Vascular endothelial-derived semaphorin 3 inhibits sympathetic axon growth

Deborah H. Damon

Department of Pharmacology, University of Vermont, Burlington, Vermont

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Damon, Deborah H. Vascular endothelial-derived semaphorin 3 inhibits sympathetic axon growth. Am J Physiol Heart Circ Physiol 290: H1220–H1225, 2006. First published October 28, 2005; doi:10.1152/ajpheart.01232.2004.—Vascular sympathetic innervation is important to the development of blood vessels and blood flow. The mechanisms that determine vascular sympathetic innervation are not well understood. Recent studies indicate that vascular endothelial cells (EC) express semaphorin 3A, a repulsive axon guidance cue. This suggests that EC would inhibit the growth of axons to blood vessels. The present study tests this hypothesis. RT-PCR and Western analyses confirmed that rat aortic vascular ECs expressed semaphorin 3A as well as other class 3 semaphorins (sema 3s). To determine the effects of EC-derived sema 3 on sympathetic axons, axon outgrowth was assessed in cultures of neonatal sympathetic ganglia grown for 72 h in the absence and presence of vascular EC. Nerve growth factor-induced axon growth in the presence of ECs was 50 ± 4% (P < 0.05) of growth in the absence of ECs. ECs did not inhibit axon growth in the presence of a control antibody, neutralizing the activity of sema 3 (P > 0.05). RT-PCR and Western analyses also indicated that sema 3s were expressed in ECs of intact arteries. To assess the function of sema 3s in arteries, sympathetic ganglia were grown in the presence of arteries for 72 h, and the percentage of axons that grew toward the artery was determined: 44 ± 4% of axons grew toward arterial carotid arteries. Neutralization of sema 3s or removal of EC increased the percentage of axons that grew toward the artery (71 ± 8% and 72 ± 8%, respectively). These data indicate that EC-derived semaphorin 3A inhibits sympathetic axon growth and may thus be a determinant of vascular sympathetic innervation.

Neural communication is mediated via axons that extend from presynaptic cell bodies to postsynaptic target cells. During the generation and regeneration of neural connections, signals provided by nontarget and target cells promote the growth and guidance of axons to their designated targets (8, 24). In the cardiovascular system, postganglionic sympathetic neurons innervate blood vessels and the heart. Vascular sympathetic innervation is an important determinant of blood pressure and blood flow, and alterations in vascular sympathetic innervation have been implicated in the development and maintenance of cardiovascular disease (3, 7, 10, 17). Our knowledge of the signals that promote the growth and guidance of sympathetic axons to blood vessels is limited.

Semaphorins are a large family of secreted and membrane-associated proteins that act primarily as repulsive cues for growing axons (1, 9, 11, 15, 18, 23, 24). Recent studies indicate that vascular endothelial cells (ECs) produce semaphorin 3A (sema 3A; Refs. 6, 19), a secreted semaphorin that repulses sympathetic axons (1, 8, 23, 24). The functional role of EC-derived semaphorins in vascular innervation has not been studied.

The present study tests the hypothesis that secreted class 3 semaphorins from ECs inhibit sympathetic axon growth. To test this hypothesis, the expression and function of class 3 semaphorins in cultured ECs and ECs in intact arteries were studied.

Materials and Methods

The use of animals in the present studies was in accordance with the National Institutes of Health guidelines for the humane care and use of animals in research and was approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Organ and tissue culture. Superior cervical ganglia were isolated from neonatal rats (2–4 days of age) and plated on 35-mm tissue culture dishes (Falcon) coated with rat tail collagen (Becton Dickinson). The nonneuronal cells in the ganglia were growth arrested with mitomycin C (1 h, 10 μg/ml). After washout of the mitomycin C, the ganglia were then grown in the absence or presence of vascular ECs or arteries. All neuronal and neurovascular cultures were grown in neuronal growth medium [DMEM-F12 supplemented with 10% NuSerum (BD Biosciences, Bedford, MA), 5% FBS (Invitrogen, Carlsbad, CA) and penicillin-streptomycin]. Rat aortic ECs were obtained from VEC Technologies (Rensselaer, NY). Before their addition to the neuronal cultures, ECs were grown in EC growth media (VEC Technologies). Carotid arteries were obtained from neonatal rats.

For the ganglia/EC cultures, ECs were added to the ganglia the day after the ganglia were plated. In these cultures, ECs were distributed throughout the dish and surrounded the ganglia. The ECs were plated at subconfluent densities. For the ganglia/artery cultures, the ganglia and artery were plated concurrently. The artery was placed ~2 mm to the right of the ganglia. Cocultures were grown for 72 h in the absence or presence of neutralizing antibodies [neuropilin-1 (npn-1; R&D Systems, catalog no. AF566) and VEGF (R&D Systems, catalog no. AF564)].

