of the blood-retinal barrier (BRB) results in extravasation of fluids from permeable capillaries, leading to diabetic macular edema (DME) (4). DME is characterized by retinal thickening and swelling of the macular area and affects more than a quarter of patients with diabetes (5). Currently, the most common intervention for DME is focal and grid laser treatment, yet this approach may lead to retinal damage and a reduced visual field (6). Several factors that drive BRB breakdown in DR, including inflammation (7) and elevated vitreous levels of VEGF (8–10), have been suggested. This has led to the clinical introduction of corticosteroids and anti-VEGFs for intravitreal injection. Regrettably, the former is associated with an increased incidence of cataracts and a harmful rise in intraocular pressure (11), while long-term intravitreal use of anti-VEGFs may lead to neuronal toxicity (12, 13) and potential geographic atrophy (14). New insights into the pathomechanisms that drive heightened permeability in DR may provide safer alternative therapeutic avenues.

While DR is primarily considered a microvascular disorder, there is accumulating evidence for early pathological events originating from the neuronal retina (15–19). Potential effector molecules with the propensity to mediate neurovascular communication in retinal health and disease are classical neuronal guidance cues and include netrins (20, 21), semaphorins (17, 18, 22–24), ephrins (25, 26), and slits (27–29). While initially identified as mediators of neuronal circuit formation during embryogenesis, these cues play equally prominent roles in vascular (30) and immune function (18, 31, 32).

We have previously shown that netrin-1 produced by retinal neurons activates a program of reparative angiogenesis within microglia and initially restores functional vasculature to ischemic tissue during retinopathy (21). Netrin-1 is a laminin-related protein (33, 34) that exerts dichotomous biological effects, depending on the receptor it binds (35) and the intracellular cAMP levels (36). Canonically, signaling through deleted-in-colorectal-cancer (DCC) leads to attraction (37), while signaling through uncoordinated 5B (UNC5B) mediates repulsion or growth inhibition (38).

Conflict of interest: The authors have declared that no conflict of interest exists.
Here, we report that cleavage and fragmentation of netrin-1 by matrix metalloprotease 9 (MMP-9), and probably other collagenases, directly impacts barrier function in the diabetic retina. Specifically, a fragment corresponding to the VI (N-terminus) and V domains of full-length netrin-1 is elevated in the vitreous fluid of patients suffering from DME and in mouse retinae during experimental type 1 diabetes. The expression dynamics of MMP-9 mirror that of the VI-V fragment, and inhibition of MMP-9 reduces processing of full-length netrin-1. In contrast to full-length netrin-1, the VI-V fragment has the capability to provoke vascular permeability via the UNC5B receptor. Together, these findings suggest that cleavage of netrin-1 may contribute to heightened vascular permeability in DR.

Results

**Truncated netrin-1 isoforms are present in the vitreous humor of patients with DME.** To assess the potential involvement of netrin-1 in DR, we first investigated protein levels in vitreous humor from patients with DME, selected according to their macular thickness as determined by spectral-domain optical coherence tomography (SD-OCT) (>250 μm) (Table 1). Representative SD-OCT 3D retinal maps and cross sections are shown in Figure 1A and B. Full-length netrin-1 is a 604-aa protein comprising the N-terminal to the C-terminal of one laminin domain (domain VI), 3 laminin EGF-like domains (domains Vα, Vβ, and Vγ), and a netrin-like domain (NTR) module also called the C domain (Figure 1C). We used a netrin-1Ab with specificity to sequences within domain V for Western blot analysis (Figure 1C). Western blot analysis of equal volumes of vitreous (Figure 1D) revealed a major band at 72 kDa corresponding to full-length netrin-1 (Figure 1D) and an increase in a 55-kDa fragment of netrin-1 in patients with DME. According to their mass and prospective fragmentation profiles, netrin-1 bands at 55 kDa were consistent with the proteolytic processing of full-length netrin-1 to remove one of its domains (Figure 1E). Densitometric analysis revealed the 55-kDa truncated form of netrin-1 to be significantly increased by approximately 8-fold in patients with DME (healthy patients 1.000 ± 0.053, DME patients 8.433 ± 3.038, P = 0.0270) (Figure 1F), coincident with a nonsignificant drop in the amount of full-length netrin-1 (healthy patients 1.000 ± 0.2474, DME patients 0.443 ± 0.1476) (Figure 1G). Importantly, higher levels of the truncated 55-kDa form of netrin-1 correlated with increased retinal edema as determined by SD-OCT (r² = 0.5235, P < 0.05) (Figure 1H), whereas we were unable to establish a significant correlation between the drop in full-length netrin-1 levels and increased retinal edema (r² = 0.2283) (Figure 1I). Together, these data suggest that patients suffering from DME show elevated vitreous levels of a 55-kDa truncated form of netrin-1.

**Truncated netrin-1 is highly expressed in retinas during streptozotocin-induced diabetes.** Given the induction of truncated fragments of netrin-1 in the vitreous of patients with DME, we next investigated the kinetics of generation of netrin-1 fragments in a streptozotocin (STZ) mouse model of type 1 diabetes. STZ was administered to 6-week-old C57BL/6J mice over 5 consecutive days (Figure 2A), and the mice were considered diabetic when their glycemic levels exceeded 17 mM (300 mg/dl) (Supplemental Figure 1A). Eight weeks after STZ treatment, retinal vascular permeability rose by more than 2-fold when compared with that of control mice injected with vehicle (Supplemental Figure 1B), and FITC-dextran perfusion corroborated the presence of leaky vessels (Supplemental Figure 1C). Physiological parameters of the STZ-treated mice are presented in a polygonal graph in Supplemental Figure 1D.

