Therapeutic Potential of Semaphorin 3E for the Treatment of Choroidal Neovascularization

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PURPOSE. We investigated the effects of semaphorin 3E (Sema3E)/PlexinD1 signaling in the development of choroidal neovascularization (CNV) and explored the therapeutic potential of the pathway.

METHODS. We used a laser-induced CNV model in the mouse. The expression of Sema3E and PlexinD1 was evaluated with immunohistochemistry, real-time RT-PCR, and Western blotting. After the intravitreal injection of Sema3E, the CNV size was measured on immunostained flat mounts. The CNV size was compared between inducible PlexinD1 homozygous mice and heterozygous knockout mice.

RESULTS. The presence of PlexinD1 was confirmed immunohistochemically in the endothelial cells of CNV. The expression of PlexinD1 mRNA was elevated after laser photocoagulation with its peak expression at day 5 (1.72 times, compared to baseline; P < 0.01). The intravitreal injection of recombinant Sema3E decreased the CNV size (16,320.9 ± 8316.0 μm² in the control eyes versus 9,487.4 ± 4424.1 μm² in the eyes administered 0.1-μg Sema3E, P < 0.01). This inhibitory effect of Sema3E in the formation of CNV was canceled in the inducible PlexinD1 knockout mice (24,920.8 ± 24,684.8 μm² in homozygous knockout mice and 13,152.6 ± 7973.0 μm² in heterozygous knockout mice, P = 0.014).

CONCLUSIONS. These results indicate that Sema3E-PlexinD1 signaling is involved in the development of CNV. Stimulation of the pathway has therapeutic potential for CNV. Further studies are needed to evaluate the effects for clinical applications.

Keywords: semaphorin, plexin, choroidal neovascularization

Age-related macular degeneration (AMD) is a major cause of visual impairment in developed countries.1,2 The wet form of AMD is characterized by the development of choroidal neovascularization (CNV), which causes hemorrhage and/or exudative changes.2 Anti-VEGF therapy is effective for the neovascularization (CNV), which causes hemorrhage and/or exudative changes.2 Anti-VEGF therapy is effective for the remission of AMD.4 and patients are required to undergo ongoing treatment as recurrences can impair vision. In addition, the chronic blockade of VEGF may result in the atrophy of choroidal capillaries,5 may increase fibrosis,6 or may result in the loss of its neuroprotective effect,7 which would damage the retinal cells. In fact, the deletion of VEGFs resulted in dysfunction of photoreceptors in mice models.5,8 Therefore, several attempts are being made to explore and evaluate new antiangiogenic agents to develop alternative or supplemental treatment strategies.9

To explore another therapeutic approach for this disease, we focused on signals mediated by semaphorin ligands and their transmembrane plexin receptors, which initially were identified as axon-guidance cues during neuronal development.10–13 Among the 9 plexin receptors (A1–A4, B1–B3, C1, and D1), PlexinD1 was shown to be highly expressed in angiogenic endothelial cells (ECs), and it was found to regulate the morphogenetic patterning of developing vascular networks.14–16 Of interest, while the soluble class 3 semaphorins stimulate plexin signals only in the presence of neuropilin coreceptors, semaphorin 3E (Sema3E) directly binds to endothelial PlexinD1, even in the absence of neuropilins, and acts as a repulsive cue for the developing vasculature.12 Furthermore, the Sema3E-PlexinD1 signal has an antiangiogenic role in pathologic conditions, such as tumor development17–19 and ischemic retinopathy.20 In the ECs of developing blood vessels, the Sema3E-PlexinD1 signal counteracts the VEGF-VEGFR2 signal in terms of cytoskeletal rearrangements and cell motility, thereby preventing filopodia projections and subsequent vessel elongation.20–25 Furthermore, a zebrafish study showed that the Sema3E-PlexinD1 signal promotes the expression of soluble VEGFR1, which acts as a VEGFR2 decoy and counteracts the effect of VEGF.24 Importantly, VEGF upregulates the expression of the Plxnd1 gene in ECs of developing retinal vessels.13,20 Thus, the Sema3E-PlexinD1 signal forms a negative feedback loop in sprouting angiogenesis.

