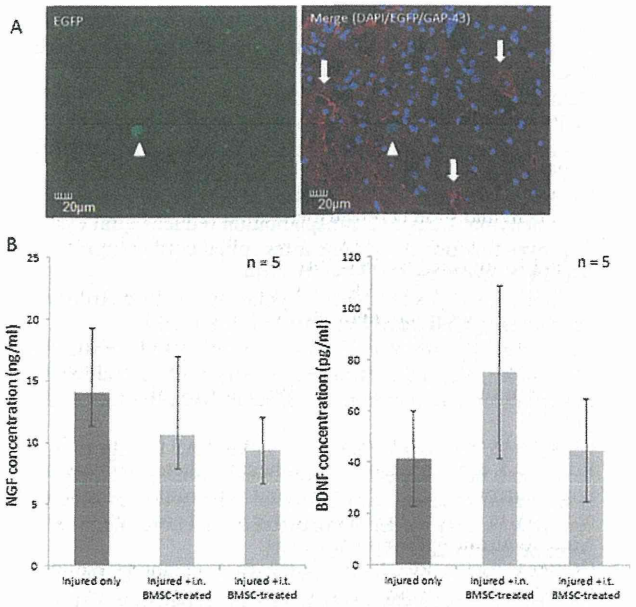

FIG. 5. A: GAP-43 *(red)* immunofluorescence staining of the transverse sections in the i.t. BMSC–treated group at 4 weeks. An EGFP *(green)*–positive cell *(arrowhead)* was found *(left)*, and some axonal regrowth *(arrows)* was confirmed around the cell *(arrowhead) (right)*. Bar = 20 μm. **B:** The NGF concentration *(left)* and the BDNF concentration *(right)* in the CSF at 2 weeks are shown in the 3 groups (n = 5). There was no statistically significant between-groups difference with respect to neurotrophins.

venously grafted MSCs even in lesion sections examined 7 days postsurgery, although neurological improvements were seen after 1 month. Therefore, we killed half of our group of rats at 2 weeks after surgery in order to to detect intralesional cells. We killed the other half of the group 2 weeks later. Thus all the rats were killed within 1 month of surgery, which is relatively early, but we predicted that there would be some neurological recovery by this point if any were going to occur. The existence of BMSCs was confirmed in spinal cord sections obtained at both time points (2 and 4 weeks after surgery) in this study. We think that these findings are important because the intranasal approach is thought to be extremely favorable from the viewpoints of invasiveness and simplicity. BMSC migration was not confirmed in sections 4 mm from the injury epicenter, where the spinal cord appeared uninjured. Although we did not clarify whether BMSCs are transferred into the brain or CNS, our study suggests that, at least locally, BMSCs will move into an inflamed area.

Cell Proportion, Limited Functional Recovery, and Possible Treatment Options With Intranasal Cell Delivery

The proportion of intranasally administered cells delivered to the spinal cord lesion was estimated as follows. Overall, in the sections taken from rats killed 2 weeks after surgery, except for those used for cavity evaluation, there was an average of 80 BMSCs within a 570-μm-thick cross-section. Because the diameter of the impactor tip is 2500 μm, approximately 350 BMSCs ([80 BSMCs × 2500 μm]/570 μm) might exist in the lesion. This accounts for 1.75% of the administered cells.

The mean BBB score of the i.n. BMSC–treated group showed significant increases as early as 1 week after surgery. However, their functional recovery was limited after 2 weeks compared with that of the i.t. BMSC–treated group. In addition, the total numbers of migrated BMSCs at both 2 weeks and 4 weeks after surgery in the i.n. BMSC–treated group were less than those of the i.t. BMSC–treated group. These findings suggest that the intrathecal application route is better than the intranasal route as far as efficiency is concerned.

In order to proceed further toward the clinical use of i.n. BMSC treatment for SCI, the timing and dosage of BMSC administration must be considered. In the present study, BMSCs penetrated to the central lesion in the i.t. BMSC–treated group in the 2-weeks section, which might influence the functional recovery. Because the i.t. BMSC–treated group had approximately 4 times as many BMSCs within the spinal cord lesion when compared with the i.n. BMSC–treated group 2 weeks postsurgery, 8×10^5 or more cells are recommended to be administered via the intranasal route.

With respect to the timing of the injection, Cizkova et al.[6] reported the interesting finding of significantly greater motor functional recovery after 3 daily injections of a total 1.5×10^6 MSCs via an intrathecal catheter than after a single injection of 5×10^5 MSCs in a rat model of contusive SCI. They attributed the greater recovery not only to an increase in the number of delivered MSCs, but also to the addition of fresh stem cells during the 3 daily applications. Furthermore, they showed different results by altering the timing of the repetitive injection.

Those finding suggest, together with our present results, that repetitive injections would be preferable, and the most effective timing should be further evaluated.

The Role of BMSCs in Tissue Protection and Functional Recovery

BMSCs are multipotent cells that have the ability to differentiate into neurons.[16,27] However, the proportions of BMSCs that differentiate or survive in CNS lesions seems small. Mezey et al. have reported that no more than 5% of grafted stromal cells differentiate into neurons within a brain lesion.[16] Others have shown the survival of less than 1% of grafted MSCs in injured spinal cord at 1 month after treatment, without any differentiation.[3] Therefore, it is the neurotrophins and cytokines that are secreted from BMSCs that are believed to be much more important than the cells themselves for neuroprotection, axonal regrowth, and tissue repair; and neurotrophins and cytokines effect these outcomes via paracrine signaling.[14,19,20,26]

For lesion cavity reduction and functional recovery after SCI, these mechanisms seem to act comprehensively. Neuroprotection, which is caused by reducing inflammation or preventing apoptosis,[4] might occur in the acute stage after injury. Statistically significant BBB score improvements occurred in both the i.n. and i.t. BMSC–treated groups by 1 week in our study, and might suggest this action. In addition, axonal regrowth was shown within the GAP-43–immunostained lesion sections in the BMSC-treated groups at 4 weeks in our study. Some studies have previously reported that the tissue repair in SCI after intrathe-

cal administration of BMSCs was due to the proliferation of nonneural tissue, including extracellular matrices that are composed of collagen fibrils.[17] In addition, others have demonstrated that the extracellular matrix that is produced by BMSCs supports neural cell growth.[1]

In our experiment, there was a tendency toward smaller lesion cyst size with more efficient delivery of BMSCs (i.e., greater numbers of BMSCs delivered to the lesion). Furthermore, the regression analysis of the final BBB scores and the cavity ratio clearly showed that cavity reduction had great influence on neurological functional recovery after SCI. Therefore, there was no doubt that our results also supported a tissue-sparing effect of BMSCs and their contributions to functional recovery.