IHC and 3D reconstruction of retinal cross sections from healthy adult mice revealed that netrin-1 was largely confined to the ganglion cell layer (GCL) (Figure 2B). Expression was quantitatively confirmed by quantitative PCR (qPCR) after laser capture microdissection (LCM) of the GCL, inner nuclear (INL), and outer nuclear layer (ONL) (GCL: 1.000 ± 0.0338, INL: 0.0258 ± 0.1236, ONL: 0.0635 ± 0.0006, P = 0.0001) (Figure 2C).

We subsequently assessed retinal expression of netrin-1 protein by Western blotting 4, 6, and 8 weeks after STZ treatment (Figure 2D). Netrin-1 protein levels were significantly increased in STZ-treated mice when compared with levels in citrate-treated control mice (4 weeks: 1.000 ± 0.032 [citrate], 1.151 ± 0.059 [STZ], NS, n = 7; 6 weeks: 1.000 ± 0.2394 [citrate], 1.944 ± 0.178 [STZ], P < 0.05, n = 3; and 8 weeks: 1.000 ± 0.064 [citrate], 1.872 ± 0.222 [STZ], P < 0.01, n = 7) (Figure 2E). In accordance with our finding in diabetic human vitreous (Figure 1D), we observed a significant induction of a 55-kDa netrin-1 fragment (4 weeks: 1.000 ± 0.045 [citrate], 1.369 ± 0.089 [STZ], P < 0.01; 6 weeks: 1.000 ± 0.031 [citrate], 2.243 ± 0.034 [STZ], P < 0.001; and 8 weeks: 1.000 ± 0.081 [citrate], 1.899 ± 0.1727 [STZ], P < 0.001) (Figure 2F). The discrepancy between netrin-1 levels, which are reduced in the vitreous of DME patients and elevated in mouse diabetic retinae, may be related to the stage of the disease.

We then purified netrin-1 by IP using an Ab specifically targeting the N-terminal extremity of netrin-1 (clone NORA-1 ALX-804-838-G100) (Figure 2G). Gel electrophoresis of the resulting immu-

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**Table 1. Characteristics of patients**

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*Type 2 diabetes.
Figure 1. Elevated levels of truncated netrin-1 in the vitreous humor of patients with DME. (A and B) 3D retinal maps and SD-OCT of control eye (left) versus eyes of patients with DME (right). The severity of retinal swelling was particularly evident in the central foveal zones. (C) Netrin-1 Ab targeting a specific peptide sequence in netrin-1 domain V (60). (D) Western blot analysis of equal volumes of vitreous humor revealed the presence of full-length netrin-1 in all samples and of an approximately 55-kDa netrin-1 fragment in a number of patients with DME (n = 4–5). (E) Schematic diagram of a 55-kDa netrin-1 fragment. (F) Quantification of signal density revealed an approximately 8-fold increase in the VI-V fragment in vitreous humor (n = 4–5). (G) Quantification of signal density revealed an approximately 50% decrease in full-length netrin-1 levels (n = 4–5, NS). (H) Correlation curve between the 55-kDa fragment and OCTs, showing that 52% of the OCT variation was associated with the 55-kDa fragment variation. (I) Correlation curve revealed no significant correlation between full-length netrin-1 and OCTs. Data are expressed as the mean ± SEM. *P < 0.05, by 2-tailed Student’s t test. Ctl, control.
noprecipitate was stained with SimplyBlue SafeStain, and the results revealed a banding pattern similar to that shown in Figure 1D (Figure 2H), demonstrating that the 55-kDa murine truncated netrin-1 contains domain VI — and likely corresponds to netrin-1 devoid of the netrin-like domain (NTR) module — or the VI-V fragment.