Since CNV is considered a pathologic angiogenesis of the choroid, and VEGF has a major role in the development of CNV, we hypothesized that activation of the Sema3E-PlexinD1 signal can have a role in the treatment of CNV. In the present study, we investigated the role of Sema3E-PlexinD1 signaling in the development of experimental CNV and assessed whether manipulation of the signal has inhibitory effects on CNV.
Semaphorin 3E for Treatment of CNV

MATERIALS AND METHODS

Mouse Strains

All animals were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the Guideline for Animal Experiments of Kyoto University (Kyoto, Japan). All experimental procedures were approved by the Institutional Review Board at Kyoto University Graduate School of Medicine (Kyoto, Japan). Except where otherwise indicated, all mice were maintained under a 12-hour light/12-hour dark cycle.

Female C57BL/6 mice six weeks old were purchased from SLI (Kyoto, Japan). PlexinD1 mutant mice that carried null alleles, wild-type (WT) alleles, or flox alleles (provided by Yutaka Yoshida at the Cincinnati Children's Hospital Medical Center of Cincinnati, OH, USA) were mated with CAG-MerCreMer transgenic mice (RIKEN CDB accession No. CDB0404T; available in the public domain at http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) to obtain tamoxifen-inducible Plxnd1 conditional knockout mice. To confirm the conditional knockout system works in the retina, we used CAG-CAFlacZ:CAG-MerCreMer double transgenic mice.

Genotyping

For the isolation and purification of genomic DNA from the tail biopsy tissues, we used the DirecPT Lysis Reagent (Viagen Biotech, Los Angeles, CA, USA). Isolation and purification were performed in accordance with the manufacturer's instructions. We used the following primers for genotyping:

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P_{\text{Plxnd1-loxP}}:  
\begin{align*} 
5'-(\text{ACAGGTTGTGTCTAAGGCCACCTC}) & -3' \quad \text{(forward)} \\
5'-(\text{CAGCCCTATAGTTCTCCACAAAGA}) & -3' \quad \text{(reverse)} \\
\end{align*} \\
C_{\text{reX}}:  
\begin{align*} 
5'-(\text{AAATTGCCAGGATCAGGGTTAAAG}) & -3' \quad \text{(forward)} \\
5'-(\text{AGAGTCATCCTTAGCGCCGTAAAT}) & -3' \quad \text{(reverse)} \\
\end{align*} \\
P_{\text{Plxnd1-null}} (\text{GFP}):  
\begin{align*} 
5'-(\text{AGCAAGGGCCAGGACGTTAAG}) & -3' \quad \text{(forward)} \\
5'-(\text{TGCGGTCTGGTGAAGAAGATG}) & -3' \quad \text{(reverse)} \\
\end{align*} \\
CAG-CAGFlacZ:  
\begin{align*} 
5'-(\text{TTGAGTTCAGGCGCAAGCTTAC}) & -3' \quad \text{(forward)} \\
5'-(\text{TCAACACCGCAGGACTAGTAGTCC}) & -3' \quad \text{(reverse)} \\
\end{align*}

Induction of CNV

The mice were anesthetized with 10 mg/kg of xylazine and 80 mg/kg of ketamine. Their pupils were dilated with 5% phenylephrine hydrochloride/0.8% tropicamide (Sandol P; Nitten, Nagoya, Japan). Cover glasses placed on the cornes of the mice were used as contact lenses. Laser photoocoagulation was performed 4 to 5 disc diameters away from the optic disc, with the setting of 75 μm, 0.05 seconds, and 150 mW on a laser photoocoagulator (DC-3000; Nidek, Gamagori, Japan). The formation of bubbles was considered a sign of successful perforation of Bruch’s membrane, and only burns with a bubble were included in this study. Four laser spots were placed for the measurement of the CNV area, and 10 spots were applied for Western blotting and cDNA expression analyses.

