Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuroscience Letters 380 (2005) 284–288
Neuroscience Letters
www.elsevier.com/locate/neulet

Oxidized galectin-1 advances the functional recovery after peripheral nerve injury

Toshihiko Kadoya [a,*], Kiyomitsu Oyanagi [b], Emiko Kawakami [b], Mitsuhiro Hasegawa [c], Yoshimasa Inagaki [d], Yoshiaki Sohma [d], Hidenori Horie [e]

[a] *CMC R&D Laboratories, Pharmaceutical Division, Kirin Brewery Co. Ltd., Hagiwara, Takasaki, Gunma 370-0013, Japan*
[b] *Department of Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Musashidai, Fuchu, Tokyo 183-8526, Japan*
[c] *Department of Neurosurgery, Division of Neuroscience, Graduate School of Medical Science, Kanazawa University, Takara-machi, Kanazawa, Ishikawa 920-0934, Japan*
[d] *Pharmaceutical Research Laboratory, Pharmaceutical Division, Kirin Brewery Co. Ltd, Miyahara, Takasaki, Gunma 370-1295, Japan*
[e] *Advanced Research Center for Biological Science, Waseda University, Higashi-Fushimi, Nishi-Tokyo, Tokyo 202-0021, Japan*

Received 12 October 2004; received in revised form 28 December 2004; accepted 19 January 2005

Abstract

Oxidized galectin-1 has been shown to promote axonal regeneration from transected-nerve sites in an in vitro dorsal root ganglion (DRG) explant model as well as in in vivo peripheral nerve axotomy models. The present study provides evidence that oxidized galectin-1 advances the restoration of nerve function after peripheral nerve injury. The sciatic nerve of adult rats was transected and the distal nerve was frozen after being sutured into a proximal site with four epineurial stitches. An osmotic pump delivered oxidized galectin-1 peripherally to the surgical site. Functional recovery was assessed by measurement of the degree of toe spread of the hind paw for 3 months after the sciatic nerve lesion. The recovery curves of toe spread in the test group showed a statistically significant improvement of functional recovery after day 21 by the application of oxidized recombinant human galectin-1 (rhGAL-1/Ox) compared to the control group. This functional recovery was supported by histological analysis performed by light microscopic examination. The regenerating myelinated fibers at the site 21 mm distal to the nerve-transected site were quantitatively examined at 100 days after the operation. The frequency distribution of myelinated fiber diameters showed that exogenous rhGAL-1/Ox increased the number and diameter of regenerating myelinated fibers; the number of medium-sized (6–11 μm in diameter) fibers increased significantly ($P < 0.05$). These results indicate that oxidized galectin-1 promotes the restoration of nerve function after peripheral nerve injury. Thus, rhGAL-1/Ox may be a factor for functional restoration of injured peripheral nerves.

Keywords: Oxidized galectin-1; Axonal regeneration; Sciatic nerve injury; Remyelination; Myelinated fiber; Functional recovery; Toe spread

Initiation of neural restoration after axotomy has been thought to be regulated by neurotrophic factors [1,17], however, it remains unclear what signal prompts the axons to send out new processes in response to nerve injuries [1]. Recently, we discovered that oxidized galectin-1 promotes initial axonal growth after axotomy in peripheral nerves [6,7,10]. Galectin-1 is a member of a family of β-galactoside-binding lectins and is intensely expressed in dorsal root ganglion (DRG) neurons, spinal cord motoneurons and Schwann cells in normal sciatic nerves of adult rodents [3,6,9,16]. Galectin-1 contains six cysteine residues and exhibits lectin activity in its reduced form [11,15]. However, our structural–activity relationship study revealed that galectin-1 promotes axonal regeneration only in its oxidized form, which contains three intramolecular disulfide bonds [10]. Therefore, it is necessary to distinguish between

Abbreviations: rhGAL-1, recombinant human galectin-1; rhGAL-1/Ox, oxidized recombinant human galectin-1; PBS, phosphate-buffered saline; DRG, dorsal root ganglion

* Corresponding author. Tel.: +81 27 353 7381; fax: +81 27 353 7400.
E-mail address: tkadoya@kirin.co.jp (T. Kadoya).

0304-3940/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.01.054

oxidized galectin-1, which promotes axonal regeneration, and galectin-1, which shows lectin activity.

