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Netrins Promote Developmental and Therapeutic Angiogenesis

Brent D. Wilson, **Masaaki Ii**, **Kye Won Park**, **Arminda Suli**, **Gerhardus A.H. Kock**, **Lise K. Sorensen**, **Wonhee Suh**, **Fréderic Larrieu-Lahargue**, **Lisa D. Urness**, **Kirk R. Thomas**, **Chi-Bin Chien***, **Douglas W. Losordo***, and **Dean Y. Li***

METHODS

Migration Assays

Migration assays were performed as described (3-5). Briefly, 16 h before the assay, 80% confluent 75 cm^2 flasks (Corning Costar) of human microvessel endothelial cells (HMVEC; Cambrex, Walkersville, MD), human coronary artery endothelial cells (HCAEC; Cambrex), human umbilical artery endothelial cells (HUAEC; Promocell, Heidelburg, Germany), or human umbilical vein endothelial cells (HUVEC; Promocell), were washed with Hank's Balanced Salt Solution (HBSS, Invitrogen) and serum-starved overnight in endothelial basal media (EBM-2, Cambrex) with 0.1% fatty-acid-free BSA (Sigma) and 0.5% fetal calf serum (FCS, Hyclone). The following day cells were lifted with Trypsin/EDTA solution (Promocell), mixed with an equal volume Trypsin Neutralization Solution (Promocell), and washed 3 times in migration media (EBM-2 with 0.1% fatty-acid-free BSA and 0.2% FCS). Cells were resuspended at a density of 1.5×10^6 cells/ml and were allowed to recover for 1 h at 37°C (5% CO_2). 3.75×10^4 cells were plated into each well of a 48-well Boyden chamber apparatus (NeuroProbe, Cabin John, MD), and the wells were overlayed with an 8 μm pore polycarbonate membrane (NeuroProbe) that had been previously coated with 50 μg/ml human fibronectin (Biomedical Technologies, Inc., Stoughton, MA). Experiments performed with membranes coated with acetylated 1% gelatin from porcine skin (Sigma, St. Louis, MO) gave similar results. The apparatus was assembled and stored inverted at 37°C (5% CO_2) for 2 h. The apparatus was then re-inverted and 52 μl of purified chemoattractants [murine netrin-1 (R&D Systems, Minneapolis, MN), chicken netrin-2 (R&D Systems), murine netrin-4 (R&D Systems), murine netrin-G1a (R&D Systems), human $VEGF_{165}$ (R&D Systems), or control/ migration media (EBM-2 with 0.1% fatty-acid-free BSA and 0.2% FCS) were added to the upper chambers, and the migration was allowed to proceed for 2 h at 37°C (5% CO_2). The membranes were then removed, fixed in methanol, stained with a Hema 3 stain set (Fisher Scientific, Pittsburgh, PA), and placed (migrated-side down) onto 50 × 75 mm glass slides. Before 90% mounting medium (in xylenes) and coverslips were applied, the non-migrated cells were removed from the exposed (non-migrated) side of the membrane with a moistened swab. Cells present on the migrated side of the membrane were manually counted (three random 200× fields per well), and data points for each experiment represent the average number of migrated cells from six separate wells (three 200× fields counted per well).

Another method was employed in a separate laboratory to evaluate the effects of the netrins on mouse (MS1) endothelial cells (ATCC, Manassas, VA) using a modified Boyden chamber assay as described previously (6). Briefly, a 5 μm-polycarbonate filter (Poretics) was placed between upper and lower chamber. Cell suspensions (5×10^4 cells/well) were placed in the upper chamber, and the lower chamber was filled with serum-free medium containing

* To whom correspondence should be addressed. E-mail: dean.li@hmbg.utah.edu (D.Y.L.); douglas.losordo@tufts.edu (D.W.L.); chi-bin.chien@neuro.utah.edu (C.B.C.).

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recombinant mouse netrin-1 and -4 at concentrations of 0, 10, 50, 100 and 200 ng/ml. Recombinant mouse VEGF (50 ng/ml) was used as a positive control. The chamber was incubated for 4 hours at 37°C and 5% CO_2. Migration activity was evaluated as the mean number of migrated cells in 3 high power fields (400×) per chamber.