To investigate the inhibitory effects of Sema3E on CNV formation, the mice were injected intravitreally with Sema3E (R&D Systems, Minneapolis, MN, USA) or with BSA (Takara Bio, Inc., Otsu, Japan) as the negative control. Soon after the laser photoocoagulation, 0.01 μg, 0.05 μg, 0.1 μg, or 0.2 μg of recombinant human Sema3E (R&D Systems), or 0.2 μg BSA was injected using a 34-G syringe (Hamilton, Reno, NV, USA). To compare the effect of Sema3E with the well-established anti-VEGF effect, VEGFR1-Fc (0.1 μg) was used as the comparative positive control (R&D Systems).

Furthermore, we used Plxnd1 conditional knockout mice to confirm that the effect of Sema3E involved the PlexinD1 receptor. To perform inducible Cre-loxP-mediated genetic recombination in Plxnd1 conditional knockout mice, we intraperitoneally injected the mice with 4 ml/kg of 10 mg/ml 4-hydroxytamoxifen (4OHT; Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in 1:9 ethanol/peanut oil (Sigma-Aldrich Corp.) once daily after laser photoocoagulation. Siblings with the loxP/null and loxP/WT genotypes were compared.

Real-Time PCR

Total mRNA was prepared from freshly dissected RPE/choroid complex of mice, using the RNeasy Mini kit (Qiagen, Tokyo, Japan), according to the manufacturer’s instructions with some modifications. The mRNA was reverse-transcribed using a First-Strand cDNA synthesis kit (Applied Biosystems, Tokyo, Japan), and real-time PCR was performed using TaqMan Universal master mix (Applied Biosystems). To investigate the gene expressions of Sema3e and Plxnd1, the TaqMan Gene Expression assay kit was used. All samples were normalized to β-actin (Applied Biosystems). Real-time PCR was performed on the ABI Prism 7300 sequence detection system (Applied Biosystems) for 40 cycles.

Protein Analysis

The eyes were enucleated and sectioned at the limbus, and the cornea and lens were discarded. The RPE/choroid/sclera complex of four eyes at each time point was placed in 600 μl of ice-cold tissue lysis buffer (RIPA buffer, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 1 mM DTT), containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), followed by homogenization using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The lysates were cleared of debris by centrifugation at 19,000g for 30 minutes at 4°C. Total protein content of the supernatant was determined by Protein Assay Bicinchoninate Kit (Nacalai Tesque).

Western blotting was carried out using a standard method. We used SDS-PAGE to separate 0.5 μg of protein with a 7.5% gel, which then was transferred to a polyvinylidene difluoride (PVDF) membrane. The anti-PlexinD1 antibody (R&D Systems) or the anti-Sema3e antibody (Abcam, Cambridge, UK) was used as the primary antibody and a horseradish peroxidase-conjugated antibody (GE Healthcare Japan, Tokyo, Japan) as the secondary antibody. After treatment with ECL Western blotting detection reagents (GE Healthcare Japan), images were acquired with the ChemiDoc XRS+ System and analyzed with Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was repeated twice to ensure the reproducibility.

