Nerve Regeneration in the Central Nervous System by a Semaphorin Inhibitor

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Kazuo KUMAGAI
Kaoru KIKUCHI
Akiyoshi KISHINO
Nobuo HOSOTANI
Akira ITO
Ikutaro SAJI
Toru KIMURA

It is well known that injured neurons in the central nervous system have only limited capability to regenerate and recover neurological function. We identified a low molecular weight compound, SM-216289, from cultured broth of a fungus isolated from the soil of the Osaka Castle Park as a new semaphorin inhibitor. SM-216289 completely inhibits semaphorin activity at less than 1 μM *in vitro*. The compound could accelerate neuro-regeneration in the central nervous system *in vivo*. Semaphorin inhibitors may become innovative drugs to enhance nerve regeneration after spinal cord or brain injury[1, 2].

This paper is translated from R&D Report, "SUMITOMO KAGAKU", vol. 2005-I.

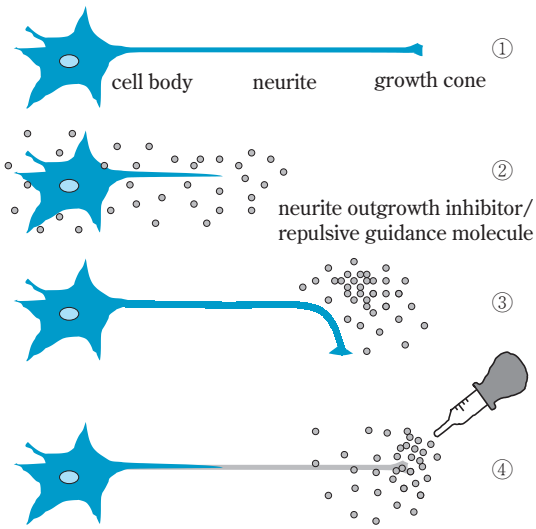
Introduction

It is well known that it is difficult to recover from a functional impairment caused by traumatic damage to the central nervous system, such as the brain or spinal cord. There are 3,000 to 4,000 people in Japan and several tens of thousands of people worldwide who incur spinal cord injuries caused by traumatic events such as automobile accidents and the like. What renders recovery difficult is a lack of axon regeneration, a fact that been known since the 19th century. Axons form the fiber structure that transmits nerve signals in the central nervous system. However, at the beginning of the 1980s, the Canadian researcher Aguayo ascertained that the axons themselves retained the capacity for regeneration even in the central nervous system. The reason they do not regenerate *in vivo* is not because there is a deficit of neuron capabilities, but rather because of the presence of a substance that inhibits their regrowth in the brain and spinal cord (nerve growth inhibitors).[3] Since then, these growth inhibitors have been identified and a great deal of research has been carried out with the hopes of clearing a pathway that would lead to the treatment of untreatable disorders by developing methods for controlling these inhibitors. So far, there have been many reports identifying axon regeneration inhibitory substances in the brain or spinal cord.[4] However, it is still unclear which of them should be controlled to obtain the kind of central nerve regeneration suggested by Aguayo.

While research on substances that inhibit regeneration in the central nervous system is progressing, there has been progress in the field of developmental biology with research on the neural network formation mechanism during the fetal period. It has been found that factors that inhibit the extension of axons (simply called neurites when in an immature state) are important in the process of forming the neural network. Research is moving forward on what are called repulsive guidance molecules.[5] We focused on the fact that semaphorin 3A (Sema 3A), which is one type of repulsive guidance molecule, demonstrates very strong nerve extension inhibitory activity and is strongly expressed even in matured central nervous systems where the formation of the neural network should have been complete. We have continued this research for ten years with the hypothesis that by controlling Sema 3A, it might be possible to promote the regeneration of nerves in the central

nervous system. (**Fig. 1**) (In the following, Sema 3A is indicated when mentioning semaphorin.)