Proliferation Assays

HMVEC (Cambrex), HCAEC (Cambrex), HUAEC (Promocell), and HUVEC (Promocell) were cultured according to manufacturer's recommendations and were used between passages 3−7. A total of 2.5×10^3 cells were plated into each well of a 96-well plate in complete growth media [EGM-2 MV (Cambrex) for HMVEC and HCAEC, EGM-2 (Promocell) for HUAEC and HUVEC, and allowed to adhere for 4 hours. Complete growth media was then removed, and the cells were washed with HBSS and then serum-starved for 16 h in EBM-2 (Cambrex) containing 0.2% FCS. Factors to be assayed (netrin-1, netrin-2, netrin-4, $VEGF_{165}$, BSA) were then diluted in EBM-2 + 0.5% FCS and added to each well. Assayed factors were re-applied every 24 hours for three days. After 72 h, cells were fixed in Zamboni's Fixative (Newcomer Supply, Middleton, WI). Stained with Gill-1 hematoxylin, overlayed with 80% glycerol, and manually counted. An alternative, more rapid method of assessing final cell number was also utilized. In this method, the number of cells present after 72 hours was quantified according to manufacturer's protocols by using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) and measuring absorbance at 450 nm with a Thermomax plate reader (Molecular Devices, Menlo Park, CA). Assays counted manually or with the cell counting kit gave similar results.

An alternate method was employed in another laboratory to assess the effects of netrins on proliferation of mouse MS1 endothelial cells (ATCC). The number of viable mouse endothelial cells was determined using a validated non-radioactive cell proliferation assay kit (CellTiter 96; Promega, Madison, WI) as described previously (6). Briefly, cells were seeded in 96-well plates at 80% confluence (1×10^4 cells/well) and cultured in 10% FBS medium with recombinant mouse VEGF (13ng/ml), netrin-1, or netrin-4 proteins (R&D Systems, 50ng/ml). After 48 hours in culture, cells were incubated with MTS reagent for 4 hours, and proliferation activity was determined by the measurement of absorbance at OD 490 nm.

Tube Formation Assays

Matrigel (200 μl, BD Biosciences) was applied to each well of a 48-well plate and incubated at 37°C for 30 minutes before adding 25×10^3 endothelial cells per well in serum free media. After 45 minutes at 37°C (5% CO_2), serum free media was removed and replaced with media containing 0.5% FCS and indicated treatments. Tube formation was assessed beginning 4 hours later. Photographs of representative 50 × fields were taken, and endothelial tubes were quantified by counting length and branches using Image J software (NIH). Experiments were repeated three times independently, and statistical analyses were performed using Student's *t* test. A p value <0.05 was considered statistically significant.

Adhesion

96-well plates (non-tissue culture treated plate, Falcon) were coated with 0.1 % BSA or with 10 μg/ml netrin-1, netrin-2, netrin-4, netrin-G1, or fibronectin for 16 h at 4°C. Wells were washed with PBS and then blocked with PBS containing 5% BSA for 1 h at room temperature. Endothelial cells were harvested, washed three times with, and then resuspended in basal media (serum-free) containing 0.5% BSA. A total of 5×10^4 cells (in 100μl) were added to each well and allowed to attach for 30 min at 37°C. After washing three times with PBS, cells were fixed in Zamboni's fixative, and stained with Hemacolor (EMD chemicals, Gibbstown, NJ). Five 100× fields were manually counted per well. Data points represent mean number of adherent wells for duplicate wells (five 100× fields counted per well).

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Migration Following Treatment with Adenosine A2b Receptor Antagonists

HMVEC (Cambrex, passage3–7) were pre-incubated for 2h in endothelial basal media (EBM-2) containing 0.1%FCS and 1.2%DMSO or in endothelial basal media (EBM-2) containing 0.1%FCS, 1.2%DMSO, and the A2b receptor antagonists [1 μM DPSPX (1,3-dipropyl-8-sulfophenylxanthine; Sigma Chemical Co) or 600 μM enprofylline (3-propylxanthine; Sigma Chemical Co)]. Migration towards bovine serum albumin (control), netrin-1, netrin-4, and VEGF, was assessed utilizing a NeuroProbe Boyden Chamber apparatus as described above. DMSO or DMSO-containing A2b inhibitors were also added to the chemotactic factors used during the migration assay. This method was based on that used previously by Stein *et al* (7).