Evaluations of NGF and BDNF

Neurotrophins, such as NGF and BDNF, are known to play a significant role in paracrine effects.[7,15,25] NGF expression by MSCs in vitro has been shown previously,[7] and to a lesser extent, BDNF secretion has also been confirmed.[7,25]

However, Quertainmont et al.[19] have reported increased NGF levels, determined by ELISA, within the injured spinal cord tissue after a MSC graft and have described the contribution of NGF to lesion repair. Furthermore, according to Donega et al.,[11] BDNF and NGF mRNA expression from MSCs significantly increases after co-culture with hypoxic-ischemic brain extract.

In contrast, there have been very few reports of neurotrophic factor expression in CSF after BMSC administration for CNS disease. In one report, BMSC infusion into the CSF resulted in a significant increase in neuronal density and neurite length in cultured hippocampus neurons through its trophic effects.[17] According to Wang et al.,[23] BDNF levels in CSF were significantly higher 15 days after the intraventricular administration of BMSCs and BDNF-overexpressing BMSCs in a rat model of brain injury.

In the present study, we did not find statistically significant differences in NGF and BDNF levels in the CSF among the injured-only group and the BMSC-treated groups at 14 days. It might be worth investigating the effects of neurotrophins in an earlier period in our model because the BBB scores of the BMSC-treated groups significantly increased by 1 week postsurgery. Through further investigation, we can clarify whether NGF and BDNF in CSF can be useful biomarkers for BMSC treatment in SCI.

Conclusions

We present a new BMSC administration route for the treatment of acute SCI. The results of our study show, for the first time, BMSC migration to the spinal cord lesions in a rat model of acute SCI. However, in order to be a viable alternative to other treatment routes, this route needs further investigation. In addition, the mechanisms underlying the effectiveness of BMSCs in neurological functional recovery should be further clarified.

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Author Contributions

Conception and design: Ninomiya. Acquisition of data: Ninomiya. Analysis and interpretation of data: Ninomiya. Drafting the article: Ninomiya. Reviewed submitted version of manuscript: Ohnishi. Approved the final version of the manuscript on behalf of all authors: Iwatsuki. Statistical analysis: Ninomiya. Administrative/technical/material support: Ohkawa. Study supervision: Iwatsuki, Yoshimine.

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Review

Consensus of Clinical Neurorestorative Progress in Patients With Complete Chronic Spinal Cord Injury

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Currently, there is a lack of effective therapeutic methods to restore neurological function for chronic complete spinal cord injury (SCI) by conventional treatment. Neurorestorative strategies with positive preclinical results have been translated to the clinic, and some patients have gotten benefits and their quality of life has improved. These strategies include cell therapy, neurostimulation or neuromodulation, neuroprosthesis, neurotization or nerve bridging, and neurorehabilitation. The aim of this consensus by 31 experts from 20 countries is to show the objective evidence of clinical neurorestoration for chronic complete SCI by the mentioned neurorestorative strategies. Complete chronic SCI patients are no longer told, "nothing can be done." The clinical translation of more effective preclinical neurorestorative strategies should be encouraged as fast as possible in order to benefit patients with incurable CNS diseases. This manuscript is published as part of the International Association of Neurorestoratology (IANR) special issue of *Cell Transplantation*.

Key words: Consensus; Clinical neurorestoration; Complete chronic spinal cord injury; Translational neuroscience; Neurorestoratology

THE PROBLEM AND CHALLENGE

Restoring function for people with spinal cord injury (SCI) is one of the most challenging tasks in clinical practice, especially for chronic complete SCI. Now more than 60% of patients can be partially or fully restored by standard surgical management, medical therapy, and neurorehabilitation for acute and subacute incomplete SCI. Regretfully, facing chronic complete SCI, clinical studies often ignore previous physicians' efforts and their significant findings and state that there are no effective therapeutic methods to restore, even partially, neurological functions for intractable damage or diseases of the central nervous system (CNS) (1,19). We are glad to see that neurorestorative strategies with positive preclinical results have been translating to the clinic and have achieved some clinical neurorestoration. These strategies include cell therapy, neurostimulation or neuromodulation, neuroprosthesis or related advanced assistive devices, neurotization or nerve bridging, neurorehabilitation, and other novel treatments. In this review, we summarize the data based on the scientific and professional information available up to April 2013. We also address questions and issues about the aforementioned experimental strategies. The aim of this consensus is to show the objective evidence to the medical community. The Beijing Declaration of the International Association of Neurorestoratology (IANR) states that "Neurorestoratology recognizes the importance of small functional gains that have significant effects on quality of life" (26). Hopefully, more people can understand new developments in this field. We should highlight the value of clinical neurorestoration for incurable CNS diseases and damage from a scientific viewpoint and encourage more clinical translational studies in neurorestoratology.

SEARCH STRATEGY AND SELECTION CRITERIA

We searched PubMed and ScienceDirect with a combination of search terms: "spinal cord injury," "injured spinal cord," "spinal cord trauma," "treatment," "therapy," "transplantation," and "human." We also searched the reference lists of key articles and consulted experts who published important papers on neurorestoration, neural regeneration and sprouting, neuromodulation, neurostimulation, neuroprosthesis, neuroprotection, neuroplasticity, nerve bridging, controlling anti-inflammatory responses, neurogenesis, angiogenesis, remyelination or neurorepair, and neuroreplacement. Literature was excluded if it was not in English or if it was not relevant to complete chronic spinal cord injury. Abstracts and reports from meetings and articles dating prior to 1950 were excluded.