After all this time, we have recently been successful in finding that a new filamentous fungus (mold) isolated from the soil in Osaka Castle Park produces a semaphorin inhibiting substance, SM-216289. We are reporting this here because we have proven for the first time worldwide that axon regeneration in the central nervous system is promoted by inhibiting semaphorin *in vivo* using SM-216289. [1), 2)]



1. Neurons extend long neurites from their cell bodies. A fan-like structure called growth cone is observed at the tip of each neurite. 2. In the presence of neurite outgrowth inhibitor or repulsive axon guidance molecules, neurite growth is inhibited. 3. When growing neurites encounter a gradient of repulsive molecules, the neurites make a turn to avoid higher concentration of repulsive molecules. 4. Growth cones are collapsed and neurites are retracted by exposure of neurite outgrowth inhibitor or repulsive axon guidance molecules onto growing neurite.

Fig. 1 Effect of neurite outgrowth inhibitor and repulsive axon guidance molecules to neurite extension

Screening Semaphorin Inhibitors and the Discovery of Active Compounds

When we first postulated that nerves might regenerate if semaphorin were inhibited, no low molecular weight inhibitors were known to have neurite growth inhibitory factors, not limited to semaphorin, and we did not even know if such a substance even existed. Only a neutralizing antibody for a neurite growth inhibitory protein called Nogo had been reported,[6)] and this was a high molecular weight protein. With our aim being the development of a pharmaceutical product, we naturally set out to find a low molecular weight compound. Thus, we screened the Sumitomo Pharmaceuticals chemical compound and natural substances libraries to try to find a low molecular weight compound that would inhibit semaphorin. In the actual screening for finding a substance that completely inhibits the activity of semaphorin, we used the time-consuming method of culturing neural tissue (tissue samples of 5,000 to 10,000 sensory neurons under 1 mm) called dorsal root ganglia (DRG) collected under a microscope from developing chick embryos in a 96-well plate and adding semaphorin protein prepared using recombinant cells and a test sample. Allowing them to act for one hour at 37°C, we screened over 100,000 samples observing each well with a microscope for growth cone collapse. As a result, we found strong semaphorin inhibitory activity in a culture medium extract from a fungus isolated from soil collected in Osaka Castle Park. There were hits for several samples from the compound library, but all of them had weak activity, and the activity of this natural substance was conspicuous. When we applied a secondary screening system allowing semaphorin to act for 48 hours with the goal of further evaluating the activity, we found extremely clear semaphorin inhibitory activity. In addition, what was amazing was our finding no cytotoxicity even at concentrations 1,000 times those exhibiting activity. Therefore, we set out to isolate the active component from this SPF-3059 strain of fungus. Since we predicted that this active component would be soluble in an organic solvent and have a molecular weight of several thousand or less, it could be assumed that rather than a macromolecule such as a protein or a peptide, it would be, as expected, a low molecular weight organic compound. A large-scale culture was carried out, and as a result of isolation and purification, 32 new active compounds were isolated including three known components. Since there were also trace components, it required more than a year to isolate all of them. The component with the strongest activity exhibited a 100 nM IC_{50} value. We were able to determine the structural formulae of almost all of the components in the end through structural analysis using UV, IR, MS and NMR, with all of compounds having xanthone and chromone structures. As a typical example of an active component, the structure of SM-216289 (xanthofulvin) is shown in **Fig. 2**. More-

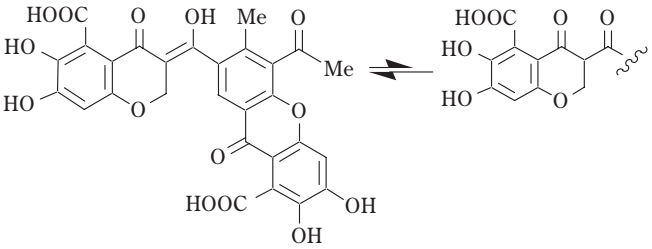


Fig. 2 Structure of SM-216289 (xanthofulvin)