Immunohistochemistry. Ganglia were fixed (4% formaldehyde in PBS for 20 min at room temperature) and permeabilized (0.2% Triton X-100 in PBS). The ganglia were incubated for 30 min with 5% FBS (in PBS) to block nonspecific labeling and then incubated with GAP43 primary antibody (1/1,000; Chemicon, Temecula, CA) overnight at 4°C. Unbound primary antibody was removed with three washes (PBS). Ganglia were then incubated with secondary antibody (1/500 goat anti-rabbit IgG; Alexa Fluor 568, Molecular Probes, Eugene, OR) and washed (3 times with PBS). Ganglia were visualized on an upright fluorescence microscope (Olympus BX50) with a ×4 objective (numerical aperture = 0.13). Images were recorded digitally with an Olympus camera (model U-ULH) and Magnifire Software and viewed with Adobe Photoshop.

Morphological analyses. For the cultured EC experiments, representative axon lengths (at least 5 per ganglia; in μm) and densities (number of axons/μm) were measured (Metamorph) (Fig. 1). For the carotid artery experiments, the length (mean of at least 5 per ganglia)

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and number of axons growing toward and away from the arteries were measured. The number of axons was determined by multiplying axon densities (number/\(\mu m\)) by the perimeter of axon growth (\(\mu m\)). Axon growth (number \(\times\) mean length) was then determined. A schematic depicting the morphological analyses is shown in Fig. 1.

**RT-PCR analysis.** We isolated RNA using Qiagen kits. The RNA was reverse transcribed (RetroScript, Ambion), and the cDNA was amplified (Amplitaq Gold, Applied Biosystems). PCR reactions were carried out for 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR primers and product sizes are indicated in Table 1. PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide and visualized with UV light. Amplified PCR products were sequenced by the University of Vermont DNA facility to confirm the identity of the DNA.

**Western analysis.** Cells and tissues were lysed and homogenized in enhanced RIPA 1640 buffer. Samples (10 \(\mu l\)) were diluted with equal volumes of electrophoresis running buffer, boiled for 5 min, and electrophoresed on 4–20% gradient acrylamide gels. Samples were then transferred to nitrocellulose membranes. After transfer, the gels were stained (GELCODE Blue stain reagent, Pierce, Rockford, IL) to verify that equal amounts of protein were loaded for each sample. The membranes were blocked with PBS containing 0.05% Tween 20 and 3% nonfat dry milk (20 min at room temperature) and then incubated overnight at 4°C in PBS-Tween 20 containing 3% nonfat dry milk and primary antibodies (1/3,000 sema, catalog no. SC-10720, Santa Cruz Biotechnology, Santa Cruz, CA; 1/1,000 GAPDH, BioDesign, Saco, ME). Unbound primary antibody was removed with three 5-min washes (PBS-Tween 20). The membranes were then incubated in PBS-Tween 20 containing 3% nonfat dry milk and 1/3,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at room temperature. The horseradish peroxidase was detected with enhanced chemiluminescence (Pierce) and documented on autoradiographic film.

**Statistical analyses.** Data are presented as means \(\pm\) SE and were compared with two-tailed unpaired \(t\)-tests assuming unequal variances. Differences were considered significant if \(P\) values were \(<0.05.\)

**RESULTS**

RT-PCR and Western analyses were performed to characterize class 3 secreted semaphorin expression in cultured vascular EC. Sema 3A, sema 3B, sema 3E, and sema 3F mRNA...
were detected in EC (Fig. 2A). Sema 3c mRNA was not detected. Semaphorin 3 protein was also present in EC (Fig. 2B). The representative Western analysis indicated that 95- and 65-kDa forms of semaphorin are expressed in rat aortic EC (1). These molecular masses are comparable to those reported for sema 3A and comparable to the predicted molecular masses of sema 3B, sema 3E, and sema 3F. Smaller forms were also detected, which are most likely proteolytic degradation products. The antibody used for these Western analyses was raised against amino acids 103–402 of sema 3A of human origin. An analysis of protein sequence homologies (BLAST search) for these amino acids indicates that this antibody should recognize all class 3 semaphorins.