EVIDENCE

Cell Therapy

Preclinical studies of over 30 types of cells have been confirmed to restore function of the animal CNS (20). Clinical evidence suggests that cell therapy or tissue transplantation is safe and feasible (2,5,12,21,34,43,45,48,49). Partial functional recovery and the quality of life improved for patients with chronic complete SCI following transplantation of cells into cord parenchyma (7,11,15,22,24, 25,31,32,59), administration of cells intrathecally (lesion area or lumbar subarachnoid space) (30) (Fig. 1), infusion of cells intravascularly (9,39,40), and by multiple routes of administration (14,44,47,50). Neurological function and daily life functions were assessed by one or more of the following scales: American Spinal Injury Association (ASIA), Frankel, International Association of Neurorestoratology (IANR) Spinal Cord Injury Functional Rating Scale (IANR-SCIFRS), Barthel, and Ashworth scales. Patients showed marked improvement in neurological and daily life functions (Table 1).

Neurostimulation/Neuromodulation and Neuroprosthesis and/or Related Advanced Assistive Devices

Task-specific training with epidural stimulation might reactivate previously silent spared neural circuits or promote plasticity. These interventions could be a viable clinical approach for functional recovery for patients with complete chronic SCI (16,37,56). Transcranial direct current stimulation and visual illusion can be effective in the management of neuropathic pain following chronic

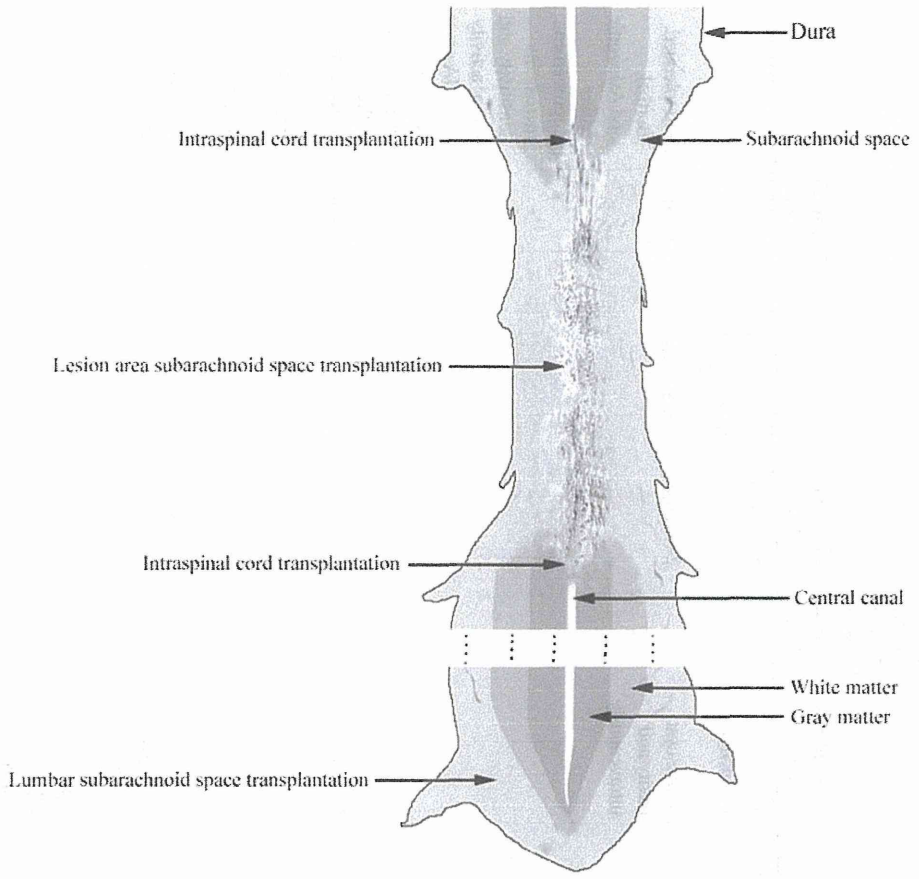

Figure 1. Diagrammatic drawing of cell transplantation into spinal cord parenchyma or subarachnoid space.

SCI, with minimal side effects and can be well tolerated (13,52). Functional electrical stimulation of permanently deinnervated muscle in patients with complete chronic lower motor neuron lesions is an effective therapy, which results in rescue of muscle mass, function, and perfusion. Additional benefits are improved leg cosmetic appearance and enhanced cushioning effect for seating (28,29).

Brain–machine interfaces with neuroprosthetic limbs can help patients with long-term paralysis to recover the natural and intuitive command signals for hand placement, orientation, and reaching, allowing them to perform several of the required activities of daily living (8,18,42,51). Sensory afferentation, feedback input, and related cerebral voluntary motor commands—the latter by electroencephalography-brain–computer interface (EEG-BCI)—may thus contribute to wireless informational powering of the respective robotic suit engine for bionic standing and gait assistance. In this respect, the first conceptual and practical related insights/contributions of this are available (42).

Neurotization or Nerve Bridging

Neurotization or nerve bridging can restore some function for complete chronic SCI in patients, especially if associated with physical rehabilitation after transferring axons to a deinnervated target. Three forms of neurotization are currently being practiced in China (33,60,62,63) and Italy (4,55) and are successful in generating some functional recovery. The first involves taking a peripheral nerve from above the injury site, such as the accessory nerve or intercostal nerve, and bridging it to nerve roots or peripheral nerves for paralyzed muscles below the injury site (62,63). The second involves taking the ventral root from lumbar 5 or the sacral 1 segment above or below the injury site and connecting it to the ventral root of sacral 2 or 3 segments that normally innervate the bladder (6,33,60). The third involves taking a peripheral nerve and inserting the central stumps 4–5 mm into the ventral–lateral bundles of the thoracic cord (the corticospinal tract) just above the complete cord lesion and the distal stump of the grafts connecting to the muscle nerve of the lower limb (4,55).