Choroidal Flat Mounts, Immunohistochemistry, and CNV Quantification

Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes at 4°C and sectioned at the limbus; the cornea, iris, and lens were discarded. For CNV quantification, the RPE/choroidal flat mounts were stained according to previously described standard immunohistochemical procedures. The primary antibodies used were goat polyclonal anti-PlexinD1 (1:50; R&D Systems), anti-lacZ (1:500; Millipore, Billerica, MA, USA), and rat anti-CD102 (1:250; BD Pharmingen, Billerica, MA, USA).
San Diego, CA, USA). The secondary antibodies used were Alexa 488- or 594-conjugated antibodies (1:1000; Invitrogen, Eugene, OR, USA). The RPE/choroidal complexes were laid flat, and radial relaxing incisions were made to obtain a whole-mount preparation. The images were obtained by confocal microscopy (LSM 5 Pascal; Carl Zeiss, Tokyo, Japan). For a sectional view, eyes were fixed in 4% PFA in PBS for 40 minutes, sequentially immersed in 10%, 20%, and 30% sucrose, and then embedded in OCT compound (Miles, Elkhart, IN, USA). Cryostat sections (14 µm thick) were mounted on Matsunami platinum-coated glass slides (Matsunami Glass, Osaka, Japan). After antigen retrieval for 1 minute with 10 mM sodium citrate (pH 8.5) at 85°C, frozen sections were blocked with 10% bovine serum/0.3% Triton-X/0.1 M PB for 1 hour at 4°C. The sections were incubated with the primary antibodies, followed by incubation with the secondary antibodies. The sections were counterstained by 4',6-diamidino-2-phenylindole (DAPI) dye (Sigma-Aldrich Corp.), and the images were obtained by fluorescence microscopy BIOREVO BZ-9000 (Keyence, Otsu, Japan).

Statistics
All values are reported as the mean ± SE. The 1-way ANOVA analysis and post hoc Bonferroni correction were used to investigate the difference. All statistical analysis was performed using IBM SPSS Statistics ver. 20.0 (IBM Japan, Tokyo, Japan). A P < 0.05 was considered statistically significant.

Results
PlexinD1 Was Upregulated After the Induction of CNV
To evaluate the changes in the expression of Sema3E and PlexinD1 during CNV formation, we examined the expression levels of their mRNA by real-time RT-PCR and the expression level of their protein by Western blotting at different time points in the RPE/choroid complex in mice models of laser-induced CNV. Because laser photoocoagulation may induce inflammation and alter the protein expression profile in the retina, we removed the retina and evaluated the expression level of PlexinD1 and Sema3E in the RPE-choroid complex. In addition, we investigated the localization of PlexinD1 and Sema3E protein in the retina by immunofluorescence staining. Immunohistochemistry revealed the presence of PlexinD1, which had localized to the ECs in laser-induced CNV, 5 days after laser photocoagulation (Fig. 1). The fluorescence within the inner plexiform layer (IPL) and photoreceptor region of the retina appears brighter than in the control, although the expression of PlexinD1 was not evident in normal retinal vessels. To confirm the elevated expression of Sema3E and PlexinD1 during CNV formation, we also analyzed their mRNA expression. Quantitative RT-PCR analyses revealed upregulation of PlexinD1 mRNA, which reached its peak on day 5 (1.7 times higher than the baseline control; P < 0.01, Fig. 2A). The expression of Sema3E mRNA also was elevated on day 5, but the increase was not significant (Fig. 2B). In addition, Western blotting showed a 5.5-fold higher expression of PlexinD1 protein in the RPE-choroid complex than in the control (n = 4 in each group; Figs. 3A, 3B).

The Administration of Sema3E Reduced CNV Size
To evaluate the inhibitory effects of Sema3E on CNV formation, we injected various doses of Sema3E or BSA intravitreally and compared the size of the CNV. The intravitreal injection of recombinant Sema3E decreased the CNV size at a dose greater than 0.1 µg. The average size of the CNVs was 16,320.9 ± 8316.0 µm² in the BSA arm and 9487.4 ± 4424.1 µm² in the 0.1-µg Sema3E arm (P < 0.01). An injection of 0.1-µg Sema3E resulted in approximately a 50% decrease in CNV size, compared to the control (Fig. 4). We also compared the effect of Sema3E to that of VEGFR1-Fc. The mean size of CNVs after intravitreal injections of VEGFR1-Fc was 8091.6 ± 3947.6 µm². No significant differences in the decreased CNV sizes were observed between 0.1-µg or 0.2-µg Sema3E and VEGFR1-Fc.
The CNV-Inhibitory Effect of Sema3E Was Lost in Plxnd1 Knockout Mice

To evaluate whether the inhibition of CNV by Sema3E was dependent on the PlexinD1 receptor, we intravitreally injected Sema3E into Plxnd1 knockout mice. Since previous studies have shown that Plxnd1 knockout mice die immediately after birth because of an anomaly of the cardiovascular system, we used the tamoxifen-inducible conditional knockout model.