If functional class 3 semaphorins were produced by ECs, ECs placed in culture with sympathetic ganglia should collapse the growth cones and thereby inhibit the growth of axons. To test this hypothesis, sympathetic ganglia were grown in the presence of 50 ng/ml nerve growth factor (to stimulate axon growth) and in the absence and presence of ECs for 72 h. Immunohistochemistry for GAP43 was then performed, and axon outgrowth was assessed. Representative ganglia are shown in Fig. 3. The average length of axons extending from ganglia grown in the presence of EC (1,157 ± 55 μm) was less than that of axons extending from ganglia grown in the absence of EC (2,085 ± 107 μm; \( P < 0.05 \)). ECs did not alter axon density (data not shown; \( P > 0.05 \)). These data suggest that ECs inhibit sympathetic axon growth.

To determine whether class 3 semaphorins mediate the effects of ECs on sympathetic axon growth, the effects of ECs were studied in the presence of a semaphorin 3 inhibitor. Sema 3A and sema 3B, but not sema 3E or sema 3F, mediate their biological effects by binding to npn-1 (8, 14, 15, 23, 24). ECs grown in the presence of an antibody that blocks binding to npn-1 (npn-1 antibody; 5 g/ml) do not inhibit sympathetic axon growth (Fig. 4), suggesting that sema 3A and/or sema 3B mediate EC inhibition of sympathetic axon growth. The addition of the npn-1 antibody marginally decreased axon growth in the absence of ECs (\( P = 0.08 \); two tailed t-test).

VEGF also binds to npn-1 (2, 25), and the npn-1 antibody used to obtain the data in Fig. 4 also prevents VEGF binding to npn-1. To distinguish semaphorin effects from potential effects of VEGF, the effects of EC on sympathetic axon growth were
also studied in the presence of an antibody that neutralizes the activity of VEGF but not semaphorin (Fig. 5). In the presence of 50 ng/ml nerve growth factor and 5 μg/ml VEGF antibody, axon growth in the presence of ECs was significantly less than that in the absence of ECs.

The carotid artery of the rat is not innervated by postganglionic sympathetic neurons (19). Does vascular-derived semaphorin contribute to this lack of innervation? RT-PCR and Western analyses indicate that semaphorins are expressed in vivo in neonatal carotid arteries (Fig. 6). Removal of ECs from the arteries by rubbing the luminal surface with surgical wire markedly decreased semaphorin protein expression (Fig. 6B). This suggests that most, if not all, of the semaphorin was in the ECs.

To assess the potential role of class 3 semaphorins in blood vessels, the effects of neonatal carotid arteries on sympathetic axon growth were studied before and after inhibition of semaphorin. The effects of the arteries on axon guidance were studied by assessing the percentage of axons that grew toward the artery. These experiments were done in the absence of nerve growth factor but in the presence of a caspase inhibitor to maintain the survival of the neurons (26). In the presence of neonatal carotid arteries, 44 ± 5% of axon growth was toward the artery (Fig. 7, left). This suggests that the arteries neither attracted nor repelled the growing axons. Inhibition of semaphorins with npn-1 antibody (5 μg/ml) increased the percentage of axon growth toward the arteries (71 ± 7.5%; Fig. 7, left; n = 8). This suggests that class 3 semaphorins were limiting the number of axons that grew toward the arteries. Removing ECs from the arteries, which decreased semaphorin (Fig. 6B), also increased axon growth toward the arteries from 49 ± 8% to 72 ± 8% (Fig. 7, right; n = 13). This increase was statistically significant in a one-tailed (P = 0.03) and marginally significant in a two-tailed (P = 0.06) unpaired t-test. These two sets of experiments support the hypothesis that vascular-derived class 3 semaphorins inhibit the growth of axons toward blood vessels. Total axon growth was not affected by class 3 semaphorin inhibition with npn-1 antibody or by removal of ECs (data not shown; P > 0.05).

DISCUSSION

ECs are present in all blood vessels and are important determinants of cardiovascular function. The present study indicates that ECs inhibit the growth of sympathetic axons and thereby suggest a novel role for ECs in determining the growth and guidance of sympathetic axons. EC inhibition or repulsion of sympathetic axons may limit sympathetic innervation to the abluminal surface of innervated blood vessels and may prevent innervation of noninnervated blood vessels.