Table 1. Clinical Literature on Cell Therapy for Complete Chronic SCI up to April 2013

Group	Country	Year	Case Numbers	Damaged Level	Injured Period	Cell Type	Transplant Route	Time Between Transplant and Initial Effect	Observation Time	Result	Follow-Up Period	Outcome
Huang et al. (22)	China	2003	171	C (114) T (52) T12–L1 (5)	6 months–18 years	Allogenic OEC	Cord parenchyma	–	2–8 weeks	Improved motor, sensation, and quality of life	–	–
Chernykh et al. (7)	Russia	2007	18	C4–6 (12) T1–9 (2) T10–12 (4)	>6 months (mean 34 months)	Autologous bone marrow stem cell	Into the cyst cavity and intravenously		2–4 months	No side effects	9.4 mo	Well tolerated, Barthel, and Ashworth scales improvement, increase in sensitivity and motor activity by ASIA score compared with historical control
Cristante et al. (9)	Brazil	2009	39		≥2 years	Autologous peripheral blood stem cell	Intra-artery				2.5 years	Somatosensitive evoked potentials recovery in twenty-six (66.7%)
Deda et al. (11)	Turkey	2008	9			Autologous BM-derived hematopoietic progenitor stem cell	Cord parenchyma, spinal cord covered with stem cell storage material (gel foam), subarachnoid space, intravenous		3 weeks		1 year	All patients had ASIA grade B or C

Geffner et al. (14)	Ecuador	2008	52			Autologous bone marrow stem cells	Multiple routes: directly into the spinal cord, directly into the spinal canal, and intravenous					Improved in ASIA, Barthel (quality of life), Frankel, and Ashworth scoring
Guest et al. (15)	US	2006	1	C3	18 months	Allogenic OEC	Cord parenchyma		11 days	Improved one ASIA motor grade Descent of sensory level		
Huang et al. (24)	China	2006	222	–	6 months–31 years (mean 3.1 years)	Allogenic OEC	Cord parenchyma	2–3 days	2–8 weeks	Improved motor, sensation and quality of life	–	–
Kang et al. (27)	S. Korea	2005	1			Allogenic multipotent stem cells from human UC blood	Open procedure, subarachnoid space, intradural and extradural space		41 days	Improved sensory perception and movement		
Huang et al. (25)	China	2012	108	C (51) T (42) T12–L1 (15)	0.5–30 (mean 3.48 ± 4.73) years	Allogenic OEC	Cord parenchyma	–	–	–	3.47 ± 1.12 yr	Improved motor, sensation, and quality of life
Kumar et al. (30)	India	2009	297			Autologous bone marrow-derived mononuclear cell	Lumbar puncture					Sensory and motor improvements in 32.6% of patients
Lima et al. (31)	Portugal	2010	20		18 to 189 (mean 49) months	Autologous olfactory mucosal autografts	Cord parenchyma		4 months	AISA grades improved in 11, 6 (A --> C), 3 (B --> C), and 2 (A --> B), and declined in 1 (B --> A). Improvements included new voluntary EMG responses (15 patients) and SSEPs (four patients)	–	–

(*continued*)

Table 1. (*continued*)

Group	Country	Year	Case Numbers	Damaged Level	Injured Period	Cell Type	Transplant Route	Time Between Transplant and Initial Effect	Observation Time	Result	Follow-Up Period	Outcome
Lima et al. (32)	Portugal	2006	7	C4–T6	6 months to 6.5 years	Autologous olfactory mucosa	Cord parenchyma			Two ASIA A patients became ASIA C. Every patient had improvement in ASIA motor scores.		
Moviglia et al. (39)	Argentina	2006	2	T8 C3–C5		Autologous BM mesenchymal stem cells (MSCs) were cocultured with the patient's autoimmune T (AT) cells to be trans-differentiated into NSCs	Forty-eight hours prior to NSC implant, patients received an IV infusion of 5×10(8) to 1×10(9) AT cells. NSCs were infused via a feeding artery of the lesion site.			Patient 1 received two AT-NSC treatments and neurorehabilitation for 6 months. His motor level corresponds to his first sacral metamere (S1) and his sensitive level to the fourth sacral metamere (S4). Patient 2 motor and sensitive levels reached her first and second thoracic metameres (T1–T2)		
Moviglia et al. (40)	Argentina	2009	8 (five with jeopardized brachial plexus and three without)			(1) Autologous bone marrow mononuclear cell (2) Effector T-cell (3) Autologous neural stem cell	(1) Artery infusion (2) IV infusion (3) Feeding artery infusion			No side effects. Five patients evolved from ASIA A to D and regained the ability to stand up and, with varying effectiveness, to walk; two patients remained in the same condition, but exhibited motor and sensitive improvements; and one patient could not be evaluated.		

Park et al. (44)	S. Korea	2012	10 A or B			Autologous BMSCs	MSCs (8×10^6) were directly injected into the spinal cord, and 4×10^7 cells were injected into the intradural space of 10 patients. After 4 and 8 weeks, an additional 5×10 MSCs were injected into each patient through lumbar tapping.		30–39 months	Three patients improved in the motor power of the upper extremities and in activities of daily living, significant magnetic resonance imaging and electrophysiological changes		
Rabinovich et al. (46)	Russia	2003	15	C (7) T (8)	1 month to 6 years	Allogenic hemopoietic tissues (2×10^8)+ Allogenic OEC (2×10^5)	Subarachnoid implant	–	–	–	1.5–3.0 years	Six cases improved from ASIA A to C Five cases (ASIA A to B) Remaining four cases no improvement
Seledtsova et al. (50)	Russia	2010	43	C (22) T (12) T11–L4 (9)	<1 year (3) >1 and <5 years (37) >5 years (3)	Fetal nervous and hemopoietic cells (9:1 ratio, $2–2.5\times10^8$)	Subarachnoid implant				>3 years	A decrease in neurological deficit with improvement of function in 48.9% cases
Wu et al. (59)	China	2012	11	Thoracic cervical	≥6 months	Autologous OEC	Cord parenchyma				14 months (range 1.0–1.5 years)	Sensation improved moderately, locomotion recovery was minimal

OEC, olfactory ensheathing cell.

Neurorehabilitation

A phenomenon called "learned nonuse" occurs after CNS injuries, and intensive, repetitive exercise can reverse atrophy of muscle and nervous tissues. Substantial recovery of function (two ASIA grades) is possible in a patient with severe C-2 ASIA Grade A injury by "activity-based recovery" (36). Multimodal intensive exercise can significantly improve motor function in subjects with chronic complete SCI, which might have therapeutic value for these patients as an adjunct to other restorative therapies (17). An individual with chronic SCI ASIA Grade A improved in overground walking ability following intensive physical therapy and robotic locomotor training (35). Yet these studies were all performed with small sample sizes, so more studies are necessary.