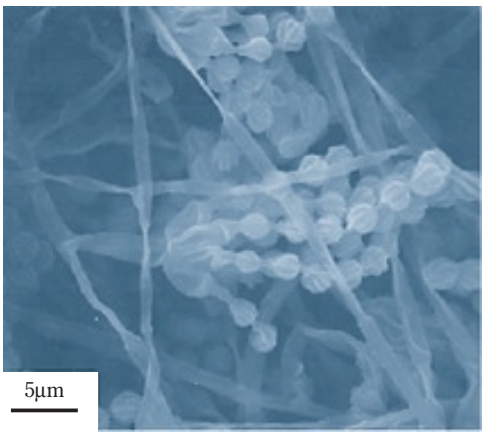


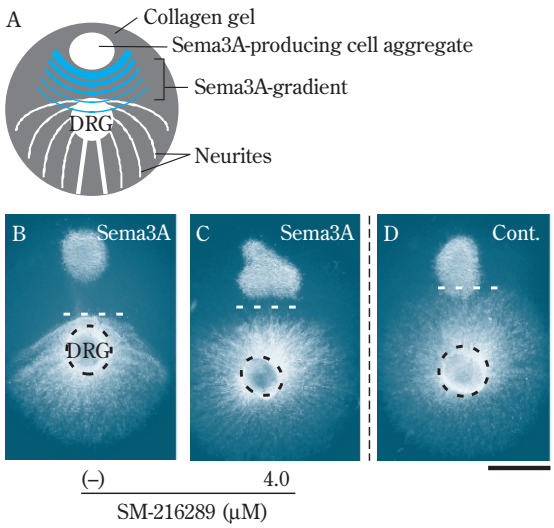
Fig. 3 Scanning electron micrograph of *Penicillium* sp. SPF-3059

over, the producing fungal strain, SPF-3059, was identified as the deuteromycete *Penicillium* sp. (**Fig. 3**) from its mycological characteristics.

In vitro investigation of the semaphorin inhibiting action of SM-216289

An *in vitro* drug efficacy reevaluation was carried out for the purpose of characterizing the compounds identified in the screening for such things as semaphorin inhibitory action with comparisons using known inhibitors.

The assay system (collapse assay) used in the first screening was an experimental system for detecting the short-term reactions arising locally at the ends of the neurites in the cultured neurons. The activity of SM-216289 was investigated using a collagen gel co-culture assay,[7] which is an experimental system closer to that in a living organism. The growth of the neurites or axons stop or are repelled (Fig. 1) near the location where semaphorin is present in the extension process *in vivo* but this can be reproduced with a collagen gel co-culture assay. If DRG explant, which consists of sensory neurons, and an aggregate of cells expressing semaphorin are cultured with a spacing of 0.5 – 1.0 mm in collagen gel, a concentration gradient is formed in the collagen gel. This limits the spread of the semaphorin secreted by the aggregate of cells expressing semaphorin and the neurites extending from the DRG are repelled by the action of the semaphorin and only extend on the side opposite to the semaphorin expressing cells (**Fig. 4**-A and B). When no semaphorin is present, the neurites extend concentrically as shown in Fig. 4-D. When SM-216289 is added to the culture, the repulsion by semaphorin is suppressed and the concentric form of the neurite extension is observed (Fig. 4-C). Even in a state of constant exposure to semaphorin during a two-day culture, there is an almost complete inhibition of the repulsive action the whole time. In other words, we can see that the SM-216289 that we discovered has a strong and continuous semaphorin inhibiting activity.



