In Vitro and in Vivo Characterization of a Novel Semaphorin 3A Inhibitor, SM-216289 or Xanthofulvin*

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The adult central nervous system (CNS)\(^1\) in higher vertebrates shows only a limited capacity for anatomical and functional recovery after nerve injury. Many studies suggest that the limited capacity for nerve regeneration is due to the presence of axonal growth inhibitory components in the CNS tissue (1–4). A number of molecules that may contribute to this inhibition have been reported, such as myelin-associated glycoprotein, tenascins, and some chondroitin sulfate proteoglycans, etc. One of the in vitro activities of these molecules is growth cone collapse or retraction of growing neurites (1–4). Schwab and co-workers (5–7) have identified a neurite growth inhibitory molecule, Sema3A-induced growth cone collapse of dorsal root ganglion neurons in vitro was completely abolished in the presence of SM-216289 at levels less than 2 \(\mu\)M (IC\(_{50}\) = 0.16 \(\mu\)M). When dorsal root ganglion explants were co-cultured with Sema3A-producing COS7 cells in a collagen gel matrix, SM-216289 enabled neurites to grow toward the COS7 cells. SM-216289 diminished the binding of Sema3A to its receptor neuropilin-1 in vitro, suggesting a direct interference of receptor-ligand association. Moreover, our data suggest that SM-216289 interacted with Sema3A directly and blocked the binding of Sema3A to its receptor. We examined the efficacy of SM-216289 in vitro using a rat olfactory nerve regeneration model, in which strong Sema3A induction has been reported around regenerating axons. The regeneration of olfactory nerves was significantly accelerated by a local administration of SM-216289 in the lesion site, suggesting the involvement of Sema3A in neural regeneration as an inhibitory factor. SM-216289 is an excellent molecular probe to investigate the function of Sema3A, in vitro and in vivo, and may be useful for the treatment of traumatic neural injuries.

SM-216289 (xanthofulvin) isolated from the fermentation broth of a fungal strain, Penicillium sp. SPF-3059, was identified as a strong semaphorin 3A (Sema3A) inhibitor. Sema3A-induced growth cone collapse of dorsal root ganglion neurons in vitro was completely abolished in the presence of SM-216289 at levels less than 2 \(\mu\)M (IC\(_{50}\) = 0.16 \(\mu\)M). When dorsal root ganglion explants were co-cultured with Sema3A-producing COS7 cells in a collagen gel matrix, SM-216289 enabled neurites to grow toward the COS7 cells. SM-216289 diminished the binding of Sema3A to its receptor neuropilin-1 in vitro, suggesting a direct interference of receptor-ligand association. Moreover, our data suggest that SM-216289 interacted with Sema3A directly and blocked the binding of Sema3A to its receptor. We examined the efficacy of SM-216289 in vitro using a rat olfactory nerve regeneration model, in which strong Sema3A induction has been reported around regenerating axons. The regeneration of olfactory nerves was significantly accelerated by a local administration of SM-216289 in the lesion site, suggesting the involvement of Sema3A in neural regeneration as an inhibitory factor. SM-216289 is an excellent molecular probe to investigate the function of Sema3A, in vitro and in vivo, and may be useful for the treatment of traumatic neural injuries.

EXPERIMENTAL PROCEDURES

Production of SM-216289 by Fermentation of Penicillium sp. Strain SPF-3059—The detailed procedure for the fermentation and purification of SM-216289 (50). Briefly, the fermentation broth of Penicillium sp. SPF-3059 that was cultured for 8 days at 27 °C with reciprocal shaking at 110 rpm was centrifuged to harvest the cell mass and the supernatant, which were extracted with acetone and ethyl acetate containing 1% formic acid, respectively. The crude extracts were combined and purified by a combination of gel filtration chromatography and reverse-phase high pressure liquid chromatography to isolate SM-216289.