Using in vivo peripheral nerve regeneration models, we have demonstrated that the application of oxidized recombinant human galectin-1 (rhGAL-1/Ox) to the injured region promotes axonal growth [6]. Conversely, treatment with a functional blocking galectin-1 antibody strongly inhibits the restoration. These experiments show that oxidized galectin-1 is an essential factor for initiating axonal regeneration in injured peripheral nerves.

In the present study, we examined whether or not local administration of exogenous rhGAL-1/Ox advances the restoration of nerve function using a rat injured sciatic nerve model. The degree of toe spread [4] was measured for 3 months after the sciatic nerve lesion in order to assess functional recovery. Histological and quantitative studies [14] were also conducted after the functional assessment to evaluate the regeneration of myelinated fibers.

rhGAL-1/Ox was prepared as described previously [6,10]. Briefly, *Escherichia coli* expressed rhGAL-1 was purified by DEAE-HPLC and rhGAL-1 was oxidized by the air oxidation method using $CuSO_4$ as a catalyst. rhGAL-1/Ox was purified by reversed phase HPLC. A total of 36 adult male Sprague–Dawley rats (10 weeks) were used. The animals were randomly assigned to one of three groups of 12 animals each. In accordance with the guidelines of our institutional Animal Research Committee, we placed the animals two to a gage with a 12-h light:12-h dark cycle, and rat chow and water were available ad libitum.

In a previous study, we have shown that oxidized galectin-1 promotes axonal regeneration together as well as the Schwann cell migration into the acellular nerve or a grafted silicone tube filled with collagen gel [6]. In order to reproduce the appropriate acellular nerve conditions, we introduced a cut and freeze-killed sciatic nerve model. The operation was carried out according to the method previously described [13], with some modification. Briefly, the animals were anesthetized, to avoid unnecessary pain, with intraperitoneal sodium pentobarbital (60 mg/kg). The left sciatic nerve was exposed and transected at the mid-thigh level with microscissors. The distal stump was sutured into the proximal stump with four epineurial stitches of 8–0 Nylon, then 7 mm of distal nerve section was frozen for 10 s with forceps that had been chilled in liquid nitrogen. An osmotic pump (Alza Corp., 2 ml reservoir) was used to deliver test solutions peripherally, either rhGAL-1/Ox or PBS for control. The solutions were delivered at 2.5 μl/h from the polyethylene tube connected to the osmotic pump, which was implanted subcutaneously on the back, for a period of 4 weeks. Rats were divided into three groups of 12 rats each: a control group which was treated with phosphate buffered saline (PBS), and two test groups, one of which received 5 μg/ml of rhGAL-1/Ox solution applied to the surgical site, and one of which received 100 μg/ml of rhGAL-1/Ox solution. These concentrations of rhGAL-1/Ox solution were chosen because 5 μg/ml of rhGAL-1/Ox was found to be effective in a mouse model in our previous studies [3,6], and because a higher dose would be expected to advance functional recovery. No rats showed any toxic effects in reaction to the administration of rhGAL-1/Ox.

Functional recovery was evaluated by measuring the degree of toe spread [4], which is defined as the maximum distances between the first and fifth toes (toe spread) and between the second and fourth toes (intermediary toe spread) of the hind paw. Both toe spreads were measured at 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days postoperatively with calipers at the tips of the toes by holding the rat's back from behind and pushing the paw slightly to the floor. The distance of the toe spread were not dependent on the pushing strength and reproducibility of the data was very good; note that the pressure exerted on the hind paw remains constant regardless of pushing strength because the rat's pelvis absorbs any extra pressure beyond that which produces the toe spread. All measurements were carried out at least three times, and the values were averaged ($n = 12$ for each group).

After the functional assessment, 14 rats (four in the PBS group, four in the 100 μg/ml rhGAL-1/Ox group, two in the 5 μg/ml rhGAL-1/Ox group and four in an unoperated group) were perfused through the heart and fixed with 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate. The fixed sciatic nerve was dehydrated through a graded ethanol series and embedded in EPON 812. Cross sections (1 μm-thick) were cut 21 mm distal to the transection site, then stained with toluidine blue and examined under a light microscope. The quantitative analysis of regenerating myelinated axons was performed as described previously [14]. Briefly, photographs of three randomly chosen areas (16,870 μm2 each) of the cross sections were taken (×200 magnification). Enlarged prints (×2200 magnification) were made, and a digitizer was used to obtain the mean diameter of the myelinated fibers by averaging the longest and shortest diameters (the latter being perpendicular to the former). The data for the three areas were summed and converted to numerical values per total area of the cross section, and the frequency distribution of the myelinated axon diameters, in 1-μm increments, was determined. Statistical analysis of the experiments was performed using StatView for Macintosh (SAS Institute, Cary, NC). Significant differences between groups were determined by two-way ANOVA. Data are presented as the mean ± S.E.M. $P < 0.05$ between any two groups was considered significant according to the Bonferroni procedure.