(A) A schematic representation of collagen gel co-culture assay. When a cell aggregate of Sema3A-producing cells (Sema3A-Cell) and DRG are cultured in collagen gel matrix, concentration gradient of Sema3A is formed around the Sema3A-Cell due to difficulty of diffusion of the Sema3A in the matrix. Neurites extending from DRG explant are repelled by Sema3A and can only grow avoiding Sema3A-Cell. (B)-(D) DRG and cell aggregate were co-cultured in collagen gel matrix. (B) Repulsion of the neurites was observed without SM-216289. (C) In the presence of SM-216289 neurites could grow even toward Sema3A-Cell, and radial extension of the neurites was observed. (D) Radial extension of the neurites was observed, when DRG was co-cultured with control (non-Sema3A producing) cell-aggregate. Dashed-circle indicates the location of explanted DRG. Dashed-line indicates the position of neurite tips extending in the direction of cell-aggregate. Scale bar: 0.5 mm.

Fig. 4 SM-216289 inhibits the repulsive activity of Sema3A.

The intracellular signaling pathway involved in the expression of the semaphorin action has not been sufficiently elucidated, but recently there have been reports that several kinase inhibitors block semaphorin signaling.[8] However, these have cytotoxicity, only partially inhibit the activity of semaphorin and do not have the characteristics of candidates for drugs like those demonstrated by SM-216289.

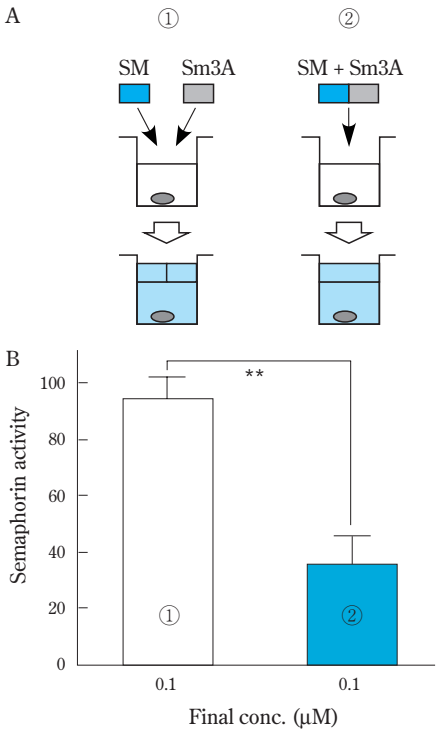
Analysis of the Action Mechanism

We investigated the mechanism by which SM-216289, which causes very little cell cytotoxicity, exerts its 100% semaphorin inhibiting activity.

The expression of semaphorin action (growth cone collapse and axon repulsion) begins with the semaphorin proteins binding to the receptors on the nerve cells. The signal from the receptors is transduced into the cell, further amplified and finally induces cytoskeletal depolymerization. SM-216289 should be operating somewhere in this series of processes and inhibiting the semaphorin function. We will omit the details here, but from preliminary experiments on analysis of the action mechanism, we have obtained results allowing us to conjecture that SM-216289 acts outside the cells. Putting it another way, this means that the target molecule is either the semaphorin protein itself or its receptors. The experiments described in the following were carried out using a collapse assay to identify which of these it is (**Fig. 5**). As is shown in Fig. 5-A, either (1) SM-216289 and semaphorin were added to the culture separately or (2) a mixture of SM-216289 and semaphorin was added to the culture, and the semaphorin inhibition in (1) and (2) were compared. (However, the final concentration of SM-216289 in (1) showed no semaphorin inhibition.) Despite the fact that the final culture conditions for (1) and (2) were set up to be the same, the results were that no semaphorin inhibition was found in (1) but was found in (2). These results can be assumed to mean that the semaphorin inhibition arises with the mixing of Sema 3A and SM-216289 (with the concentration of SM-216289 in the mixture exhibiting inhibitory activity) and that the inhibitory effect continues after the addition to the culture (SM-216289 being diluted to a concentration where it is inactive). In other words, we can assume that the target molecule for SM-216289 is the semaphorin protein.