Migration Following Immunodepletion of Netrin Protein Preparations

Polyclonal goat antibodies (20 μg/ml) against mouse netrin-1 and netrin-4 (R&D Systems) or non-specific goat anti-mouse IgG antibodies (Jackson ImmunoResearch) were incubated with Protein G+ agarose beads (Santa Cruz Biotechnology) in endothelial basal media (EBM-2) containing 0.1% FBS for 14 hours at 4° C. The Protein G+ beads were then pelleted and washed 3 times at 4° C in EBM-2 containing 0.1%FBS. The beads were resuspended in 1 ml EBM-2 containing 0.1%FCS and 50 ng Netrin 1 or Netrin 4 and gently rocked at 4° C for 2 hours. The beads were then pelleted, and the remaining media was added to the appropriate well of a NeuroProbe Boyden Chamber apparatus, and migration was carried out as described above.

Expression of Netrin Receptors in Endothelial Cells using Real Time RT-PCR

RNA was isolated from multiple primary endothelial cells [human aortic endothelial cells (HAEC, Cambrex), human microvessel endothelial cells (HMVEC, Cambrex), human umbilical vein endothelial cells (HUVEC, Cambrex), and human umbilical artery endothelial cells (HUAEC, Cambrex)] using TRIZOL (Gibco BRL, Gaithersburg, Maryland). Human total brain RNA (BD Biosciences) was used as a positive control. Total RNA was used as template to make random primed cDNA (RetroScript kit, Ambion). Assays for *DCC, neogenin, Unc5a, Unc5b, Unc5c, Unc5d, Flk1, PECAM-1* and *Robo4* expression MIGHT BE NICE TO INCLUDE SEQUENCES (Assays-on-Demand, Applied Biosystems) were performed according to manufacturer's instructions on an Applied Biosystems 7900HT thermal cycler (University of Utah Genomics Core Facility). Transcripts were normalized in relation to *GAPDH* expression (human GAPDH, Applied Biosystems). All reactions were performed in triplicate.

Intracellular cAMP Measurement

cDNAs encoding an irrevelant G protein-coupled receptor [arginine vasopressin receptor-2 (mock/control)] and the A2b receptor were cloned into a modified pcDNA3.1 expression construct. Lipofectamine 2000 reagent (Invitrogen) was used to transfect 293T cells with these expression constructs. The following day 100,000 cells were plated into each well of a 96-well plate. At 48 hours after transfection, the cells were treated for 15 minutes with 0–1,000 μM 5′-(N- ethylcarboxamido) adenosine (NECA) or with 0–25 nM (0–2,000 ng/ml) netrin-1 or netrin-4, and cAMP levels were then determined. The experiment was repeated multiple times with lengths of agonist treatment ranging from 5 minutes to 150 minutes, with 25,000–150,000 cells assayed per well, and with and without 3-isobutyl-1-methylxanthine (IBMX). Two different cAMP measurement systems were utilized (Mediomics and Assay Designs); similar results were obtained with each assessment.

Immunoprecipitation of DCC and Unc Receptors with Netrins

Immunoprecipitation was carried out using purified netrin-1 or netrin-4 containing a carboxy-terminal 10 histidine tag (R&D Systems) and purified proteins containing the Fc region of

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IgG_1 fused to the extracellular region of the deleted in colon carcinoma receptors (DCC and neogenin) or the Unc5 receptors (Unc5a, Unc5b, Unc5c, Unc5d; R&D Systems). 25 μl (50% slurry) Protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were incubated with 2 μg of receptor-Fc fusion proteins and 1μg of His-tagged netrins at 4 °C for 90 min. The protein A/G beads were collected by centrifugation, washed three times with PBS containing 0.1% NP-40, resolved on a 10% acrylamide gel by SDS-PAGE, and analyzed by immunoblotting with an anti-His antibody (Clone BMG-His-1, Roche).

Immunoprecipitation of A2b Receptor with Netrins

A cDNA encoding the A2b receptor was cloned into pcDNA3.1 and into pcDNA3.1-HA. These constructs allowed expression of the A2b receptor with and without a carboxy terminal tag in mammalian cells. These A2b expression constructs were transiently transfected into HEK 293T cells (ATCC) using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared with Lysis Buffer 1 [50 mM HEPES, 0.5 % NP-40, 250 mM NaCl, 2 mM EDTA, 10 % glycerol and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN)] (8). The cell lysates were centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4°C. Supernatants were incubated with 1 μg netrin-1-His or netrin-4-His and then immunoprecipitated with an anti-HA affinity matrix (Roche). Western blot analysis using anti-His antibody was performed. Multiple conditions of immunoprecipitation to detect interaction of netrins with A2b produced similar results.