Motor nerve conduction velocity (MNCV) was measured using 18 rats with the same operation mentioned above: 10 rats were received PBS and eight were received 100 μg/ml rhGAL-1/Ox. MNCV was measured on left sciatic nerve at day 84 after the operation using MEB-7102 instrument (Nihon Koden, Osaka, Japan). Measurements were performed under general anesthesia using halothane and the body temperature of the animals was kept constant at 37 °C. The comparisons between the groups for MNCV were performed using an unpaired t-test.

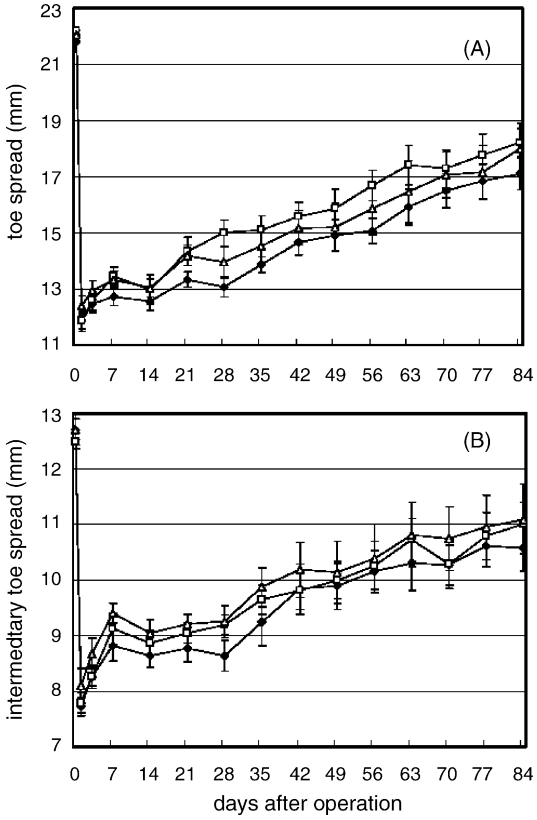

Fig. 1. Rates of functional recovery after sciatic nerve injury. Functional recovery was evaluated by the degree of toe spread (A) and intermediary toe spread (B) of the hind paw. (♦) PBS control group; (△) 100 μg/ml rhGAL-1/Ox group; (□) 5 μg/ml rhGAL-1/Ox group. Data are mean ± S.E.M. ($n = 12$).

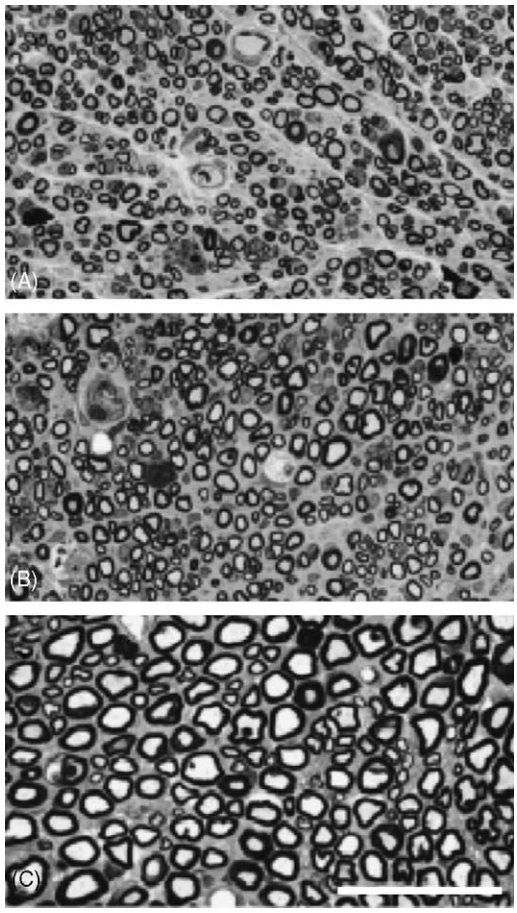

Fig. 2. Light micrographs of the sciatic nerves. At 100 days after operation, the cross sections at a site 21 mm distal to the nerve-transected site in the operated rat and the exact same position in the normal control rat were stained with toluidine blue. (A) PBS control; (B) rhGAL-1/Ox 100 μg/ml; and (C) unoperated control. Scale bar: 50 μm.