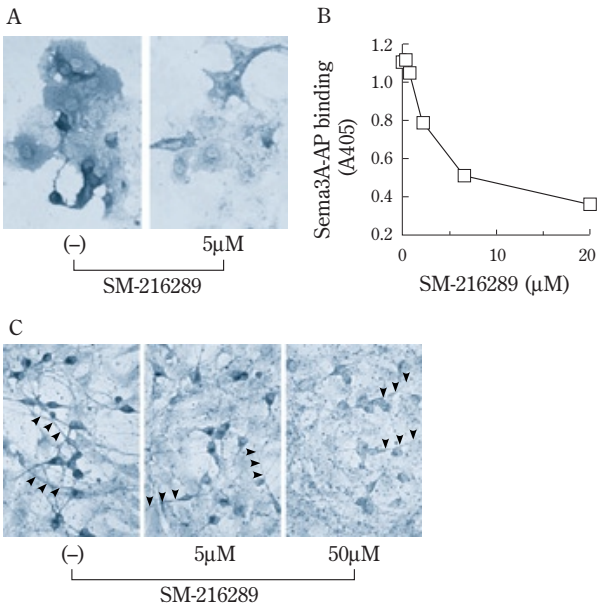
Next, we investigated whether SM-216289 actually inhibits the semaphorin receptor binding. As a result of examinations into the action on binding for COS 7 cells (NP1-COS) expressing Neuropilin-1, which is an essential binding component of semaphorin receptor, it was clear that SM-216289 inhibits the binding of semaphorin to Neuropilin-1 (**Fig. 6**-A and B). Furthermore, when we investigated whether there was inhibition of binding for a primary culture of neurons expressing functional semaphorin receptors including receptor components other than Neuropilin-1, the binding of semaphorin to neurons was also inhibited by SM-216289 in this case (Fig. 6-C).

As a result of the investigations above, it was clear that SM-216289 exerts its inhibitory action by directly binding to the semaphorin protein and reducing the binding capacity for receptors.



(A) A schematic representation of experimental procedures. ①: SM-216289 and Sema3A (Sm3A) are independently added to the assay medium. ②: Sema3A was pre-incubated with 0.5μM of SM-216289 before addition to the assay medium. Final concentration of Sema3A and SM-216289 were identical in both cases (3 units/ml Sema3A and 0.1μM SM-216289). (B) Collapse activities of Sema3A observed in procedure-① and procedure-② are shown. In the procedure-①, Sema3A was not inhibited. However, in the procedure-②, significant Sema3A-inhibition was observed, although final condition was identical to the procedure-①. Thus, incubation of Sema3A with higher concentration of SM-216289 was crucial to inhibit activity of Sema3A, suggesting that SM-216289 directly targets Sema3A. ** Student's *t*-test: $P<0.01$.

Fig. 5 SM-216289 directly targets Sema3A.