Cell lysates were also prepared in two additional buffers as follows: Lysis Buffer 2 [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5 % NP-40, 1% Triton X-100, 5 mM EDTA, Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN)] and Lysis Buffer 3 [50 mM HEPES (pH 7.5), 125 mM NaCl, 5 mM EDTA, 0.1 % NP-40, and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN)] (9) followed by sonication. Experiments utilizing Lysis Buffers 2 and 3 yielded identical results to those performed with Lysis Buffer 1.

Cell-Surface Binding of Netrins

COS-7 cells were transfected with a pcDNA3.1 expression construct alone (empty vector) or with pcDNA3.1 constructs containing the Unc5b or A2b receptors using Lipofectamine 2000 (Invitrogen) and were seeded into 12-well culture dishes (Corning Costar). A netrin-1-AP expression construct containing alkaline phosphatase fused to the carboxy terminus of human netrin-1 was kindly provided by Robert P. Kruger and Kun-Liang Guan of the University of Michigan. We replaced netrin-1 from this construct with a mouse netrin-4 cDNA to produce a plasmid encoding alkaline phosphatase fused to the carboxy terminus of netrin-4. Netrin-AP binding was carried out as described (10). Pretreatment (10 times molar excess) with unlabelled netrin before incubation with netrin-AP was performed to demonstrate specificity of interaction. Representative fields were photographed with a Zeiss Axiovert 200 microscope.

Fish Stocks and Embryo Raising

Adult fish were bred according to standard conditions. Heterozygous Tg(*fli1:egfp*)^{y1} transgenic carriers [a gift from Joseph Yost; (11)] were incrossed, and embryos were raised at 28.5°C in E2/GN embryo medium with 0.003% phenylthiourea to inhibit pigment formation and staged according to (12). For *in situ* hybridization, embryos were fixed in 4% PFA (in PBS) for 1−2 hr at room temperature (RT) or overnight (O/N) at 4°C, washed briefly in PBS, dehydrated, and stored in 100% methanol at −20°C until use.

In Situ *Hybridization*

Standard *in situ* hybridization procedures were used. Antisense RNA probe was prepared from a plasmid containing a partial *netrin1a* clone [a gift from Uwe Strähle; (13)], using T7

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polymerase and Dig RNA labeling kit (Boehringer Mannheim). Embryos were rehydrated to PBS, washed briefly with PBST (0.1% Tween-20 in PBS), digested with 0.1% collagenase (Sigma) for 15 min, washed with PBST, postfixed for 10 min in 4% PFA, treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 7.0) for 30 min at RT, hybridized with RNA probe O/N at 65°C, washed with SSCT (SSC in 0.1% Tween-20) at 65°C, treated with 10 μg/ml RNase A at 65°C for 30 minutes to digest unbound probe, and incubated for at least 2 hr with 2% Blocking Reagent (Boehringer Mannheim). Embryos were then incubated O/N with alkaline phosphatase-conjugated anti-DIG Fab fragment (1:5000, Boehringer Mannheim), washed with PBST and developed by incubating in AP substrate (Boehringer Mannheim).

Morpholino Oligonucleotide (MO) Sequence and Reverse Transcription

Control MO was a standard control from Gene Tools, sequence 5′-CCTCTTACCTCAGTTACAATTTATA-3′. The *netrin1a* splice blocking MO, sequence 5′-ATGATGGACTTACCGACACATTCGT-3′, was synthesized by Gene Tools and was a gift from Iain Shepherd. It was designed to bind to the last 11 bp of exon 1 and first 14 bp of intron 1. For RT-PCR analyses, morpholino-injected and wt embryos were placed in Trizol, triturated through a 25 gauge needle and stored at −20°C. Total RNA was extracted using the Qiagen RNeasy kit, treated with DNase, and cDNA prepared using SuperScript™ First-Strand Synthesis (Invitrogen) and random hexamer primers. Primers F1 (5′-CTTTCGGAGACGAAAACGAG-3′) and R1 (5′-GTAGGCGCTTTCCAGAGATG-3′) were used to amplify *netrin1a* pre-mRNA, while primers F1 and R2 (5′-CTTTGCAGTAGTGGCAGTGG-3′) were used to amplify normal mRNA; primers beta-actin FP (5′-CCCAAGGCCAACAGGGAAAA-3′) and beta-actin RP (5′-GGTGCCCATCTCCTGCTCAA-3′) were used to amplify *beta-actin* mRNA. PCR conditions: 2 min 94°C; followed by 31 cycles (*netrin1a*) or 20 cycles (*beta actin*) of 94°C, 30 s/59°C, 30 s/72°C, 60 s; followed by 5 min 72°C.