At 12 weeks after the operation, the nerve repair sites and pump delivery systems were intact and there was no difference between the PBS group and the two rhGAL-1/Ox groups. Recovery curves drawn from the toe spread and intermediary toe spread data are shown in Fig. 1. Statistical analysis of the recovery curves in the early period until postoperative day 14 revealed no significant differences among the three groups. After postoperative day 21, however, the recovery curves of intermediary toe spread showed a significant difference between the PBS group and the 5 and 100 μg/ml rhGAL-1/Ox groups (P value <0.05 by analysis of variance, ANOVA). The recovery curves of toe spread after postoperative day 21 also showed significant differences between the PBS group and the two rhGAL-1/Ox groups (P value <0.05 by ANOVA), with a significantly improved rate of recovery in injured nerves supplemented by the delivery of exogenous rhGAL-1/Ox.

Histological analysis was performed by light microscopic examination after the functional assessment. The regenerating axons at 100 days after operation were observed at a site 21 mm distal to the nerve-transected site (Fig. 2), and the numbers of regenerating myelinated fibers were quantitatively examined (Fig. 3). The frequency distribution of myelinated fiber diameters showed that the number of

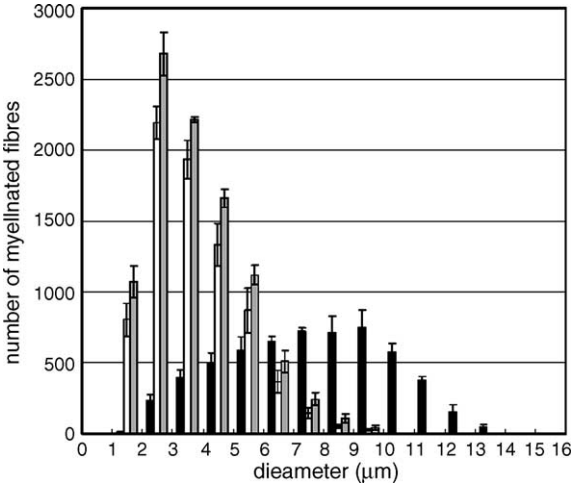

Fig. 3. Fiber-size distribution of the myelinated fibers in the cross sections at a site 21 mm distal to the nerve-transected site 100 days after operation. Unoperated group (closed bar), PBS control group (open bar), 100 μg/ml rhGAL-1/Ox group (striped bar). Data are means ± S.E.M. ($n = 4$).

Table 1
MNCV in the sciatic nerve at 84 days after the operation

PBS group ($n = 10$)		100 μg/ml rhGAL-1/Ox ($n = 8$)	
Animal number	MNCV (m/s)	Animal number	MNCV (m/s)
1	23.8	1	19.6
2	20.6	2	29.8
3	27.7	3	20.1
4	23.9	4	30.3
5	19.9	5	21.5
6	23.1	6	27.1
7	24.5	7	35.3
8	26.0	8	30.9
9	21.9		
10	23.6		

regenerating myelinated fibers was significantly higher in the 100 μg/ml rhGAL-1/Ox group than in the PBS control group (P values <0.05 by ANOVA), and the total number of regenerating myelinated fibers was approximately 6 and 26% higher in the 5 and 100 μg/ml groups, respectively, than in the PBS control group. The numbers of medium-sized myelinated fibers (6–11 μm in diameter) were especially increased in the rhGAL-1/Ox groups, averaging 983.4 ± 168.8 (100 μg/ml, $n = 4$) and 846.8 ± 136.2 (5 μg/ml, $n = 2$); on the other hand, in the PBS group, only 580.6 ± 128.4 ($n = 4$) was the average.