Collapse Assay—Mouse Sema3A cDNA was cloned into the pUC19 expression vector, pUC19-mSema3A (14, 33). The expression plasmid was transfected into COS7 cells using the FuGENE 6 transfection

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\(^1\) The abbreviations used are: CNS, central nervous system; DRG, dorsal root ganglion; WGA-HRP, wheat germ agglutinin-horseradish peroxidase; PBS, phosphate-buffered saline; PBS, fetal bovine serum; AP, alkaline phosphatase; Sema3A, semaphorin 3A; NP-1, neuropilin-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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reagent (Roche Diagnostics), as described in the manufacturer's protocol. Two days after transfection, the conditioned medium was collected and used for the growth cone collapse assay after determination of Sema3A activity. The growth cone collapse assay was performed as described by Luo et al. (19), Briefly, DRG explants derived from E7–8 chick or E14 rat embryos were cultured for 16–20 h in F12 medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 20 ng/ml nerve growth factor (Promega, Madison, WI) in 96-well plates (Sumitomo Bakelite, Tokyo, Japan) pre-coated with poly-l-lysine and laminin. In order to measure the Sema3A inhibitory activity of SM-216289, the compound was added to the cultures 30–60 min before Sema3A. A small amount of Sema3A-containing conditioned medium was added to the cultures to give final concentration of 300 pM, followed by a 1-h incubation. Then the DRG explants were fixed with 1% glutaraldehyde, and collapsed growth cones were counted under a light microscope (×100). According to the unit definition of Sema3A by Luo et al. (19), 1 unit corresponded to 100 µM and 1.0 nM of purified mouse Sema3A and Sema3A-AP, respectively, in our assay condition.

Cell Growth Assay—A cell growth assay was performed to test the cytotoxicity of SM-216289. Approximately 2000 COST7 cells were seeded onto a 96-well plate (Sumitomo Bakelite), and the compound was added to the culture medium (Dulbecco's modified Eagle's medium (Invitrogen) containing 10% FBS) at various concentrations. After 48 h of cultivation, cell growth was measured using the MTT Cell Growth Assay Kit (Chemicon, Temecula, CA), according to the manufacturer's protocol.

Co-culture Assay in Collagen Gel—Collagen co-culture experiments were performed as described previously (34), with a slight modification. COST7 cell aggregates were prepared as follows. COST7 cells (102 cells) grown in a 35-mm dish were transfected with 1 µg of pUCSRmSema3A or the vector plasmid using FuGENE 6. Four hours after transfection, the cells were trypsinized and resuspended in 250 µl of Dulbecco's modified Eagle's medium containing 1% FBS. Droplets of 20 µl of the cell suspension were suspended under the surface of a culture dish lid and incubated for 12 h (55). COST7 cell aggregates and DRG explants derived from E8 chick embryos were co-cultured in 0.2% collagen gel (Koken, Tokyo, Japan) separated by a distance of 1 mm. The collagen gel containing the cell aggregate, and the DRG explant was incubated for 2 days in F12 medium containing 10% FBS and 20 ng/ml nerve growth factor, with or without SM-216289.

Pre-mix Experiment—The "pre-mix" experiment we performed was based on the growth cone collapse assay. Usually the inhibitor was pre-incubated with the culture, before addition of Sema3A. In the pre-mix experiments, Sema 3A was incubated with the inhibitor for 30 min before being used to treat the DRG explant. Alternatively, cultivated DRG was incubated for 30 min with a relatively high concentration of compound, before the DRG was mixed with a large volume of Sema3A containing medium in the counteractive pre-mix experiment.