Combination Therapies

The degree of clinical neurorestoration by a single neurorestorative therapy is limited. Preliminary results of combination therapies for complete chronic SCI are promising for more functional neurorestoration, which include identical cell transplantation by two or more routes (14,39,40), two kinds of cells being transplanted in combination (40,46,50), cell therapy with neurorehabilitation (25), cell therapy with laser puncture, and neurorehabilitation (3). Therefore, combination therapy studies pose major challenges in terms of logistics and design in the future.

Mechanisms of Neurorestorative Therapies

The mechanisms of neurorestorative therapies for SCI include neuromodulation, neuroprotection, remyelination or neurorepair, neuroplasticity, axonal regeneration and sprouting, nerve bridging, controlling anti-inflammatory responses, neurogenesis, angiogenesis, and neuroreplacement (Fig. 2). Science is unraveling the mechanisms of neurorestoration; however, clinically, we can only provide supportive care for patients with SCI. Generally, the patient's functional neurorestoration originated from some or all of the complex mechanisms mentioned above. In fact, current neurorestoration or functional recovery most likely originates from neuromodulation or unmasking, neuroprotection, neuroplasticity, axonal sprouting and remyelination, nerve bridging and neural circuit reconstruction by neurotrophins, immune or inflammatory modulation, and local microenvironment change (20,41). Neurogenesis or axonal regeneration likely plays a smaller role in recovery (54,61). So the term neurorestoration is more accurate in describing functional recovery than neuroregeneration.

QUESTIONS

Study Design

Many would criticize many of the clinical trials for complete SCI for failing to be a randomized, double-blind control study. As we know, randomized, double-blind control studies are not suitable for some clinical trials or studies, for example, organ (heart, liver, and kidney) transplantation, because of ethical, lawful, and scientific considerations. On the contrary, self-comparison is likely a better assessment method for clinical studies of those diseases. This is why cardiology, liver, and urology studies are not often criticized for their failure to conduct randomized, double-blind control studies. In clinical practice, patients with complete chronic SCI are always informed that there is no way of restoring their motor function, much like patients with heart failure, liver failure, and kidney failure. Furthermore, patients with chronic complete SCI vary in structural damage, level of injury, and age (even those with clinical ASIA Grade A). Results of randomized, double-blind control studies may be influenced by such diversity. However, the results of self-comparison studies are only influenced by the intervention itself. Rama's clinical study used the self-comparison design (47). Results were encouraging and support the idea of applying this design to more clinical studies of intractable diseases, such as chronic complete SCI, heart failure, liver failure, and kidney failure.

Ethics

Some ethical issues have arisen with regards to potential treatments, such as methods being immature and their mechanisms of action being unclear. The Declaration of Helsinki offers a cogent response to such concerns: "In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures if in the physician's judgement it offers hope of saving a life, re-establishing health or alleviating suffering" (58). Obviously, one of the most important objectives for physicians is to try their best to help patients. The need of clinical neurorestoration for complete chronic SCI is known, though a cure has not yet been elucidated. Many promising therapeutic techniques have been, or are being, explored. To deliberate or question is simple; however, helping patients with complete chronic SCI to improve their neurological function and quality of life presents many challenges. The medical community should encourage any efforts to discover effective therapeutic strategies and should favor, not fear, the initial clinical endeavors and pioneering strategies for patients with complete chronic SCI according to the Beijing Declaration of the International Association of Neurorestoratology (IANR) (26). We therefore encourage that the integration and combination of current confirmed effective therapies for complete chronic SCI be carried out as soon as possible so that patients can be correctly informed, enabling them to make their own decisions on