The VI-V fragment of netrin-1 plays a role in early breakdown of the BRB. Given the early induction of the VI-V fragment of netrin-1 in diabetic retinae (Figure 2), we sought to determine whether this protein contributes to the pathogenesis of DR. The time point at which the VI-V fragment of netrin-1 appears in the retina (Figure 2D) is associated with heightened vascular permeability. We therefore generated a Myc-tagged recombinant form of the VI-V fragment and assessed its ability to mediate vascular permeability using 3 distinct models. First, we tested the ability of the VI-V fragment to induce retinal vascular permeability (Figure 3A). Intravitreal injection of the VI-V fragment significantly induced up to 2-fold more Evans blue (EB) extravasation when compared with vehicle control (1.000 ± 0.1112 [vehicle], 1.891 ± 0.1951 [VI-V fragment, 3 nM], P < 0.01; 1.297 ± 0.2423 [full-length netrin-1, 3 nM], NS; 2.961 ± 0.1151 [VEGF, 2 nM], P < 0.001 (Figure 3, A and B).
Figure 3. The VI-V fragment plays a role in early BRB breakdown. (A) Representative images of vascular leakage induced with vehicle (PBS), recombinant VI-V fragment (3 nM), full-length netrin-1 (3 nM), or recombinant VEGF (2 nM) injected into the vitreous. Intravitreal injection resulted in a robust increase of retinal vasopermeability in eyes treated with the VI-V fragment of netrin-1 and with VEGF. \( n = 3 \). Scale bars: 30 \( \mu \)m. (B) EB extravasation (vascular leakage) induced by VI-V fragment, full-length netrin-1, and recombinant VEGF, measured by overnight extraction of the dye and spectrophotometric quantification \( n = 4–6 \). (C) Representative images of the vascular leakage induced with vehicle, synthetic VI-V fragment, full-length netrin-1, or recombinant VEGF s.c. injected into adult C57BL/6J mice \( n = 5 \). Original magnification, \( \times 2.5 \). (D) EB extravasation (vascular leakage) induced by VI-V fragment, full-length netrin-1, and recombinant VEGF was assessed by overnight extraction of the blue dye in formamide and spectrophotometric quantification \( n = 5 \). (E) Representative images of the vascular leakage induced with vehicle (PBS), recombinant VEGF (2 nM), full-length netrin-1 (3 nM), or recombinant VI-V fragment (3 nM) intradermally injected into CD-1 mouse ears \( n = 5 \). Original magnification, \( \times 1.6 \). (F) Quantification of EB extravasation (vascular leakage) induced by VI-V fragment, full-length netrin-1, and recombinant VEGF \( n = 5 \). Data are expressed as the mean \pm SEM. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \), by 2-tailed Student’s t test (B and F) and 1-way ANOVA with Tukey’s post-hoc test (D).
Figure 4. Netrin-1 preserves endothelial integrity, whereas the VI-V fragment disrupts endothelial barrier function. (A) ECIS measured live barrier function by assessing the resistance of the paracellular pathway between endothelial cells. $R_{\text{medium}}$, resistance of culture medium. (B) Paracellular resistance measured in real time by ECIS demonstrated that the VI-V fragment (3 nM) compromised endothelial barrier function (1–6 hours, $n = 4$), whereas netrin-1 (3 nM) preserved endothelial integrity with HUVECs. (C) Paracellular resistance measured in real-time by ECIS demonstrated that full-length netrin-1 delayed starving-induced (8 hours) monolayer breakdown (3–8 hours, $n = 4$). (D) An endothelial cell monolayer pretreated with netrin-1 and exposed to VEGF was significantly more resistant than were endothelial cells pretreated with the VI-V fragment (2–5 hours, $n = 4$). Data are expressed as the mean ± SEM.
We next performed a modified Miles assay with s.c. injections of vehicle (PBS); the VI-V fragment (3 and 7.5 nM); full-length netrin-1 (3 and 7.5 nM); or recombinant VEGF (Figure 3C). As above, the VI-V fragment induced approximately 3-fold more vascular leakage than was seen in vehicle controls (1.000 ± 0.3110 [vehicle], 2.744 ± 0.537 [VI-V fragment, 3 nM], P < 0.05; 3.295 ± 0.336 [VI-V fragment, 7.5 nM], P < 0.01; 2.046 ± 0.402 [full-length netrin-1, 3 nM], NS; 1.708 ± 0.4346 [full-length netrin-1, 7.5 nM], NS; 5.078 ± 0.3658 [VEGF, 2 nM], P < 0.001) (Figure 3, C and D). Finally, we performed an auricular Miles assay of s.c. permeability, whereby vehicle (PBS), the VI-V fragment (3 nM), and recombinant VEGF (2 nM) were compared in auricular injections (Figure 3E). Quantification revealed that the VI-V fragment induced approximately 6-fold more permeability when compared with vehicle controls, yet less than was observed with VEGF (1.000 ± 0.3126 [vehicle], 5.832 ± 0.3658 [VI-V fragment], P < 0.001; 1.824 ± 0.3539 [full-length netrin-1], NS; 16.93 ± 2.282 [VEGF], P < 0.001) (Figure 3, C and D). Finally, we performed an auricular Miles assay of s.c. permeability, whereby vehicle (PBS), the VI-V fragment (3 nM), and recombinant VEGF (2 nM) were compared in auricular injections (Figure 3E). Quantification revealed that the VI-V fragment induced approximately 6-fold more permeability when compared with vehicle controls, yet less than was observed with VEGF (1.000 ± 0.3126 [vehicle], 5.832 ± 0.3658 [VI-V fragment], P < 0.001; 1.824 ± 0.3539 [full-length netrin-1], NS; 16.93 ± 2.282 [VEGF], P < 0.001) (Figure 3F).