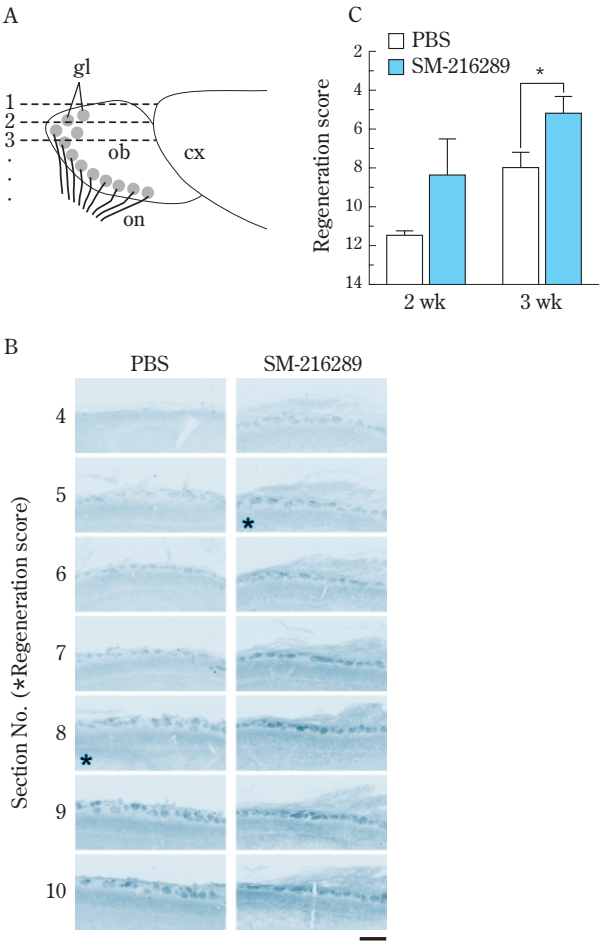


(A) Alkaline phosphatase-fused Sema3A (Sema3A-AP) could bind to COS cells expressing a Sema3A receptor component, neuropilin-1 (NP1-COS), and were stained black. But in the presence of SM-216289, the binding was significantly inhibited, which could be seen as faint staining. (B) Quantitative measurements of the inhibition of Sema3A-AP binding to NP1-COS by SM-216289. SM-216289 inhibited Sema3A-AP-binding in a dose dependent manner. (C) SM-216289 also inhibited binding of Sema3A-AP to DRG neurons, in which functional Sema3A receptor was expressed. Arrowheads in (C) indicate neurites.

Fig. 6 SM-216289 inhibits binding of Sema3A to its receptor.

Promotion of Olfactory Nerve Regeneration by SM-216289

As was discussed at the beginning of this paper, the nerves of the central nervous system in adults are vulnerable to injury after which there is almost no hope for re-growth of the axons. Starting at the end of the 1990s, there has been one report after another stating that the expression of semaphorin is induced at the injury sites, and the idea that semaphorin may be involved in this inability to regenerate has become global.[9] Therefore, in order to examine whether the nerve regeneration is promoted by semaphorin inhibition by administration of SM-216289, we used an olfactory nerve axotomy model in which the expression of semaphorin at the injury site has been reported, from among several nerve injury models. (Olfactory nerve: indicates the nerve fibers of olfactory epithelial neurons on the nasal mucosa project to the olfactory bulb of the brain. They are classified as peripheral nerves but the fibers run to central nervous system tissue (the olfactory bulb). A remarkable increase in semaphorin at injury site of olfactory nerve axotomy model has also been reported. Furthermore, it has been known that fetal olfactory epithelial neurons are sensitive to semaphorin.) Since almost



Olfactory nerves of rats were axotomized, and PBS or SM-216289 was locally administered into injury sites for two or three weeks. Regenerated olfactory nerves were stained and the level of regeneration was quantified by scoring. (A) Schematic representation of our scoring method. Regenerating nerves grow from bottom to top in this figure. Horizontal sections (30μm thickness) were prepared from top to bottom of the olfactory bulb, and the number of the first section in which regenerated nerves were used as regeneration score. Note that smaller scores indicate faster regeneration. (B) A series of sections from PBS- (left) and SM-216289- (right) treated rats (3 weeks) are shown. Asterisks indicate the first section in which regenerating nerves were detected in each treatment. Scale bar: 300μm. gl; glomerulus, ob; olfactory bulb, on; olfactory nerve (growing bottom to top), cx; cerebral cortex. (C) Graphical view of the results. Acceleration of olfactory nerve regeneration by SM-216289 treatment was statistically significant. Regeneration scores are presented as the mean ± SEM (2 wk: PBS; n = 4, SM-216289; n = 3, 3wk: PBS; n = 4, SM-216289; n = 5). * Student's *t*-test: $P<0.05$.