MNCV was measured to confirm the recovery promoting effect of rhGAL-1/Ox. Table 1 shows the results of MNCV measurement at 84 postoperative days. MNCV in PBS treated group was 23.5 ± 0.7 ($n = 10$), indicating that the operation was reproducible enough to perform the evaluation. Compared to the control group, the data in rhGAL-1/Ox treated group was divided into two groups; five of the 8 rats showing large MNCV (29.8, 30.3, 27.1, 35.3, and 30.9), and the other three rats showing small MNCV (19.6, 20.1, 21.5). The average of the small MNCV group (20.4 ± 0.6, $n = 3$), which was seemed to be similar to that of the control group, was clearly different from that of the large MNCV group (30.7 ± 1.3, $n = 5$). This division might be due to insufficient supply of the factor into the operated region. MNCV of the large MNCV group was specifically different from that of the control group (P value <0.005 by an unpaired t-test).

In our previous study [6], the acceleration of axonal regeneration by oxidized galectin-1 was shown by the application of rhGAL-1/Ox to in vivo injured peripheral nerve models. Recently, Fukaya et al. [3] investigated the effects of oxidized galectin-1 on the regeneration of rat spinal nerves using acellular autografts and allografts during the period of one to 2 weeks after the injury, with special attention to the relationship between axonal regeneration and Schwann cell migration. The administration of rhGAL-1/Ox was found to promote axonal regeneration from motoneurons as well as from DRG neurons; this was confirmed by a fluorogold tracer study [3]. Moreover, the migration of Schwann cells from both proximal and distal stumps was enhanced, and Schwann cell migration was found to precede axonal growth in the presence of exogenous rhGAL-1/Ox in the grafts. These results strongly suggest that oxidized galectin-1 is a key factor in the initial stages of axonal regeneration.

The present study provides evidence in favor of the idea that oxidized galectin-1 advances the restoration of nerve function after peripheral nerve injury. Functional recovery was evaluated by measuring the degree of toe spread because this examination is a simple and easily available method of evaluating the effects of given factors on functional recovery after sciatic nerve injury [2,4]. Fig. 1 shows that the rhGAL-1/Ox groups experienced functional recovery 1–2 weeks earlier than did the PBS group, which is consistent with data provided by studies on treatment with brain-derived neurotrophic factor (BDNF) or other trophic factors [5,12,13,18]. The functional recovery of both toe spread curves began to show a significant difference between the rhGAL-1/Ox groups and the PBS control group after postoperative day 21. Although further improvements were expected, the effect was limited to the initial advancement of recovery. This may be due to the method of application of rhGAL-1/Ox, which was supplied using a mini-osmotic pump. The operated site was quickly covered with connective tissues, preventing diffusion of rhGAL-1/Ox. A constant delivery system of rhGAL-1/Ox to the injured sites may make it possible to achieve further improvements in recovery.

The reconstruction of myelin in regenerating fibers is an important step for the restoration and functional recovery of injured nerves. Therefore, histological analysis was performed with special attention to the remyelination of the regenerating fibers after the functional assessment. Based on the fiber-size distribution of the myelinated fibers of unoperated axons (Fig. 3), it is clear that reconstruction of myelin in the operated groups is insufficient at 100 postoperative days. However, administration of rhGAL-1/Ox to the nerve injury site was found to increase both the number and the diameter of regenerating myelinated fibers, especially of medium-sized fibers. These histological and quantitative studies support the data obtained from our tests on functional recovery. The promotion of MNCV recovery by the treatment of rhGAL-1/Ox confirmed it.

The mechanism which controls how oxidized galectin-1 promotes peripheral nerve regeneration remains unclear. Recently, however, we have shown that macrophages are the target cells and that oxidized galectin-1 stimulates macrophages to secrete a factor that promotes axonal growth and Schwann cell migration [8]. This essential function of oxidized galectin-1 for peripheral nerve regeneration is thought to be specifically different from other known neurotrophic factors.

A factor that initiates the regeneration process is a candidate for enhancing nerve recovery, and oxidized galectin-1 seems to be one of the triggers of nerve regeneration [6–8,10]. The present study shows that administration of rhGAL-1/Ox to the sciatic nerve injury site improves functional recovery at a concentration (μg/ml order) at which no toxic phenomena were observed. Thus, rhGAL-1/Ox is potentially therapeutic for functional restoration after peripheral nerve injury.

Acknowledgements

This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports and Culture (#14580735 to KO) and the Japanese Ministry of Health, Labor and Welfare (#H16-kokoro-017 of the Research on Psychiatric and Neurological Diseases and Mental Health to KO).