In all paradigms tested, the VI-V fragment of netrin-1 significantly induced vascular permeability, albeit less potently than did VEGF. MMP-9–mediated proteolysis generates fragments of netrin-1. Given the sequence homology of netrin-1 with tissue inhibitors of metalloproteinase (TIMPs) in its NTR module (ref. 39 and Figure 5A), we sought to determine whether netrin-1 could be a substrate for collagenases. MMP-9 is a collagenase that is induced in DR and has documented roles in vascular permeability (40) (Figure 6A). We therefore incubated full-length netrin-1 with MMP-9 at 37°C and monitored fragmentation at different time points (30 and 60 min) by Western blot analysis. At 30 minutes, a fragment corresponding to the predicted molecular weight of the VI-V peptide was detected, while netrin-1 itself was being degraded (Figure 5B). A c-Myc–tagged construct of the VI-V fragment was used as a control and hence accounts for the slight shift in bands between recombinant VI-V and MMP-9–generated VI-V. Importantly, the observed patterns of fragmentation are reminiscent of those seen in murine...
diabetic retinal blots (Figure 2D). We used mass spectrometric analysis to determine the mass of full-length netrin-1 (66.62 kDa) and the masses of 2 fragments resulting from the enzymatic cleavage of netrin-1 (Figure 5B). A first fragment with a mass of 55.99 kDa corresponded to full-length netrin-1 with a cleaved NTR module, and a second one, with a mass of 34.52 kDa, corresponded to netrin-1 cleaved within the domain V (EGF-like I) (Figure 5, B and C).

**MMP-9 is induced in the vitreous humor of patients with DME.** Collagenases are abundantly produced during development or during heightened inflammation. We therefore screened for transcripts of matricial collagenases (**Mmp2**, **Mmp3**, **Mmp9**, and **Mmp12**) by qPCR at 8 weeks after induction of diabetes. We found **Mmp9** to be the most robustly induced at this time point (Figure 6A), and it remained significantly elevated at 12 and 14 weeks of disease (12 weeks: 1.000 ± 0.266 [citrate], 2.348 ± 0.440 [STZ], **P** < 0.05; 14 weeks: 1.000 ± 0.290 [citrate], 3.206 ± 0.6947 [STZ], **P** < 0.05) (Figure 6B). LCM and real-time qPCR showed that **Mmp9** increased by approximately 3-fold in diabetic GCLs and ONLs (GCL: 1.000 ± 0.0338 [citrate], 2.688 ± 0.1302 [STZ], **P** < 0.01; INL: 0.5864 ± 0.0056 [citrate], 0.4742 ± 0.0817 [STZ], NS; ONL: 0.1574 ± 0.0222 [citrate], 0.4891 ± 0.01413 [STZ], **P** < 0.01) (Figure 6C). Induction of MMP-9 was also corroborated by IHC in diabetic retinae at 8 weeks of diabetes, with elevated staining of MMP-9 in the GCL, in vessels, and partially in microglia (ionized calcium–binding adaptor molecule 1-positive [IBA1-positive] cells) (Figure 6D).

To assess the potential involvement of MMP-9 in DR, we next investigated its protein levels in vitreous from patients with DME. Western blot analysis of equal volumes of vitreous humor revealed the presence of MMP-9 in patients with DME (n = 4–5). (F) Correlation curve between MMP-9 and VI-V fragment generation showing that 90% of the VI-V fragment variation was associated with MMP-9 variation. Data are expressed as the mean ± SEM. *P < 0.05 and **P < 0.01, by 2-tailed Student’s t test.
Inhibition of collagenases or MMP-9 in vivo reduces pathological vascular permeability. To further investigate whether cleavage of netrin-1 is driving vasopermeability, we examined the behavior of HUVEC monolayers stimulated with fragments produced in vitro by incubation of netrin-1 (3 nM) with MMP-9. Fragments were generated as described for Figure 5B, collected after 30 minutes, immunoprecipitated to remove MMP-9 (Figure 7A), and exposed to the endothelial monolayer by ECIS. IP of netrin-1 or MMP-9 alone served as a control (Figure 7A, right panel). A drop in HUVEC impedance following exposure to products of MMP-9–mediated degradation of netrin-1 suggests processing of netrin-1 into vasoactive fragments that compromise endothelial barrier function. Controls were immunoprecipitated from full-length netrin-1 or MMP-9–alone immunoprecipitated with netrin-1 Ab, and they showed no effect (Figure 7B).

To validate our findings in vivo, we performed an auricular Miles assay with digested full-length netrin-1 (MMP-9–digested), VEGF, or vehicle (IP elution buffer) (Figure 7C). Our results confirmed that MMP-9–mediated digestion of full-length netrin-1 generated bioactive fragments with 3-fold greater potency in inducing vascular permeability than did the vehicle control, albeit less effectively than did VEGF (1.000 [vehicle], 3.166 ± 0.4376 [VI-V fragments], P < 0.05; 7.349 ± 0.644 [VEGF], P < 0.001) (Figure 7D).

Given the potent effects of the VI-V fragment on vascular permeability, we sought to prevent its generation by inhibiting collagenases. At 6 and 7 weeks after induction of diabetes, we performed intravitreal injections of a soluble synthetic pan-inhibitor of collagenases (2-Pro-D-Leu-D-Ala-NOH; final vitreous concentration of 1 μM) (Figure 7E). This approach led to a significant reduction in diabetes-induced permeability of approximately 31% at 8 weeks of diabetes (collagenase pan-inhibitor, 31.11%; 1.000 ± 0.0903 [STZ], 0.6869 ± 0.0881 [anti-collagenase and STZ], P < 0.05, n = 15 mice) (Figure 7F).
IHC and 3D retinal reconstruction (Figure 9E) confirmed that UNC5B was predominantly expressed in mature vasculature. We then used LCM to measure the expression of UNC5B in the different retinal layers and in the vasculature. Our data show that UNC5B expression was upregulated (~4-fold increase) in STZ-induced diabetic vasculature compared with expression in control mice that were only injected with citrate (citrate, 1.000 ± 0.342, STZ, 3.954 ± 0.6241, P < 0.05) (Figure 9F).