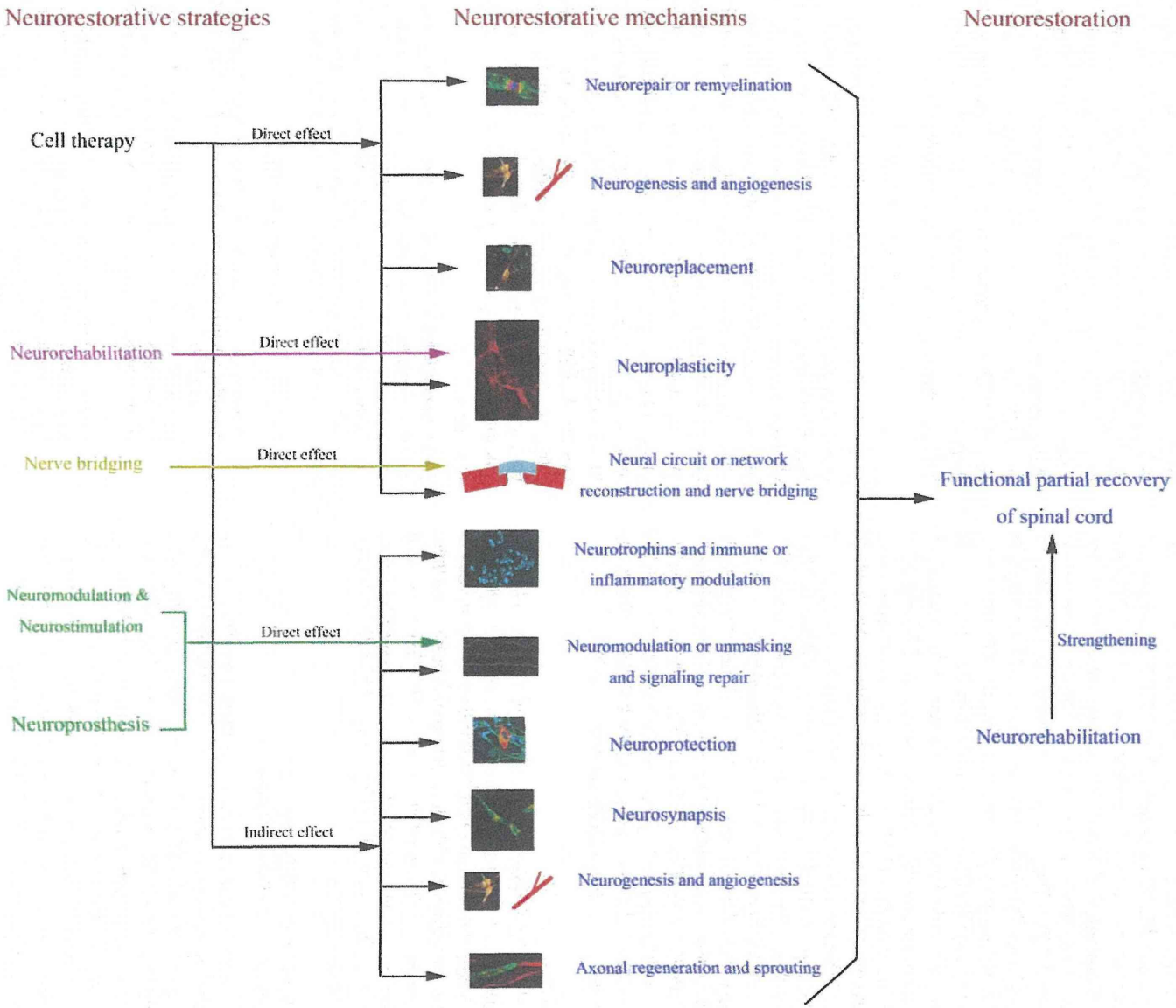

Figure 2. Neurorestorative strategies and mechanisms for chronic complete SCI. The first vertical column lists neurorestorative strategies; the second one lists neurorestorative mechanisms; the third one shows functional neurorestoration. One strategy may restore functions through some different mechanisms, and different strategies may share the same mechanisms.

receiving the maximal benefits from the achievements and advancement of neurorestoratology.

Reliability of Results

Huang et al. (20) have previously reviewed the fact that thousands of years ago (about 2500 BC), SCIs were described as "crushed vertebra," as well as symptoms of neurological deterioration without treatment in the ancient Egyptian medical papyrus known as the Edwin Smith Surgical Papyrus by the physician and architect of the Sakkara pyramids, Imhotep. Nearly 100 years ago, in 1926, Ramon y Cajal stated with certainty that there were not any ways for CNS regeneration to occur in adults. Until now, the medical mainstream community still persists that there are not any ways to restore neurological functions for patients with SCI (1,38,53). According to the assessment standards by the Center for Evidence-based Medicine at the University of Oxford, England (http://www.cebm.net/index.aspx?o=1025), a series of clinical studies or trials with over 6 months follow-up for complete chronic SCI by cell therapy (7,9,25,30,31), neurotization, or nerve bridge (63) showed strong evidence of neurorestoration with grades A-1c. Indeed, the current neurorestoration is only partial without full recovery, but evidence is reliable and is more than enough to answer "yes" or "no" for the question of whether clinical neurorestoration is able to be attained in patients with complete chronic SCI.

Safety

Many have expressed concerns about the safety of neurorestorative strategies as new methods for patients with complete chronic SCI, but fortunately, all studies or trials including cell therapy, neuromodulation and neuroprosthesis, neurotization, or nerve bridging were well tolerated and showed good results with regard to safety and feasibility (2,5,7,9,11,12,14,15,21,22,24,25,27,30–32,34,39,40,43–50,59).

Comprehension Difference From Medical Community and Patients

Current considerations in medical communities are if clinical neurorestoration is attainable for patients with complete chronic SCI. However, many patients with complete chronic SCI want to be cured. They often misunderstand academic language, such as that pertaining to function that may or may not cure diseases or damages. Currently, patients do not reach the whole clinical neurorestoration or total recovery that they expect, but most patients are satisfied with some degree of improving functions and quality of life (10,57). Thus, when patients intend to get any kind of neurorestorative therapies, they should know the methods, potential results, limitations, and risks, which should be clearly explained by physicians.

Implementing Translational Medicine

The key issue for implementing translational medicine is to do, not only to say. By comparing Huang et al. (20) and current publishing papers (2,3,7,11,12,15,21,22,24,25,27,31,32,34,43,45,48,49,59), we can see that only a few kinds of cells have so far been translated from the bench to the bedside, but we would expect more to follow as our understanding of the benefits and potential pitfalls of each cell type is identified. The time is right for doing more clinical trials as patients with a severe medical status are looking forward to garnering more positive results. With the current advances in clinical progresses in the field of neurorestoratology (23), more translational studies can really make patients benefit more.

CONCLUSION

Translating neurorestorative strategies with positive preclinical results for complete chronic SCI to the clinic has shown that patients are able to get some degree of clinical neurorestoration. These strategies include cell therapy, neurostimulation/neuromodulation, neuroprosthesis, neurotization or nerve bridging, neurorehabilitation, and combined therapies. Now it is time to change the traditional concept that there are not any effective therapeutic methods to restore neurological function for lesions of the CNS (including complete chronic SCI). From now on, complete chronic SCI patients may no longer be told that nothing can be done. In the future, more effective preclinical neurorestorative strategies should be encouraged to be translated to the clinic as soon as possible in order to benefit patients with incurable CNS disorders.

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Scientific Research

Depletion of Glial Cell Line-Derived Neurotrophic Factor by Disuse Muscle Atrophy Exacerbates the Degeneration of Alpha Motor Neurons in Caudal Regions Remote from the Spinal Cord Injury

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Abstract

We have been previously reported that disuse muscle atrophy exacerbates both motor neuron (MN) degeneration in caudal regions remote from a spinal cord injury, and decrease in glial cell line-derived neurotrophic factor (GDNF) protein level in paralyzed muscle. In this study we found that disuse muscle atrophy exacerbated the decrease in GDNF protein level in the L4/5 spinal cord, which was not immunopositive for GDNF. Our results were consistent with the fact that in the lumbar spinal cord of rats with mid-thoracic contusion, GDNF expression was not detected, while expression of GDNF receptors (GFRα1 and RET) was. Our study showed that administration of exogenous recombinant GDNF into the atrophic muscle partially rescued α-MN degeneration in the L4/5 spinal cord. These results suggest that the depletion of GDNF protein by muscle atrophy exacerbates α-MN degeneration in caudal regions remote from the injury.

Keywords

Disuse Muscle Atrophy, Motor Neuron, Degeneration, Glial Cell Line-Derived Neurotrophic Factor

1. Introduction

Neurons derive trophic support from the muscles, which are a source of trophic factors (TFs) [1]. Glial cell

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line-derived neurotrophic factor (GDNF) is crucial to the survival of spinal motor neurons (MNs) [2]. TFs derived from the target muscle (by retrograde transport), from afferent neurons (by anterograde transport), from neighboring glial cells, or even via autocrine production, support the survival of spinal MNs [3]-[5].