Receptor Binding Assay—Alkaline phosphatase-fused Sema3A (Sema3A-AP) was used as a ligand for receptor binding assays. A DNA fragment corresponding to the amino acid residues 1–758 of mouse Sema3A was cloned into the N-terminal portion of the human alkaline phosphatase (AP) gene in the pPAG-2 vector (36) in order to insert a His tag sequence at the C-terminal end of the recombinant protein. A DNA fragment for the chimeric gene, Sema3A-AP, was re-cloned into the pUCSR expression vector. Recombinant Sema3A-AP protein was expressed in COS7 cells that were transiently transfected with the expression plasmid using FuGENE 6. Sema3A-AP protein was purified from the conditioned medium by nickel-nitrioltriacetic acid column (Qiagen, Hilden, Germany) chromatography, as described by Takahashi et al. (37). In order to express NP-1, CDNA of mouse NP-1 (38) was cloned into the pUCSR expression vector, pUCSRa-NP-1. COST7 cells were transfected with the pUCSRa-NP-1 (NP-1-COS). 24 h after transfection, the cells were trypsinized, re-seeded in 24-well plates (104 cells/well, Sumitomo Bakelite), and incubated for 24 h. The cells were then treated with Sema3A-AP (10 nM) in Hank's balanced salt solution containing 10% FBS, 20 mM HEPES (pH 7.2), 0.1% NaN3 (modified HBHA buffer) to perform a receptor binding assay, as described by Flanagan and Leder (39). In order to examine the effect of SM-216289 on the binding of Sema3A-AP to NP-1 or DRG neurons, the compound was added to the cells prior to Sema3A-AP addition. For in situ detection of Sema3A-AP bound to cell surface receptors, the cells were fixed after the binding reaction and stained using 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium as substrates. For quantitative analysis, the cells were solubilized by lysis buffer containing Triton X-100, and AP activity derived from bound Sema3A-AP was measured colorimetrically (absorbance at 405 nm) using p-nitrophenyl phosphate.
as substrate (39). Dissociation culture of E7 or E8 chick DRG neurons was performed as described previously (37).

**Olfactory Nerve Axotomy**—All aspects of animal care and treatment were carried out according to the local guidelines of the experimental animal care committee.

Wistar rats (male; 6.5 weeks old) were anesthetized with pentobarbital (50 mg/kg). The left olfactory bulb was exposed by opening the region of the frontal bone covering the olfactory bulb using a dental drill. The olfactory nerves were then anasthetized by insertion of a custom knife between the cribiform plate and the olfactory bulb (40). Following the operation, SM-216289 was locally and continuously administered into the injury site at a 6-μl/day rate using a cannula and an osmotic pump (Alza, Mountain View, CA). Regeneration of the olfactory nerve was monitored histologically as follows. Two or 3 weeks after the operation, the animals were anesthetized with pentobarbital and placed on their backs. For anterograde labeling of the olfactory nerve, 100 μl of 1% wheat germ agglutinin-horseradish peroxidase conjugate (WGA-HRP) in PBS was injected slowly into nasal cavity (41). After 24 h, the animals were re-anesthetized and perfused with 4% paraformaldehyde/ PBS. The olfactory bulb was dissected and frozen at −80 °C. Cryostat sections (30 μm thickness) were prepared from the entire olfactory bulb (Fig. 5a), and every third section was transferred into a 24-well plate containing PBS. For visualization of the labeled olfactory nerve and glomerulus, the sections were treated with 0.05% dichromobenzidine, 0.03% H2O2 in PBS for 15 min. After the visualization, the sections were mounted onto glass slides. The number of the first glomeruli-stained sections from the top of the olfactory bulb was defined as the nerve regeneration score for each animal.

**RESULTS**

Identification of a Sema3A Inhibitor, SM-216289, from the Fungus Penicillium sp. SPF-3059—We found a strong Sema3A inhibitory activity in the cultured broth of a fungal strain, SPF-3059, which was isolated from soil and identified as a strain belonging to the genus Penicillium. Bioassay guided fractionation of the extract of the fungal strain led to the isolation of the active compound, SM-216289. The molecular formula of SM-216289, as determined by HRFAB-MS, was C18H20O14 and the molecular weight was 578. The structure was determined by spectroscopic analysis and was found to be identical to xanthofulvin (Fig. 1A), which was previously isolated from a strain of Eupenicillium, as appeared in patent literature but not in a paper (50).