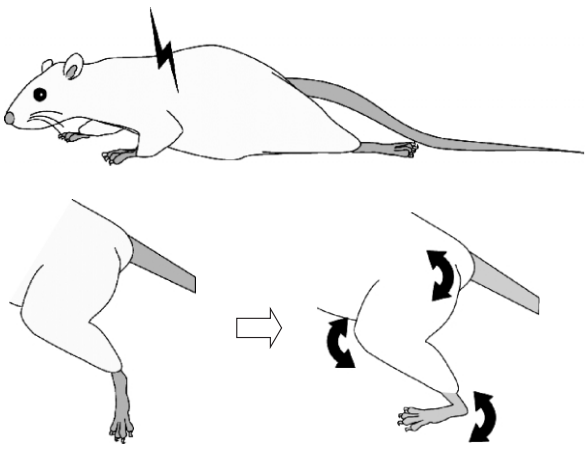
Fig. 7 SM-216289 promotes regeneration of axotomized olfactory nerves in rat

no nerve regeneration occurs in injury models for the brain and spinal cord, evaluating drug efficacy may be difficult but olfactory nerves naturally have a capacity for regeneration[10] and we thought that we could easily evaluate drug efficacy by focusing on the rate of regeneration. There was also merit in the point that evaluations in a comparatively short period (2 – 3 weeks) were possible because the regeneration rate is fast.

The olfactory nerves of rats were axotomized and SM-216289 was administered continuously to the injury site using an osmotic pressure pump. After a tracer (WGA-HRP: wheat germ agglutinin-horseradish peroxidase conjugate) was administered to the nasal cavity at two weeks and three weeks after the operation, the brain was dissected. When we examined the regeneration of the olfactory nerve by making the tracer visible, there was significantly more facilitation of regeneration in the SM-216289 group than in the vehicle-treated group (**Fig. 7**). These results show that SM-216289 suppresses the action of semaphorin, which increases following an injury, and that the olfactory nerve was actually re-extended suggesting the possibilities for the semaphorin inhibitor being a drug for treating nerve injury. Furthermore, the results of these experiments are important from the standpoint of actually confirming that semaphorin suppresses nerve regeneration *in vivo*. Up to this point, there have been a number of examples of experimental models showing that the expression of semaphorin is induced when there is an injury to the nerves but whether or not the semaphorin actually suppresses nerve regeneration had not been verified. The results introduced here is the first example worldwide demonstrating the function of semaphorin in the adult central nervous system *in vivo*.

Future Prospects for the Semaphorin Inhibitor

In this paper, we have introduced olfactory nerve regeneration in rats with a semaphorin inhibitor operating *in vivo*, but the greatest expectations for clinical applications of the semaphorin inhibitor at present are for spinal cord injuries. It goes without saying that spinal cord injuries are one of the most serious degenerative afflictions of the nerves, and if the case is serious, there is damage to the motor, sensory and autonomic nervous systems starting with quadriplegia. Even when it is minor, there are many cases where nerve functions are partially impaired. At present, there is no effective method of treatment, and when it is acute, steroid administration is the only way to control secondary inflammation. Because of this, there is a great desire for a new method for treating injuries to the spinal cord and basic research into regenerative surgical treatments using transplants of various types of neural cells is now moving forward. In recent research, it is thought that Sema 3A is expressed excessively at the sites of injury in spinal cord injuries and that it inhibits the regeneration of nerve fibers. Therefore, we examined the effectiveness of the semaphorin inhibitor for spinal cord injuries in an animal model through joint research with Prof. Hideyuki Okano of the Keio University School of Medicine. After artificially damaging the spinal cords of rats, SM-216289 was continuously administered to the injury site intra thecally and changes in the motor functions were examined. In the results, the recovery of motor functions for the group administered with SM-216289 significantly surpassed the group administered with PBS (**Fig. 8**). Furthermore, when histological investigations were carried out in the spinal cord, we observed a re-extension of many nerve fibers in the animals given SM-216289. This shows that there is a possibility for the semaphorin inhibitor to promote recovery following injuries to the spinal cord.[11] These results have been in many newspapers and have attracted a great deal of attention. In addition, a report has appeared recently saying that semaphorin contributes to retinal nerve cell death[12] and it is possible that the ailments to