Given the discrepancy in the activation kinetics of netrin-1 and its VI-V fragment to activate permeability pathways (Figure 8), we next investigated the binding affinity toward UNC5B of full-length netrin-1 and the VI-V fragment via surface plasmon resonance. 

**Figure 8. The VI-V fragment of netrin-1 activates FAK and SRC kinases.** (A) VEGF mRNA levels after VI-V fragment intravitreal injection, NS (n = 6). (B) Representative Western blot of HRMEC treatment with VI-V fragment leading to phosphorylation of SRC, FAK, and, ultimately, VE-cadherin, with a maximum response amplitude at approximately 10 minutes, while treatment with netrin-1 showed a maximum response amplitude at 2.5 minutes (n = 3). (C) Quantification of p-SRC, p-FAK, p-VE-cadherin, and β-actin signal density in double-scale graphics, which represent p-SRC/β-actin, p-FAK/β-actin, and p-VE-cadherin/β-actin at 0, 2.5, 5, 10, 20, 40, and 60 minutes (n = 3). (D) Working hypothesis. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-tailed Student’s t test (A) or 1-way ANOVA with Tukey’s post-hoc test (C).
barrier function, as has been suggested for UNC5B–roundabout guidance receptor 4 (UNC5B-ROBO4) interactions (42). SPR experiments also revealed that netrin-1 was tightly associated with the negatively charged carboxymethylated dextran surface of the sensor chip in contrast to the VI-V fragment, which showed low, nonspecific binding to the dextran matrix. By using sensor chips with different charge densities and/or chain lengths (CM4, CM3, CM5), we observed that the nonspecific interactions (SPR). We found that the affinity of the VI-V fragment was approximately 100-fold lower than that of netrin-1 (netrin-1 $K_d = 1.9$ nM, versus VI-V fragment $K_d = 267$ nM) (Figure 9G). Kinetics analysis of the full-length netrin-1–UNC5B association also revealed a rapid association and slow dissociation, which is consistent with the VE-cadherin phosphorylation patterns observed at early time points (2.5 and 5 min) (Figure 8B). Slow dissociation may suggest that full-length netrin-1 remains bound to UNC5B and reinforces barrier function, as has been suggested for UNC5B–roundabout guidance receptor 4 (UNC5B-ROBO4) interactions (42). SPR experiments also revealed that netrin-1 was tightly associated with the negatively charged carboxymethylated dextran surface of the sensor chip in contrast to the VI-V fragment, which showed low, nonspecific binding to the dextran matrix. By using sensor chips with different charge densities and/or chain lengths (CM4, CM3, CM5), we observed that the nonspecific interactions.
MMP-9 and other collagenases into the more soluble VI-V fragment. Although future work will identify the exact sequence of the netrin fragment detected in retinopathy, the current study supports the conclusion that the 55-kDa fragment observed in vitreous samples of DME patients and in the diabetic mouse retina is similar to the VI-V fragment.

While full-length netrin-1 has vasoprotective properties (21) and maintains blood-brain barrier function (45), the newly generated fragments contribute to the breakdown of the BRB and thus exacerbate retinal and macular edema. A role for the VI-V fragment of netrin-1 in pathology was previously suggested, given its presence in multiple sclerosis (MS) plaques and capacity to inhibit oligodendrocyte migration (46). While netrin-1 is required for the maintenance of axo-oligodendroglial paranodal junctions (47), ectopic expression of netrin-1 in vivo inhibited remyelination in an experimental model of MS lesions (48).

Retinal netrin-1 is produced primarily in the GCL adjacent the retinal vascular plexus affected in DR. Paradoxically, at similar time points, retinal ganglion neurons in diabetes also secrete the guidance cue semaphorin 3A, which is a potent inducer of vascular permeability (17). However, degradation of netrin-1 by collagenases and MMPs generates fragments, such as the VI-V peptide, that exacerbate retinal edema. Together, these data further suggest that cells of the ganglion layer undergo diabetes-induced

Discussion

A mechanistic understanding of the pathogenesis of DR and DME has evolved in recent years beyond microvessels and now encompasses early disturbances in the neurovascular unit as a whole (2). This view of the disease is particularly plausible, given the preponderance of neural (neuronal and glial) cells versus endothelial and mural cells in the retina and the substantial metabolic requirements of neuronal transmission (44). In this study, we provide evidence that DR is associated with the processing of the guidance cue netrin-1, a protein with the multivalent ability to influence vascular, neuronal, and immune systems (37). Contrary to several other guidance cues, expression of netrin-1 is not limited to embryogenesis, and its levels remain elevated in mature retina (Figure 2), suggesting a role in tissue homeostasis. We demonstrate that during diabetes, retinal netrin-1 is processed by MMP-9 and other collagenases into the more soluble VI-V fragment. Although future work will identify the exact sequence of the netrin fragment detected in retinopathy, the current study supports the conclusion that the 55-kDa fragment observed in vitreous samples of DME patients and in the diabetic mouse retina is similar to the VI-V fragment.