To evaluate the efficiency of tamoxifen-inducible Cre-loxP-mediated genetic recombination, we first investigated the lacZ reporter expression in laser-induced CNV in CAG-MerCreMer; CAG-CAT-lacZ double transgenic mice after daily intraperitoneal injections of 4OHT for 7 days. The immunohistochemical study suggested the ubiquitous expression of lacZ in the retina and also confirmed the reporter protein in the CNV (Supplementary Fig. S1), which indicated that these conditional knockout mice are applicable to the disruption of PlexinD1 in laser-induced CNV. We then compared the size of the CNVs between siblings with the loxP/WT and loxP/null alleles. The average size of the CNV was $24,920.8 \pm 24,684.8 \mu \text{m}^2$ in the loxP/null mice and $13,152.6 \pm 7973.0 \mu \text{m}^2$ in the loxP/WT mice ($P = 0.014$, Fig. 5). This indicated that PlexinD1 is responsible for the inhibitory effects of Sema3E on the development of CNV.

**DISCUSSION**

To our knowledge, this is the first study to show that the Sema3E-PlexinD1 pathway is associated with the development of CNV. In the present study, we showed that PlexinD1 was expressed in CNV and that it was upregulated during CNV formation. In addition, the intravitreal injection of Sema3E suppressed the CNV size. This result paves the way for development of an alternative or additive treatment strategy for AMD.

The Sema3E-PlexinD1 signal mediates a repulsive cue in angiogenic blood vessels in the development of the vascular system and in a variety of pathologic states. Accumulating evidence suggests that this pathway is involved in tumor angiogenesis, ischemic retinopathy, atherosclerosis, and immune regulation. The multifunctionality of Sema3E-PlexinD1 has attracted considerable attention, and several studies have been performed with the hope that understanding the pathway will clarify the undetermined processes of such various pathological conditions and, thereby, lead to the development of new therapies.

The results of this study exemplify one of the functions of the Sema3E-PlexinD1 pathway. The administration of Sema3E resulted in a reduction in CNV size and was comparable to that of blocking VEGF. To confirm that the effect of Sema3E is mediated by the receptor PlexinD1, we used Plxnd1 conditional knockout mice. The inhibitory effect of Sema3E on CNV was abrogated after the inducible disruption of the Plxnd1 gene. This shows that the Sema3E-PlexinD1 pathway is involved in the progression of CNV and that stimulation of the pathway may have therapeutic effects.

The findings of this study have clinical implications—Sema3E or an equivalent agonist might be used in supplemental therapy to alleviate concerns regarding the adverse effects of anti-VEGF therapy. Although anti-VEGF therapy improves the treatment outcome of AMD, frequent injections of anti-VEGF have plausible drawbacks, including the inhibition of physiologic angiogenesis or blockade of its neurotrophic effect. The deletion of RPE-derived VEGFs in mouse models resulted in choriocapillaris loss, apoptosis, or dysfunction of photoreceptors, and vision loss. A decrease in the choroidal thickness was, indeed, observed in patients treated with an alternative...
anti-VEGF agent, and monthly dosing of the drug was associated with a higher risk of developing RPE/choroid atrophy. If supplemental administration of Sema3E can successfully reduce the number of treatments with anti-VEGF agents, the long-term clinical outcome would be more favorable.