MO Injections

Lyophilized MO was diluted and stored at 4°C; concentrations were periodically checked by spectrophotometry (A265 in 0.1N HCl, as suggested by Gene Tools). Working solutions were diluted in water and phenol red (0.5% final concentration), and 1 nl was pressure-injected into the cell of 1-cell embryos or high yolk of 2-cell embryos using a Picospritzer II or an ASI MPPI-2 injector. The injected volume was calculated using a calibrated eyepiece micrometer. *netrin1a* SBMO was injected at 5.6 ng/embryo, control MO at 6.0 ng/embryo.

Microscopy and Image Analysis

Live GFP-positive embryos were embedded laterally in 1.5% low-melt agarose in E2/GN on a Petriperm dish (Greiner Bio-One). An Olympus Fluoview confocal microscope was used to acquire confocal z-stacks at the level of somites 7−12, focusing through both the near and far sides of the trunk. Presence or absence of PAV in each hemisegment was scored after examining all slices of each z-stack. Fluoview software was used to create confocal z projections, and Adobe Photoshop and Adobe Illustrator were used to compose figures.

Zebrafish Antibody Staining

Stored 32hpf embryos were rehydrated in PBS, incubated in 1% H_2O_2 for 1 hr to block endogenous peroxidase, permeabilized with 0.1% collagenase for 15 min, washed in PBST, blocked with NCST (10% heat-inactivated newborn calf serum, 0.1% Tween-20, 1% DMSO, in PBS) for 1.5 hr, and incubated with rabbit anti-GFP pAb (1:400, Molecular Probes) and either 4D9 (1:4, Zebrafish International Resource Center) or F59 (1:5, Developmental Studies Hybridoma Bank) O/N at 4°C. Embryos were then extensively washed with PBST, incubated with anti rabbit Alexa 488 (1:200, Molecular Probes) and either anti mouse HRP (1:100,

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Molecular Probes) or anti mouse Cy3 (1:100, Jackson Immunoresearch) in NCST respectively at RT for 3.5 hr. Embryos incubated with the anti mouse HRP secondary were then treated with 0.5% DMSO in PBS, and developed in Alexa-568 tyramide (1:100, Molecular Probes) in 0.0015% H_2O_2 in PBS.

Murine Ischemic Hindlimb Model

All procedures were performed in accordance with the Institutional Animal Care and Use Committee of Caritas St. Elizabeth's Medical Center. Ischemia was induced in 8- to 12-week-old male FVB/NJ mice as described previously (1). VEGF, netrin-1, netrin-4 and empty vector plasmid (50 μg/100μl saline in each group) were locally injected to the right gastrocnemius muscle immediately, 7, 14 and 21 days after surgery.

Physiological Assessment of Treated Animals

Laser Doppler perfusion imaging (Moor Instrument, Wilmington, DE) was used to record serial blood flow (LDBF) measurements over the course of 4 weeks postoperatively, as previously described (2). Before initiating laser scanning, mice were placed for 15 min on a heating plate kept at 37°C to minimize body temperature changes. After scanning, the stored images were subjected to computer-assisted quantification of blood flow, and the average flow of the ischemic and nonischemic feet was calculated. The LDBF value was expressed as the ratio of right (ischemic) to left (nonischemic) limb blood flow to avoid data variations caused by ambient light and room temperature.

Neurophysiological measurements

Sciatic nerve conduction velocity was measured using standard orthodromic surface recording techniques and a Teca TD-10 (Oxford Instruments, Chicago, IL) portable recording system in all mice at baseline (before treatment) and then at 1 week, 2 weeks, 3 weeks and 4 weeks after treatment as described previously (6). Briefly, motor nerve conduction velocity (MCV) was calculated by dividing the distance between stimulating electrodes by the average latency difference between the peaks of the compound muscle action potentials (CMAPs) evoked from two sites (sciatic notch and ankle). Sensory nerve conduction velocity (SCV) was calculated by dividing the distance between stimulating and recording electrodes by the latency of the signal from the stimulation artifact to the onset of the peak signal. For each nerve, maximal velocities were determined bilaterally. All measured data from both sides were averaged.

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