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stress and produce vasomodulatory proteins that are involved in the mediation of vascular phenotypes in DR.

The present study also provides evidence for a diabetes-induced upregulation of the netrin-1 receptor UNC5B in retinal microvessels. These findings are consistent with those of several studies describing elevated levels of UNC5B in conditions associated with chronic inflammation such as atherosclerosis, with induction of UNC5B in plaques (49), obesity, with upregulation of UNC5B in macrophages (31), and oxygen-induced retinopathy, with induction of UNC5B in retinal vessels (50). Consistent with our findings, a role for UNC5B in vascular integrity had previously been demonstrated, in which interaction between UNC5B and the extracellular domain of the ROBO4 receptor prevents VEGF-driven permeability (42). Hence, induction of UNC5B on retinal microvessels may enhance vascular integrity and function as a protective mechanism of adaptation to diabetes. Activation of UNC5B via the VI-V fragment of netrin-1 may disrupt the vasostabilizing interactions of UNC5B and thereby promote vasogenic edema. Our results point to apparently divergent effects of full-length netrin-1 and the VI-V fragment on the vasculature. The lower binding affinity of the VI-V fragment versus that of full-length netrin-1 with UNC5B may explain the differential FAK, SRC, and VE-cadherin phosphorylation kinetics observed. Yet, full-length netrin-1 dissociates less readily, suggesting a possible sequestration by the receptor. MMP-9–mediated cleavage of netrin-1 generates fragments with opposite actions, much like what has been described for cytokines (51). MMP-9–mediated proteolysis of netrin-1 may also increase the bioavailability of netrin-1, akin to VEGF distribution in tissue (52). The resulting fragments would be liberate to diffuse within the retina and increase vasopermeability.

The landscape for the treatment of DME evolved with the introduction of anti-VEGFs such as bevacizumab (Avastin), ranibizumab (Lucentis), and aflibercept (Eylea). Paradoxically, their ability to effectively block VEGF function may compromise retinal function in the long term, given the fundamental role of VEGF as a neurotrophic factor (12, 13). Because VEGF is not upregulated in the early phases of disease (17), blocking the biological activity of the VI-V fragment may be of therapeutic interest. Given that the VI-V fragment and full-length netrin-1 can both signal through UNC5B (other receptors cannot be excluded), that the UNC5B receptor is required for vascular stability (42), and considering the vasoprotective role of netrin-1 (21, 45), a successful approach may require interfering with the processing of full-length netrin-1. The positively charged NTR module has been observed to interact with extracellular matrix heparin sulfates (53) and has also been reported to control MMP activity (54). Given the induction of MMPs such as MMP-9 in diabetic retinae and their contribution to generation of the VI-V fragment, neutralizing MMPs (55) may also provide prospective pharmacological avenues once specific inhibitors are generated. To date, broad-spectrum MMP inhibitors have failed in oncology trials, largely because of the crucial roles of MMPs in several ubiquitous cellular and extracellular processes such as cell-surface receptor cleavage and release as well as cytokine and chemokine activation and inactivation (56). Further research is still needed to develop potent selective MMP inhibitors that will allow for successful clinical applications (57).

Collectively, our results demonstrate that processing of netrin-1 in the diabetic retina leads to vasoactive fragments with the capacity to exacerbate vasogenic edema. While future studies will determine the therapeutic merits of interfering with netrin-1 fragmentation, our study provides further evidence for the role of guidance cues and neurovascular interplay in DR.

Methods

Animals

C57BL/6 WT mice were purchased from the Jackson Laboratory and CD-1 mice from Charles River Laboratories.

Vitrectomy

All patients previously diagnosed with DME were followed up and operated on by a single vitreoretinal surgeon (FAR). The control patients were undergoing surgical treatment for nonvascular pathology (epiretinal membrane [ERM] or macular hole) by the same surgeon. Topical instillation of 5% povidone-iodine solution was applied to the ocular surface and used to clean the periocular skin prior to the surgical procedure. For sampling of DME vitreous, patients were then draped in a standard sterile manner with placement of a lid speculum. A 27-gauge self-retaining line (Insight Instruments) for balanced salt solution (BSS) infusion was first placed, followed by a 29-gauge chandelier placement connected to a mercury vapor light source (Synergetics). The surgical view during the procedure was provided through a surgical operative microscope and a Volk contact lens (Volk direct image ×1.5 magnifying disposable vitrectomy lens). The vitrectomy was performed in all patients using a 25-gauge sutureless retractor system (Insight Instruments). The model used in the study is a portable, battery-powered system with a maximum cut rate of 600 cpm and features a single-use retractable sheathed guillotine cutter (25-gauge) with an in-built needle (23-gauge). The needle was introduced bevel down through displaced conjunctiva in an oblique single-plane tunnel into the vitreous cavity 3–4 mm from the limbus. With the exception of the portable vitrector motor handpiece, which was placed within a sterile plastic cover when in use, all other instruments used were sterile and disposable. For patients with ERM and macular hole (controls), a standard 25-gauge 3-port pars plana vitrectomy was performed in the operating room.