References

[1] S.C. Apfel, Clinical Application of Neurotrophic Factors, Lippincott-Raven Publishers, 1997.
[2] J.R. Bain, S.E. Mackinnon, D.A. Hunter, Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat, Plast. Reconstr. Surg. 83 (1989) 129–136.
[3] K. Fukaya, M. Hasegawa, T. Mashitani, T. Kadoya, H. Horie, Y. Hayashi, H. Fujisawa, O. Tachibana, S. Kida, J. Yamashita, Oxidized galectin-1 stimulates the migration of Schwann cells from both proximal and distal stumps of transected nerves and promotes axonal regeneration after peripheral nerve injury, J. Neuropathol. Exp. Neurol. 62 (2003) 162–172.
[4] K. Hasegawa, A new method of measuring functional recovery after crushing the peripheral nerves in unaneathetized and unrestrained rats, Experientia 34 (1978) 272–273.
[5] P.-R. Ho, G.M. Coan, E.T. Cheng, C. Niell, D.M. Tarn, H. Zhou, D. Sierra, D.J. Terris, Repair with collagen tubules linked with brain-derived neurotrophic factor and ciliary neurotrophic factor in a rat sciatic nerve injury model, Arch. Otolaryngol. Head Neck Surg. 124 (1998) 761–766.
[6] H. Horie, Y. Inagaki, Y. Sohma, R. Nozawa, K. Okawa, M. Hasegawa, N. Muramatsu, H. Kawano, M. Horie, H. Koyama, I. Sakai, K. Takeshita, Y. Kowada, M. Takano, T. Kadoya, Galectin-1 regulates initial axonal growth in peripheral nerves after axotomy, J. Neurosci. 19 (1999) 9964–9974.
[7] H. Horie, T. Kadoya, Identification of oxidized galectin-1 as an initial repair regulatory factor after axotomy in peripheral nerve, Neurosci. Res. 38 (2000) 131–137.
[8] H. Horie, T. Kadoya, N. Hikawa, K. Sango, H. Inoue, Y. Inagaki, K. Takeshita, R. Asawa, T. Hiroi, M. Sato, T. Yoshioka, Y. Ishikawa, Oxidized galectin-1 stimulates macrophages to promote axonal regeneration in peripheral nerves after axotomy, J. Neurosci. 24 (2004) 1873–1880.
[9] M.A. Hynes, M. Gitt, S.H. Barondes, T.M. Jessell, L.B. Buck, Selective expression of an endogenous lactose-binding lectin gene in subsets of central and peripheral neurons, J. Neurosci. 10 (1990) 1004–1013.
[10] Y. Inagaki, Y. Sohma, H. Horie, R. Nozawa, T. Kadoya, Oxidized galectin-1 promotes axonal regeneration in peripheral nerves but does not possess lectin properties, Eur. J. Biochem. 267 (2000) 2955–2964.
[11] K. Kasai, J. Hirabayashi, Galectins: a family of animal lectins that decipher glycocodes, J. Biochem. 119 (1996) 1–8.
[12] S.L. Lewin, D.S. Utley, E.T. Cheng, A.N. Verity, D.J. Terris, Simultaneous treatment with BDNF and CNTF after peripheral nerve transection and repair enhances rate of functional recovery compared with BDNF alone, Laryngoscope 107 (1997) 992–999.
[13] J.P. Newman, A.N. Verity, S. Hawatmeh, W.E. Fee, D.J. Terris, Ciliary neurotrophic factor enhances peripheral nerve regeneration, Arch. Otolaryngol. Head Neck Surg. 122 (1996) 399–403.
[14] K. Oyanagi, E. Kawakami, T. Morita, H. Takahashi, Pursuit of the origin of the large myelinated fibers of the anterolateral funiculus in the spinal cord in humans in relation to the pathomechanism in amyotrophic lateral sclerosis, Acta Neuropathol. 98 (1999) 635–640.
[15] N.L. Perillo, M.E. Marcus, L.G. Baum, Galectins: versatile modulators of cell adhesion, cell proliferlation, and cell death, J. Mol. Med. 76 (1998) 402–412.
[16] L.J. Regan, J. Dodd, S.H. Barondes, T.M. Jessell, Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 2248–2252.
[17] G. Terenghi, Peripheral nerve regeneration and neurotrophic factors, J. Anat. 194 (1999) 1–14.
[18] D.S. Utley, S.L. Lewin, E.T. Cheng, A.N. Verity, D. Sierra, Brain-derived neurotrophic factor and collagen tubulization enhance functional recovery after peripheral nerve transection and repair, Arch. Otolaryngol. Head Neck Surg. 122 (1996) 407–413.