This study highlights another advantage of targeting this pathway for the treatment of CNV. The expression of PlexinD1 was confined mostly to the ECs of CNV and was not evident in the ECs of normal retinal vessels. This result is similar to those of previous studies that showed that elevated VEGF expression levels induce upregulation of PlexinD1. In the process of CNV formation, VEGF is upregulated and has a critical role. The local upregulation of VEGF during the formation of CNV might have induced the upregulation of PlexinD1. Considering that the expression of PlexinD1 was confirmed mainly in CNV and not in retinal vessels, the administration of Sema3E would have little effect, if any, on adjacent retinal tissues, compared to anti-VEGF therapies. In contrast, the VEGF receptor 2 is constantly expressed in Müller cells and photoreceptors. Compared to VEGF-targeted therapies, which block these physiologic pathways, Sema3E-targeted treatment is more selective with regard to pathologic changes and, thus, would be beneficial for the retina. While some fluorescence was observed within the IPL and photoreceptor layer of the retina, to our knowledge no previous study showed the expression of PlexinD1 in these regions. In addition, the photoreceptor outer segments often show autofluorescence. However, researchers still should be careful of the adverse effect of Sema3E on the retina via PlexinD1 within the IPL and photoreceptors.

To establish treatment with Sema3E, the proteolytic cleavage of the molecule should be taken into account. In general, the function of class 3 semaphorins is subject to the regulation of furin-like proprotein-convertases. The proteolytic cleavage neutralizes the effect of semaphorins in most cases; however, the proteolytic fragment of Sema3E induces the formation of a complex with tyrosine kinase erythroblastic leukemia viral oncogene homolog 2 (ErbB2) in cancer cells and promotes tumor metastasis. Although an attempt to use the furin-resistant Sema3E IgG1 Fc fusion protein failed because of the aggregation of proteins, Casazza et al. created furin-resistant mutant Sema3E and showed that the protein still has an antiangiogenic effect but no prometastatic effect. The mutant Sema3E could be applied to the treatment of CNV.

The endogenous secretion of Sema3E might be insufficient to inhibit CNV. Although Sema3E was found in the outer retina of chick embryo or human retina, we did not observe satisfactory Sema3E staining with in situ hybridization or immunohistochemistry in the adult mice used in this study. Although minimum secretion of Sema3E was confirmed with PCR and Western blotting, a higher level of Sema3E should be required to suppress the progression of CNV efficiently. An alternative treatment strategy would involve the determination of cells that endogenously secrete Sema3E and stimulation of those cells to secrete more Sema3E.

There are some limitations to this study. The first and most important limitation is that this study is based on an experimental mouse model. To apply Sema3E to the treatment of CNV in clinical settings, it is a pivotal task to confirm the expression of PlexinD1 in the CNV of human tissues or confirm the association of the Sema3E-PlexinD1 signal with the formation of CNV in humans. However, a human CNV sample is very difficult to obtain. We hope future studies using donated
eyes or genome analysis reveal the association of Sema3E-PlexinD1 in human disease. The second limitation is that CNV in this study was laser-induced. Laser-induced CNV is different from the CNV in the clinical situations in terms of the pathogenesis. In addition, we injected Sema3E intravitreally soon after laser photoacoagulation; this is the third limitation. In clinical situations, treatment cannot be initiated before or at the same time as the formation of CNV. In pilot studies, Sema3E injected 3 or 5 days after laser photoacoagulation still showed an inhibitory effect on CNV, but the effect was inferior to the inhibitory effect of an immediate injection (data not shown). Considering the effect of Sema3E was equivalent to the effect of VEGFRI-Fc, we believe that the injection of Sema3E for developed CNV would have a sufficient effect. However, to establish a therapeutic strategy using Sema3E, the effects of Sema3E on CNV in AMD must be validated.

In conclusion, we showed the therapeutic potential of Sema3E-PlexinD1 in a mouse model of wet-type AMD. Further studies are needed to evaluate their effects in primates and humans.

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