Although normal muscle is a source of TFs for spinal MNs, there is a considerable decrease in TF levels in atrophic muscles, in part due to elevated levels of ubiquitin ligases and decreased protein synthesis [6]-[8]. It has been reported that limb immobilization produces atrophic muscles [6] [9]-[11]. On the other hand, training increases TFs including GDNF in skeletal muscle [12] [13]. The effects of TFs derived from muscle on the degeneration of spinal MNs have not yet been fully clarified. We hypothesize that disuse muscle atrophy decreases the availability of muscle-derived TFs available for retrograde transport.

We have previously reported that disuse muscle atrophy within the context of spinal cord injury exacerbates not only MN degeneration in caudal regions remote from the injury but also the decrease in GDNF protein level in paralyzed muscle [11]. In this study, disuse muscle atrophy was associated with a significant decrease in GDNF protein levels in the L4/5 spinal cord. Administration of exogenous recombinant GDNF into the atrophic muscle partially rescued α-MN degeneration.

2. Materials and Methods

2.1. Animals

Six-week-old adult male Sprague-Dawley rats (n = 31; weight, 195 - 210 g; Japan SLC Inc., Hamamatsu, Japan) were randomly assigned to undergo laminectomy only (laminectomy, n = 4), spinal cord injury only (injury, n = 10), or spinal cord injury with hind limb immobilization (injury + immobilization, n = 17). Histological study was performed for laminectomy (n = 2), injury (n = 6) and injury + immobilization (n = 6) group respectively. ELISA study was performed for laminectomy (n = 2), injury (n = 4) and injury + immobilization (n = 4) group respectively. Injection study was performed for injury + immobilization group (PBS injection, n = 3; GDNF injection, n = 4). The laminectomy group was a control group in this study. The protocol was approved by the Animal Committee of Aino University, Japan. All procedures were performed in accordance with the Guidelines for Animal Experiments of Aino University and in compliance with the Japanese Regulations for Animal Welfare.

2.2. Interventions

Basic surgical procedures and postoperative care were as described previously [11] [14]-[16]. Laminectomy was performed at the mid-thoracic vertebrae 7/8. The spinal cord was crush-injured by dropping a 10-g metal rod from a height of 7.5 cm using a New York University (NYU) impactor [17]-[19].

After surgery, the right and left hind limbs were immobilized with aluminum plates on soft sponges, with the ankle and knee joints fully extended. The gastrocnemius and tibialis anterior muscles were immobilized in the contracted and extended positions, respectively. The quadriceps and hamstring muscles were immobilized, but rats could move their hind limbs at the hip joints.

Recombinant GDNF protein (R & D Systems, Inc., Minneapolis, MN) or control phosphate-buffered saline (PBS) injection was administered to the injury + immobilization rats. GDNF at a concentration of 200 μg/mL or PBS alone was injected into the gastrocnemius muscles of both legs, using a 26G needle, at a depth of 1 mm and for a duration of 3 minutes; this was done twice weekly for 3 weeks following the crush injury. The gastrocnemius muscle was removed and, prior to transcardial paraformaldehyde perfusion, the wet weight was measured (PBS group, n = 3; GDNF injection, n = 4).

2.3. Histological Evaluation

The spinal cords of the rats were cut into 2-mm lengths from the lesion site to the lumbar enlargement and were embedded in optimal cutting temperature (OCT) compound (laminectomy, n = 2; injury, n = 6; injury + immobilization, n = 6) (Tissue Tek, Sakura Finetechnical, Tokyo, Japan) at 3 weeks postintervention. The gastrocnemius muscle was removed prior to paraformaldehyde perfusion at 3 weeks postintervention. The muscles were then immediately frozen in acetone chilled with dry ice, and were embedded in OCT compound. Sections (thickness, 10 μm) were cut axially from the blocks by using a cryostat (CM1510S; Leica), and every fifth section was saved. A set of sections was mounted on 20 silane-coated glass slides (Matsunami, Tokyo, Japan) for

each animal. Sections were used for hematoxylin and eosin (HE) staining and immunohistochemical analysis. The primary antibodies were anti-neuronal nuclei (NeuN) mouse polyclonal (1:100; Chemicon International, Inc., CA), anti-glial fibrillary acidic protein mouse monoclonal (1:300; Sigma, Tokyo, Japan) for astrocytes, and anti-GDNF goat polyclonal (1:10; R & D, Minneapolis, USA). Secondary antibodies were cyanine 3 (Cy3)-labeled anti-mouse IgG (1:1000; GE Healthcare Bio-Sciences Corp., NJ) and anti-goat Alexa Fluor 488 IgG (1:1000; Molecular Probes, OR).

2.4. MN Counts

The diameter of α-MN in lumbar spinal cord distribute from 30 to 50 μm, and peak at 40 μm [20]. Sections (thickness, 10 μm) were cut axially from the blocks by using a cryostat; every 5th section was saved. A set of sections was mounted on 20 silane-coated glass slides for each animal. Ten slides from 1 animal in each group were stained with anti-NeuN antibody, and the sections were examined by fluorescence microscopy (ECLIPSE E600, Nikon, Tokyo, Japan; DP71, Olympus, Tokyo, Japan). We scanned each section at 100× magnification, and counted the identified motor neurons at 40-μm intervals to prevent from biased counting. Mean α-MN counts were calculated for each group using the counts from each animal. The number of large (diameter, ≥40 μm) cells containing a single nucleolus located in the ventral horn was recorded and measured on both sides of each section with the use of an image analysis software (ImageJ, version 1.44; http://rsbweb.nih.gov/ij/index.html) [21] [22].

2.5. Cavity Measurement

We examined the HE sections to measure cavity volume [14] [15]. Images were obtained with the aid of a digital microscope (BZ-8000, Keyence). High-resolution images were used to trace the cavity areas. Areas identified in individual sections were measured with the use of an image analysis software (ImageJ). The areas of the cavities were measured on 6 serial axial sections with a section-to-section interval of 2 mm. These six measurements were added and averaged to produce the relative quantification data for each animal.