SM-216289 Is a Potent Sema3A Inhibitor—Sema3A induces growth cone collapse in cultured chick DRG neurons. The growth cone collapse was completely abolished in the presence of SM-216289, even at less than 2 μM, where IC50 was 0.16 μM (Fig. 1B), without any prior morphological differences observed in growth cone structure. The inhibition was also observed on rat DRG neurons (Fig. 1B; IC50 = 0.12 μM). SM-216289 showed a similar inhibitory effect to human and chick Sema3A, as well as to that from mouse (data not shown). These observations suggested that the inhibitory effect was not species-specific.

When COS7 cells were cultured in the presence of various concentrations of SM-216289, no growth inhibition was detected at 500 μM (Fig. 1D). This level of SM-216289 is more than 3,000-fold higher than the IC50 value for growth cone collapse inhibition.

SM-216289 Has a Persistent Inhibitory Effect on Sema3A—Although growth cone collapse caused by Sema3A is a localized and rapid biological reaction at the tip of the neurites (42), long term exposure of growing neurites to gradients of Sema3A concentration changes the direction of the neurite growth through a process of chemorepulsion. This process is thought to be an important mechanism of axonal pathfinding during neural development. To assess if SM-216289 has the potential to inhibit the redirection of growing axons by Sema3A, we employed a collagen gel co-culture system. When neurons are co-cultured in a collagen gel matrix very close to cells producing repulsive or attractive guidance molecules, such as a semaphorin or netrin, growing neurites sense the concentration gradient and change their direction of growth accordingly (34). As shown in Fig. 2, an aggregate of Sema3A-expressing COS7 cells (Sema3A-COS) and E8 chick DRG explant were co-cultured in a collagen gel for 2 days with or without SM-216289 in the growth medium. Although the neurons in the DRG explant extended their neurites radially in the control cultures (Fig. 2A), neurites all grew away from the COS7 cell aggregate when co-cultured with Sema3A-COS (Fig. 2B). However, in the presence of 0.5 or 1.0 μM SM-216289, the repulsion was suppressed.

Furthermore, growing neurites were observed in the direction of Sema3A-COS, although their length was reduced in comparison to the control (Fig. 2, C and D). At 4.0 μM SM-216289, neurites were radially extended from the DRG explant and were indistinguishable from the control culture (Fig. 2D). As shown in Fig. 2, the length of neurites growing away from COS7 cell aggregates was similar between the SM-216289-treated DRG explant and the non-treated DRG explant, suggesting that SM-216289 did not affect neurite extension per se. When COS7 cells were transfected with the Sema3A-AP expression plasmid (see below), neither mRNA expression nor protein production for Sema3A was affected by SM-216289 (data not shown). These results indicated that SM-216289 inhibits the activity of Sema3A continuously during the culture period and suppressed the repulsive activity of Sema3A.

SM-216289 Targets Sema3A Molecule—In order to explore the mechanism of action of SM-216289, we examined the effect of the pre-treatment time of the cultured explants with SM-
216289 before the addition of Sema3A in the growth cone collapse assay. In the assay, the collapsing activity of Sema3A was inhibited regardless of the SM-216289 pretreatment time. When SM-216289 and Sema3A were applied to cultured DRG explants simultaneously, the collapsing activity of Sema3A was almost completely inhibited. However, when the compound was added 5 min after Sema3A, little inhibition was observed (data not shown). This result suggests that Sema3A may interact with SM-216289, thereby preventing Sema3A from binding to the receptor.