3D rendering of the netrin-1 structure

The Robetta 3D structure server (58) was used to generate full-chain protein structures of full-length netrin-1 and of the VI-V fragment. Prediction was performed using the Ginzu protocol, consisting of ab initio fragment assembly. Ginzu identifies homologs with an experimentally determined structure (pdb protocol), generates a multiple alignment of the highest-scoring pairs of the blast-p, and ultimately selects cut-points between domains (PSI Blast MSA).

Diabetic mouse model

Multiple low doses of STZ (Sigma-Aldrich) were administered i.p. on 5 consecutive days to 6-week-old C57BL/6J mice at a concentration of 55 mg/kg to induce a type 1 diabetic model. Control mice were injected i.p. with sodium citrate buffer.

Cell lines

HRMECs were purchased from Cell Systems and HUVECs from Lonza and were used from passages 6 to 11. HRMECs were cultured...
in EGM-2 microvascular medium from Lonza, whereasHUVECs were cultured in EGM-2 macrovascular medium fromLonza. For stimulationexperiments and for real-time ECIS, cells were previously starvedfor 4 hours with EB2 medium from Lonza plus 0.5% FBS. Recombinant
VEGF_{165} was purchased from Peprotech.

**Recombinant truncated netrin-1 VI and V**

Truncated netrin-1 containing domains VI and V with a C-terminal
Myc tag was generated by expression in Epstein-Barr virus nuclear
antigen/human embryonic kidney 293 (EBNA 293) cells with the use
of the pCEP4 vector (Invitrogen) and purified by a heparin sepharose
high-performance column (HR 5/10; Amersham Biosciences).

**Western blot analysis**

For assessment of retinal protein levels, we enucleated eyes at 4, 6, and 8 weeks of diabetes (versus citrate controls) and rapidly dissected
and homogenized retinai. Protein concentration was assessed by
bicinchoninic acid (BCA) assay (Sigma-Aldrich), and 30 μg protein was
analyzed for each condition by standard SDS-PAGE technique. For
supernatants, we loaded equal volumes of samples on gels and dete-
mined the amount of protein present by Ponceau red (Sigma-Aldrich)
staining of membranes.

Netrin-1 rabbit polyclonal Abs against netrin-1 VI-V antigen were
generated as described previously (59). MMP-9 Ab was purchased from
Abcam (catalog ab119906). Anti-VE-cadherin Ab (p-Y731) was
purchased from Cell Signaling Technology. β-Actin was purchased from Medimabs (catalog MM-0164P).

**Netrin-1 processing in vitro**

Netrin-1 fragments were generated in vitro as follows: 3 nM synthetic
netrin-1 was digested in 100 μl of active MMP-9 (1 μg) from Abcam
(catalog ab168863) for 30 minutes at 37°C. The reaction was then
stopped with proteases inhibitors and the mix stored at –80°C for sub-
sequent Western blot analysis and IP. For mass spectrometric analysis,
we used a larger quantity of netrin-1 (10 μg).

**Netrin-1P**

Netrin-1 mAb (ALX-804-838-C100, clone NORA-1) used for IP was
purchased from Enzo (Enzo Life Sciences). To avoid contamination
of the Ab for permeability assays, we used a Pierce Crosslink IP Kit
(Thermo Fisher Scientific).

**qPCR**

Extraction of mRNA was performed by standard TRIzol extraction.
DNase digestion to prevent amplification of genomic DNA was then
performed and was followed by a retrotranscription step to generate
cDNA using a Bio-Rad iScript cDNA Synthesis Kit. Gene expression
was then quantified using SYBR Green in an ABI Biosystems Real-
Time PCR system. *Actb* was used as a reference gene.

**ECIS assay**

Real-time analysis of trans- and interendothelial impedance was per-
formed by plating 10^4 HRMECs and HUVECs onto 8W10E+ 8-well
arrays (Applied Biophysics) (40 electrodes per well). Cells were
allowed to grow until resistance stabilized at approximately 2,000 Ω.
Cells were then starved for 4 to 6 hours in basal medium (EBM2) and
stimulated for 10 hours. The results were then normalized to the vehi-
cle control and expressed as relative resistance.

**LCM**

Eyes were enucleated from C57BL/6J mice at 4 and 8 weeks of diabetes
(versus citrate controls) and flash frozen in OCT. We then cut 12-μm sec-
tions using a Leica cryostat at -20°C and air dried the sections for 10 min-
utes. For LCM, retinal layers were dissected using a Zeiss Observer micro-
scope equipped with a Palm MicroBeam device. mRNA was isolated from
these sections and qPCRs were performed as described above.

**Mass spectrometry**

One hundred microliters of full-length netrin-1 and digested net-
rin-1 was dried in a Speed-Vac (Thermosavant), resolubilized in 0.2%
formic acid (FA), and analyzed on an Agilent 1100 LC coupled to an
Agilent 6510 Q-TOF instrument. Samples were loaded on a C5 Phe-
nomenex precolumn (360 × 500 μm) and separated on an analytical
column (150 μm × 15 cm), with a 56-minute gradient from 0% to 30%
acetonitrile (0.2% FA) and a 600 nl/minute flow rate.