Semaphorins are a family of proteins that repel axons by inducing collapse of growth cones (1, 8, 11, 15, 23, 24). In
vertebrates, class 3 semaphorins are secreted proteins that repel sympathetic axons (8, 23, 24). RT-PCR analyses (Fig. 2A) indicate that the ECs used in the present study express mRNAs encoding for class 3 semaphorins. Western analyses using an antibody that recognizes class 3 semaphorins also indicate that the ECs express one or more class 3 semaphorins. These cells express 95- and 65-kDa forms of class 3 semaphorins, which have been reported to be active (1). The functional data in Figs. 2 and 3 support the RT-PCR and Western analyses and indicate that ECs inhibit sympathetic axon growth and that an antibody that prevents class 3 semaphorin binding to neuropilin-1 on the neurons prevents this inhibition. In the presence of neuropilin-1 antibody, axon growth in the presence of ECs (1,542 ± 108 μm) was not significantly different from that in the absence of EC (1,632 ± 144 μm; P > 0.05). Furthermore, axon growth in the presence of ECs and neuropilin-1 antibody (1,542 ± 108 μm) was greater than that in the presence of EC alone (1,156 ± 55 mm; P < 0.05). The effects of sema 3A (14, 15, 23) and sema 3B (23) but not sema 3E and sema 3F (4, 11, 13) are mediated by binding to neuropilin-1. This suggests that EC-derived sema 3A and/or sema 3B inhibits sympathetic axon growth.

This is not the first report that ECs express functional class 3 semaphorins. Deroanne et al. (6) presented RT-PCR analysis that human ECs express sema 3A mRNA and presented Western analysis that media conditioned by human EC contained the 95-kDa form of sema 3A. These investigators also reported sema 3A activity that inhibited VEGF binding to neuropilin-1. The present study, however, is the first report that EC-derived class 3 semaphorins modulate axon growth and guidance in vitro.

VEGF is produced by sympathetic neurons (20), and like sema 3A and 3B, binds to neuropilin-1 and neuropilin-2 (2). VEGF binding to neuropilin-1 modulates cell function directly (25) or indirectly by enhancing VEGF binding to VEGF receptor 2 (2). The neuropilin-1 antibody used in the present studies inhibits VEGF as well as class 3 semaphorin binding to neuropilin-1. To verify that the effects of the neuropilin-1 antibody (Fig. 4) were attributable to inhibition of class 3 semaphorin binding and not VEGF binding, the effects of EC on sympathetic axon growth were also assessed in the presence of an antibody that neutralized VEGF and not class 3 semaphorins (Fig. 5). In the presence of VEGF antibody, sympathetic axon growth in the presence of EC (732 ± 94 μm) was less than that in the absence of EC (1,010 ± 96 μm; P < 0.05). This indicates that EC inhibited sympathetic axon growth in the presence of VEGF antibody, suggesting that the inhibition of VEGF binding did not mediate this effect. VEGF antibody also reduced axon growth in both the presence and absence of ECs (P < 0.05). In the absence of ECs, VEGF antibody reduced mean axon length from 2,085 ± 107 μm (Figs. 3 and 4) to 1,010 ± 96 μm (Fig. 5). In the presence of EC, VEGF antibody reduced mean axon length from 1,157 ± 55 μm (Fig. 3) to 732 ± 94 μm (Fig. 5). Sondell and Kanje (20) reported that VEGF is produced by postganglionic sympathetic neurons, and Sondell et al. (21) reported that VEGF promotes the growth of sympathetic axons. These studies and the data presented in Figs. 4 and 5 suggest that ganglia-derived VEGF promotes axon growth that was inhibited by VEGF antibody. The data in Fig. 4 indicate that the neuropilin-1 antibody, like the VEGF antibody, reduced axon growth in the absence of EC. This effect is most likely due to neuropilin-1 antibody inhibition of VEGF-induced axon growth.

Vascular smooth muscle cells produce neurotrophic factors that stimulate the growth of axons (10). The data presented in Figs. 2, 3, and 4 suggest that EC produce class 3 semaphorins that inhibit axon growth. The net effect of a blood vessel on axon growth would be determined by the balance between stimulation and inhibition. The carotid artery in the rat is not innervated by postganglionic sympathetic neurons. This lack of innervation could be due, at least in part, to the axon repellant activity of carotid artery-derived class 3 semaphorins. Figure 6 indicates that class 3 semaphorins are produced by ECs in carotid arteries. These arteries stimulated the growth of axons, but the growth of axons was not directed toward the artery. When class 3 semaphorins were inhibited, however, more axons grew toward the carotid arteries (Fig. 7). These data suggest that, in the carotid artery, class 3 semaphorins oppose neurotrophic stimulation of axon growth and limit the growth of axons to the artery. Exogenous semaphorin was not added to these cultures, and thus any semaphorin was derived from the artery. Blood vessels, smooth muscle cells and endothelial cells in blood vessels, and innervation of blood vessels are heterogeneous (6, 13, 22). It is likely that vascular production of axon growth stimulators and inhibitors and thus the effects of arteries on sympathetic axon growth will also be heterogeneous.