In order to examine whether SM-216289 directly acts on Sema3A, we performed a pre-mix experiment as follows. As described above, the inhibitory effect of SM-216289 was dependent on its concentration. In our general experimental procedure in which SM-216289 and Sema3A were added to the culture medium separately (“one-by-one” procedure), 0.5 μM SM-216289 largely suppressed 300 pm Sema3A activity, whereas little inhibition was observed at 0.1 μM SM-216289 (Fig. 3). In the pre-mix experiment, 1500 pm Sema3A was mixed with 0.5 μM of SM-216289 and incubated for 30 min. A small volume of the mixture was then added to a four times larger volume of culture medium in which DRG explants were cultured. This gave a final concentration of Sema3A (300 pm) and SM-216289 (0.1 μM) which was equivalent to that used in the one-by-one experiment. Although 0.1 μM SM-216289 resulted in little inhibition of Sema3A in the one-by-one procedure, the collapsing activity of Sema3A was significantly inhibited in the pre-mix experiment. The critical difference between the two experiments is that in the pre-mix scenario, Sema3A came into contact with a higher concentration of SM-216289 causing inhibition. This is also supported by the competitive pre-mix experiment. In this procedure, a cultured DRG explant was incubated with 0.5 μM SM-216289 followed by dilution with a large volume of Sema3A-containing medium to give a final concentration of 0.1 μM SM-216289. Interestingly, no inhibition of Sema3A was observed in this case (Fig. 3). It should be noted that the final concentration of SM-216289 and Sema3A in the culture medium was the same in all these experiments. Therefore, SM-216289 inactivated Sema3A in an experimental procedure-dependent manner.

SM-216289 Inhibits Binding of Sema3A to Neuropilin-1—In order to examine the mode of action of SM-216289 more directly, we studied the effect of SM-216289 on the binding of Sema3A to neuropilin-1 (NP-1), an essential component of the Sema3A receptor complex (43–45). We employed alkaline phosphatase-fused Sema3A (Sema3A-AP) for the following binding experiments (39). As described previously (37), Sema3A-AP fused protein had a lower growth cone collapsing activity (EC50 = 1.0 nM) than the native Sema3A (Fig. 1C). Likewise, SM-216289 inhibited the collapsing activity of Sema3A-AP but at a higher concentration (IC50 = 1.7 μM) (Fig. 4A). The binding of Sema3A-AP to NP-1 in the presence or absence of SM-216289 was examined by using COST cells that were transiently expressing NP-1 (NP-1-COST). As shown in Fig. 4B, Sema3A-AP binds to NP-1-COST, and the binding was significantly inhibited by the presence of SM-216289. Quantitative analysis of the binding by alkaline phosphatase activity revealed that the inhibitory effect of SM-216289 was dose-dependent (Fig. 4A). The effect of SM-216289 on direct interaction between Sema3A and plexin-A1, which is another component of Sema3A receptor (46), could not be examined because Sema3A does not bind to plexin-A1 itself (46). However, binding of Sema3A-AP to DRG neurons was also inhibited by SM-216289 at a similar concentration (Fig. 4C). SM-216289 did not affect the AP activity itself nor the protein expression level of NP-1 (data not shown). These results strongly suggest that SM-216289 inhibits the collapsing activity of Sema3A by diminishing its receptor binding ability.

SM-216289 Accelerates Nerve Regeneration in Vivo in a Rat Olfactory Nerve Axotomy Model—Pasterkamp et al. (30) have recently demonstrated that axotomy of the olfactory nerve leads to an induction of Sema3A mRNA at the injured site. Because NP-1 is expressed on the olfactory nerve, induced Sema3A may inhibit extension of olfactory axons (30). By using this animal model, we examined the in vivo efficacy of SM-216289. Following olfactory nerve axotomy, SM-216289 was locally and continuously administered between the cribiform plate and the olfactory bulb of operated animals at the rate of 6 μg/6 μl/day through a cannula using osmotic pumps. Two or 3 weeks after the axotomy, olfactory nerves were anterogradely
labeled with wheat germ agglutinin-horseradish peroxidase conjugate (WGA-HRP) (41), and the length of regenerated olfactory nerve was determined by the methods described under “Experimental Procedures.” A schematic representation of our scoring method. Horizontal sections (30 μm thickness) were prepared from top to bottom of the WGA-HRP-labeled olfactory bulb (broken lines), and every 3rd section was used for the score estimation. The number of the first glomeruli-stained sections (sections with asterisk in B, from the top of the olfactory bulb) was defined as the nerve regeneration score for each animal. Smaller numerical values for the regeneration score indicate that regenerated nerves extended to a more distant level. gl, glomerulus; ob, olfactory bulb; on, olfactory nerve (growing bottom to top); cx, cerebral cortex. B, the series of sections from PBS (left) and SM-216289-treated (right) rats (3 weeks), which were derived from animals that gave median score in each experimental group, are shown. The lateral side of the olfactory bulb is shown. Scale bar, 300 μm. C, graphical view of the results. Regeneration scores are presented as the mean ± S.E. (2 weeks: PBS, n = 4; SM-216289, n = 3; 3 weeks: PBS, n = 4; SM-216289, n = 5). The score estimation was performed in a blind manner. Acceleration of the olfactory nerve regeneration by SM-216289 treatment was statistically significant. *, Student’s t test, p < 0.05.