2.6. Protein Isolation

Fifty micrograms of spinal cord at L4/5 from each animal (injury, n = 4; injury + immobilization, n = 4) was homogenized in 1000 μL of ice-cold lysis buffer (137 mM NaCl, 1% NP40, 20 mM Tris-HCl [pH, 8.0], and a protease inhibitor (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific Inc., Kanagawa, Japan) using a mechanical homogenizer. Spinal cord homogenates were centrifuged at 1500 × *g* for 15 min at 4˚C. The resulting supernatant was divided into several portions and frozen at −80˚C.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

For measurement of GDNF protein levels, spinal cord samples (laminectomy, n = 2; injury, n = 4; injury + immobilization, n = 4) were examined by ELISA with acid treatment procedure (GDNF kit, Novus Biologicals, CO). ELISA was performed twice for each sample.

2.8. Statistical Analyses

Analysis of variance was used to characterize group differences. In the case of a significant F ratio by a one-way analysis of variance, a Tukey's post-hoc test was performed. In all cases, the level of significance was set at $P < 0.05$ or $P < 0.01$. Data are presented as the mean ± standard deviation, unless otherwise indicated.

3. Results

3.1. Decrease in GDNF Level at L4/5 Spinal Cord Segment

We previously reported that the injury + immobilization group had lower GDNF levels in muscles compared to the injury group. In this study, intramyocellular GDNF proteins were significantly decreased in the injury and injury + immobilization groups at 3 weeks postintervention (**Figures 1(A)-(C)**).

GDNF content at the L4/5 spinal cord segment was measured using ELISA. The injury + immobilization

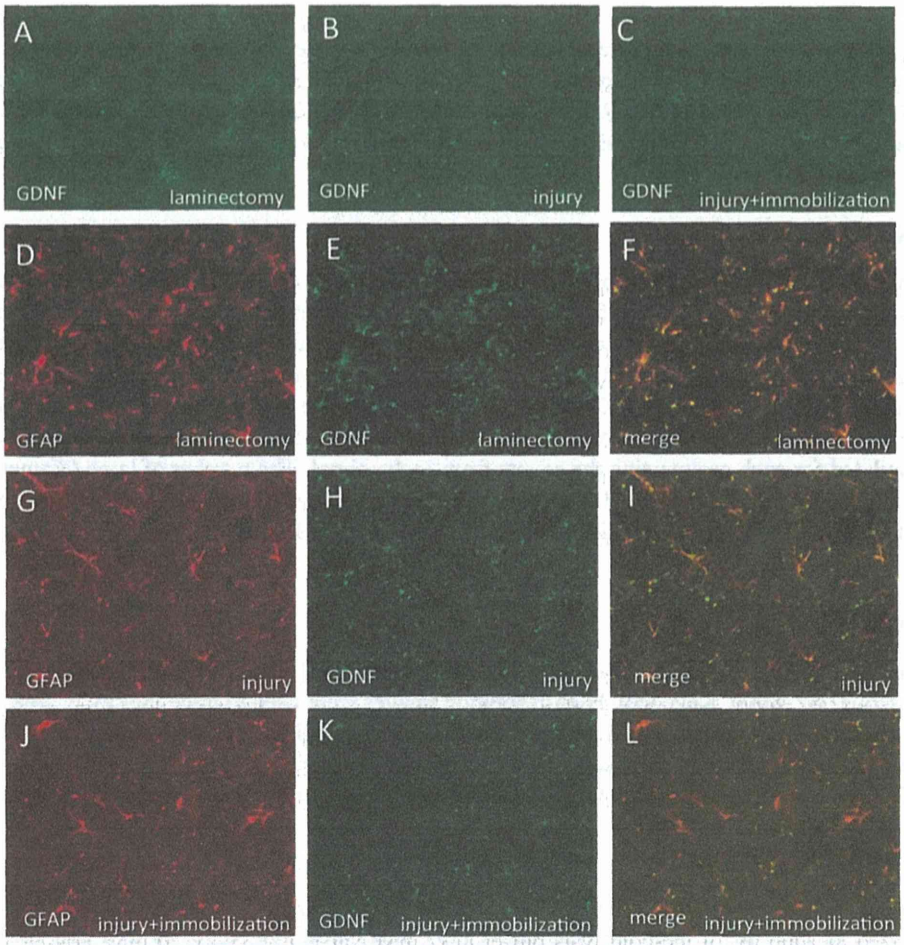

Figure 1. Immunohistochemical analysis for GDNF and GFAP proteins in the gastrocnemius muscle (A)-(C), and in the L4/5 segment of the spinal cord (D)-(L) at 3 weeks postintervention. (A) (D)-(F) laminectomy group, (B) (G)-(I) injury group, (C) (J)-(L) injury + immobilization group; GDNF (A)-(C) (E), (H), (K), GFAP (D), (G), (J), merge (F), (I), (L). Original magnification: 200×.

group exhibited a significant decrease in GDNF proteins at 3 weeks postintervention compared to the injury and laminectomy group (**Figure 2(A)**). L4/5 spinal cord segments were not immunopositive for GDNF in injury + immobilization group, but slightly immunopositive in injury group (**Figures 1(D)-(L)**) compared to the laminectomy group.

3.2. α-MN Rescue by GDNF

We administered the recombinant GDNF proteins into the gastrocnemius muscle of rats in the injury + immobilization group. We measured the wet weight of muscle as the degree of disuse muscle atrophy. The average of wet weight of gastrocnemius muscle in PBS group (n = 3) and GDNF injection group (n = 4) was 1.13 ± 0.35 and 1.16 ± 0.25 respectively. The wet weight of gastrocnemius muscle in GDNF injection group did not differ from that of the PBS group at 3 weeks postintervention. The number of L4/5 α-MNs was 3.0 ± 1.41 in the control PBS injection group and 6.25 ± 1.25 in the GDNF injection group (**Figure 3(A)**, **Figure 3(B)**). α-MN degeneration was partially rescued by exogenous GDNF proteins.

The mean volume of these cavities around the lesions did not differ significantly between the injury and injury + immobilization group (**Figure 2(B)**) or the PBS injection group and GDNF injection group at 3 weeks postintervention (**Figure 3(C)**, **Figure 3(D)**).