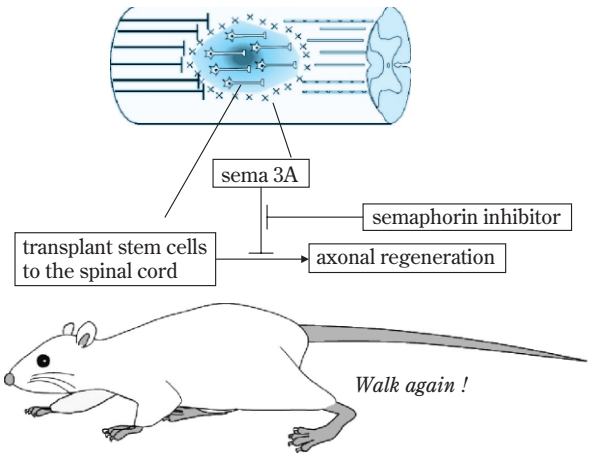


In rat spinal cord injury models, SM-216289 was administered in the proximity of the injury site by osmotic mini-pump. SM-216289 promoted recovery in the motor function of hind limb.

Fig. 8 Functional recovery by SM-216289

which the semaphorin inhibitor is applied may increase even more.

On the other hand, the transplant treatments for ES cells and neural stem cells for which research has been active in recent years, are thought of as being promising methods for treating spinal cord injuries in the future. Medical treatments for regeneration that directly replenish the lost nerve cells are expected to be basic methods for the treatment of neurodegenerative diseases. We think that the semaphorin inhibitor will be particularly effective in these medical treatments for regeneration. In other words, even if stem cells are transplanted to the sites of injuries and to nerves in the central nervous system, nerve functions will not be recovered unless new nerve fibers extending from these cells and neural circuits to the host are formed. However, the effects of the injury will remain in the neighborhood of the transplants and there is a possibility that there will be expressions of factors inhibiting nerve extension starting with semaphorin. In such cases, the nerve fibers that start to grow from the transplanted cells will not be able to extend to the neural parenchyma of the host because of the inhibiting factors. If a semaphorin inhibitor is used jointly with cell transplants, we can expect that the regenerated fibers will cross the places where semaphorin is expressed and extend to the host tissue and new neural circuits will be formed. The semaphorin inhibitor introduced here can be expected to exhibit superior functions in medical treatments for regeneration accompanying these new transplant treatments (**Fig. 9**).



Semaphorin inhibitor promotes the axonal elongation from the transplant cells into the spinal cord by inhibits the activity of semaphorin in the injury site.

Fig. 9 Application of semaphorin inhibitor in the stem cell implantation

Since the semaphorin inhibitor is a compound with a new mechanism of action targeting ailments for which there have been almost no effective treatments up to now, there are many problems that must be solved. However, we believe that the superior characteristics of the semaphorin inhibitor not available in existing pharmaceutical products will lead to new treatments for incurable diseases and we will proceed with multifaceted investigations aimed at the development of actual pharmaceutical products.

Acknowledgements

We would like to thank Prof. Okano of Keio University who investigated the effectiveness of the semaphorin inhibitor in an animal model with spinal cord injuries.

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PROFILE

Kazuo Kumagai

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Senior Research Scientist
Ph. D.

Akira Ito

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Discovery Research Laboratories I
Senior Research Scientist
Ph. D.

Kaoru Kikuchi

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Genomic Science Laboratories
Senior Research Scientist
Ph. D.

Ikutaro Saji

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Chemistry Research Laboratories
Senior Research Scientist
Ph. D.

Akiyoshi Kishino

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Discovery Research Laboratories I
Senior Research Scientist
Ph. D.

Toru Kimura

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Genomic Science Laboratories
Senior Research Scientist
Ph. D.

Nobuo Hosotani

Sumitomo Pharmaceuticals Co., Ltd.
Research Division
Research Scientist