DISCUSSION

We isolated SM-216289 from the cultured broth of Penicillium sp. SPF-3059, and we identified this compound as a potent Sema3A inhibitor. SM-216289 inhibited Sema3A from collapsing growth cones and repulsing growing neurites in vitro. The binding of Sema3A to its receptor was abolished by SM-216289, which may be attributed to direct interaction with Sema3A. Moreover, SM-216289 promoted nerve regeneration in vivo in a rat olfactory nerve axotomy model.

As shown in Fig. 1, growth cone collapse was almost completely suppressed in the presence of 2 μM SM-216289 (IC50 = 0.16 μM). However, higher concentrations were needed to inhibit the repulsive activity of Sema3A in the gel assay (0.5 and 4.0 μM gave partial or complete inhibition, respectively). The apparent discrepancy in the inhibitory concentration for growth cone collapse and neurite repulsive activity may be explained in terms of the nature of the two phenomena. Once Sema3A binds to a receptor and induces growth cone collapse or retraction, the neurite stop extending and may even shorten. If the neurite is to grow normally in the presence of Sema3A, its activity needs to be continuously and completely blocked during the culture by a relatively high concentration of inhibitor as compared with that for the growth cone collapse assay. Alternatively, the local concentration of Sema3A in the collagen gel matrix may be higher than the concentration of Sema3A used for the collapse assay (300 μM). Indeed this difference in the local concentration of Sema3A is highly likely because growing neurites on an ascending gradient of inhibitory molecules are desensitized by a mechanism called habituation (47). As indicated in Fig. 1, a higher Sema3A concentration will require more SM-216289 to suppress the Sema3A activity. Thus, we can rationalize the elevated concentration of SM-216289 needed to suppress Sema3A in the collagen gel assay.

The binding of Sema3A-AP (a chimera of Sema3A and alkaline phosphatase) to both NP-1-expressing COS cells and DRG neurons was suppressed by SM-216289. Thus the compound would not only inhibit binding of Sema3A to NP-1 but also to the receptor complex that includes NP-1 and plexin-A1. Ten times more SM-216289 was needed to inhibit binding of Sema3A-AP to the receptor than was needed to suppress...
growth cone collapse of native Sema3A. This observation may be due to different assay conditions and/or that Sema3A-AP was used for the binding assay instead of native Sema3A. In accordance with previous reports (19, 37), the specific activity of Sema3A-AP for growth cone collapse was 10 times lower than that of wild-type Sema3A in our experiments (Fig. 4A). Furthermore, a higher concentration of SM-216289 was needed to inhibit the collapsing activity of Sema3A-AP, IC50 = 1.7 μM. Thus there was a good correlation between the concentration of SM-216289 required to inhibit binding of Sema3A-AP and the IC50 value for growth cone collapse by Sema3A-AP. The reason why Sema3-AP shows lower activity than native Sema3A is unclear. The structural differences between Sema3A-AP and the wild-type Sema3A may reduce its affinity for the receptor, as well as the inhibitor.