**SPR**

SPR analyses were performed using a Biacore T200 instrument (GE
Healthcare). UNC5B recombinant protein was immobilized on car-
boxymethylated dextran CM5 (for VI-V) and CM4 (for netrin) sensor
chips (GE Healthcare) using an amine-coupling strategy. Briefly, the
sensor chip surface was activated with a 1:1 mixture of N-hydroxysuc-
cinimide and 3-(N,N-dimethylamino)-propyl-N-ethylcarbodiimide.
UNC5B solution (solubilized in acetate buffer, pH 4.5) was injected at
a flow rate of 20 μl/minute in PBS running buffer (pH 7.2; 0.025% [v/v]
Tween-20) to reach a level of immobilization of 400 and 800 relative
units (RU) on CM5 and CM4 sensor chips, respectively. Surfaces (pro-
tein and reference) were blocked by the injection of an ethanalamine
solution. Binding kinetics of netrin and VI-V over the UNC5B sensor
chip was evaluated in PBS buffer (pH 7.2; 0.025% [v/v] Tween-20),
with concentrations ranging from 12.5 to 100 nM VI-V and netrin,
respectively. All tests were performed at 25°C using a flow rate of 40
μl/minute. Sensor chip surfaces were regenerated by twice inject-
ing 20 μl of a 50 mM NaOH solution at a flow rate of 40 μl/minute.
Binding sensograms were obtained by subtracting the reference flow
cell. Data analysis was performed using BIA Evaluation Software (GE
Healthcare) and fit to a one-site Langmuir binding model.

**Collagenase and MMP-9 inhibitors**

Collagenase inhibitor (EMD Millipore Calbiochem; catalog CAS
234140) was injected intravitreally into diabetic C57BL/6J mice at 6 and
7 weeks of diabetes. Type I MMP-9 inhibitor (EMD Millipore Cal-
biochem; catalog CAS 11777 49-58-4) was injected in the same way
into diabetic mice at 6 and 7 weeks of diabetes. For both experiments,
1 μl (5 μM for the collagenase inhibitor, 25 nM for the MMP-9 inhibi-
tor) was injected into the vitreous.

**Permeability assays**

We performed 3 different tests of permeability, all based on fluores-
cent dye extravasation.

**Retinal EB permeation assay.** EB (45 mg/kg; Sigma-Aldrich) was
injected i.v. into C57BL/6J mice and allowed to circulate for 2 hours.
Eyes were then enucleated, fixed for 1 hour in 4% paraformaldehyde

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(PFA), and the retina dissected. EB was extracted overnight in formaldehyde at 65°C and quantified by spectrophotometry (620 nm maximum, 740 nm minimum absorbance) (17).

* Auricular Miles assay.* EB (45 mg/kg) was injected i.v. into CD-1 mice. After 10 minutes, the mice were injected with PBS (vehicle) into the control ear, and PBS VI-V (3–7 nM), PBS VEGF (2 nM), or PBS purified fragments (~3 nM) into the contralateral ear. EB extravasation was photographed 3 hours after EB injection and evaluated by ImageJ software (NIH) (60).

* Modified Miles assay.* EB (45 mg/kg) was injected i.v. into C57BL/6 mice. After 10 minutes, the mice were injected s.c. with PBS (vehicle), PBS VI-V fragment (3 nM), or PBS VEGF (2 nM). EB extravasation was evaluated 3 hours after EB injection.

Retinal angiography was performed as previously described (61). Briefly, C57BL/6J mice were anesthetized, and the descending aorta was clamped and then perfused via the left ventricle with 1 ml warm PBS containing 50 mg high-molecular-weight (2 × 10^6 Da) FITC (Sigma-Aldrich). After 2 minutes of circulation, the eyes were enucleated and placed in 4% PFA for 2 hours. Retinas were then dissected, stained overnight with lectin-MgCl₂ and flat-mounted in fluorescent mounting medium. Leakage was observed and photographed under a confocal microscope at 488 nm (×10 objective).

**IHC**

Eyes were enucleated from mice and fixed in 4% PFA at room temperature for 4 hours, incubated in 30% sucrose overnight at 4°C, and frozen in optimal OCT compound. Sections of 10-μm thickness were then prepared using a cryostat (Leica), keeping the temperature at −20°C. IHC experiments were performed and cross sections visualized with an Olympus FluoView confocal microscope.

UNCSB Ab was purchased from Abcam (catalog ab54430). IBA1 Ab was purchased from Wako (catalog 019-19741). Rhodamine isoclectin B4 was purchased from Vector Laboratories (catalog RL-1102). DAPI was purchased from Molecular Probes (Thermo Fisher Scientific).

**Statistics**

Data are presented as the mean ± SEM. One-way ANOVA with Tukey’s post-hoc tests were used to compare differences between groups. A 2-tailed Student’s t test was used when only 2 groups were compared. The criterion for statistical significance was a P value of less than 0.05.

**Study approval.** Approval of the human clinical protocol was provided by the ethics committee of Maisonneuve-Rosemont Hospital Research Centre, 5415 Assomption Boulevard, Montreal, QC, H1T 2M4, Canada. Phone: 514.252.3400, ext. 7711; E-mail: mike.sapieha@umontreal.ca.

**Author contributions**

KM designed the research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript. FB and AW wrote the manuscript. KM, KL, and SH analyzed data. TEK provided vitreous samples. SB analyzed data. TEK provided reagents, contributed to the study design, and wrote the manuscript. PS designed the research studies and wrote the manuscript.

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