In our pre-mix experiment, when 0.5 μM of the compound was pre-incubated with Sema3A in culture medium, the growth cone collapse activity was suppressed (Fig. 3). However, no inhibition was observed when 0.5 μM compound was exposed to DRG in culture. Thus, direct contact of SM-216289 with Sema3A was essential for inactivation. Sema3A is activated by proteolytic cleavage. The mechanism of action of SM-216289 must be independent of this proteolytic processing because the reported cleavage sites (48) were deleted in our Sema3A-AP construct, and the observed molecular weight of Sema3A-AP by Western blot analysis was unaffected by incubation with cultured DRG. Taken together, our results strongly suggest that SM-216289 directly interacts with Sema3A to inhibit its activity.

In the pre-mix experiments, the inhibition was always partial (30–36% collapse observed in five independent experiments). This observation suggests that the inhibition was reversible and that a proportion of the compound was released after the pre-mixed solution was added to the DRG explants and diluted to a non-inhibitory concentration (Fig. 3). This was further confirmed by an experiment in which the collapsing activity of Sema3A was recovered from inactivation by an inhibitor level (1.0 μM) of SM-216289 after dialysis against an inhibitory culture medium (data not shown).

Interestingly, SM-216289 also showed efficacy in vivo as well as in vitro Sema3A inhibition. Olfactory nerve axotomy induces Sema3A mRNA expression in cells between the cribiform plate and the olfactory bulb, where regenerating axons grow, in this animal model (30). Although Sema3A protein distribution remains to be confirmed because of the lack of a reliable antibody for immunohistochemistry, it is reasonable to suppose that secreted Sema3A is distributed widely in the injured area. As shown in Fig. 5, olfactory nerve regeneration was significantly enhanced when SM-216289 was administered directly onto the injury site. Because pharmacokinetic property of the compound remains to be studied, the route of administration was chosen to minimize the effect and to simplify the interpretation of the result. We confirmed the distribution of the administered solution in our system by a control experiment using a dye (Evans Blue) in place of SM-216289. After 2 days of administration of the dye solution, the blue color was widely distributed in the olfactory bulb. Thus the enhanced nerve regeneration is most likely attributable to the inhibition of Sema3A by SM-216289. It should be noted that the specificity of SM-216289 to other semaphorins remains to be studied.

Our preliminary experiment suggested that SM-216289 inhibits Sema6C but at much higher concentration compared with Sema3A (data not shown). Recently, Shirvan et al. (49) reported that anti-Sema3A antibodies rescued retinal ganglion cells from cell death following optic nerve axotomy. It would be very interesting to examine whether the antibody promotes regeneration of olfactory nerves.

The essential issue that remains for future study is to appraise the recovery of function after axotomy. As regenerating axons reach the olfactory glomerulus, synapse formation with secondary neurons may be established. However, a careful physical and functional examination is required to establish that the regenerating axons reach their correct targets.

This is the first evidence to support the hypothesis that the suppression of Sema3A activity in glial scar tissue promotes the regeneration of axons in adult animals. Although olfactory primary neurons are peripheral neurons, the regenerating axons traveled in the olfactory bulb of the CNS. Interestingly, in many CNS nerve injury models, the regeneration of injured nerves may be affected by Sema3A expressed in glial scars (23, 30–32). If semaphorin, especially Sema3A, in scar tissue is the major obstacle to the regeneration of CNS axons, SM-216289 might promote re-growth of the axons.

SM-216289 is the first Sema3A inhibitor showing both in vitro and in vivo efficacy and is an excellent molecular probe to investigate the mechanism of Sema3A function. In addition, SM-216289 may be clinically useful as a pharmaceutical to promote neural regeneration, especially in therapy of some types of anosmia. Growing evidence suggests an involvement of Sema3A in the inhibition of regeneration of CNS axons after traumatic injury. Future studies will examine the effects of SM-216289 on CNS regeneration using animal models with other clinical relevance, such as spinal cord transections.

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