Neuroscience Research xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Immediate elimination of injured white matter tissue achieves a rapid axonal growth across the severed spinal cord in adult rats

Takeshi Nishio[a,*], Hiroshi Fujiwara[b], Isaku Kanno[c]

[a] Department of Integrative Brain Science, Kyoto University Graduate School of Medicine, Yoshida-Konoe, Sakyo, Kyoto 606-8501, Japan
[b] Department of Obstetrics and Gynecology, Kanazawa University Graduate School of Medical Sciences, Takara-Machi 13-1, Kanazawa 920-8641, Japan
[c] Department of Mechanical Engineering, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan

ARTICLE INFO

Article history:
Received 18 July 2017
Received in revised form 8 October 2017
Accepted 26 October 2017
Available online xxx

Keywords:
Regenerative pioneering axon
Regenerative follower axon
Axon-glial complex
Debridement
Fascicle formation
Axon segments (fragments)

ABSTRACT

In general, axonal regeneration is very limited after transection of adult rat spinal cord. We previously demonstrated that regenerative axons reached the lesion site within 6 h of sharp transection with a thin scalpel. However, they failed to grow across the lesion site, where injured axon fragments (axon-glial complex, AGC) were accumulated. Considering a possible role of these axon fragments as physicochemical barriers, we examined the effects of prompt elimination of the barriers on axonal growth beyond the lesion site. In this study, we made additional oblique section immediately after the primary transection and surgically eliminated the AGC (debridement). Under this treatment, regenerative axons successfully traversed the lesion site within 4 h of surgery. To exclude axonal sparing, we further inserted a pored sheet into the debrided lesion and observed the presence of fascicles of unmyelinated axons traversing the sheet through the pores by electron microscopy, indicating *bona fide* regeneration. These results suggest that the sequential trial of reduction and early elimination of the physicochemical barriers is one of the effective approaches to induce spontaneous and rapid regeneration beyond the lesion site.

1. Introduction

After a transection of the white matter in adult mammalian central nervous system (CNS), severed axons show a rapid retraction within 0.5–1 h of axotomy and then attempt regeneration in 6 h (Ramon y Cajal, 1928; Kerschensteiner et al., 2005; Nishio et al., 2008). However, axon regeneration is very limited in adult CNS because regenerative axons hardly grow across the lesion site (Silver and Miller, 2004; Busch and Silver, 2007; Schwab and Strittmatter, 2014).

Regarding the cause of limited regeneration, the presence of several types of axon growth inhibitory molecules have been shown in adult mammalian CNS, such as myelin-associated inhibitors (Schwab and Strittmatter, 2014) or glial scar-related extracellular matrix molecules (Silver and Miller, 2004; Busch and Silver, 2007; Sharma et al., 2012). However, the real cause remains elusive. The evidence that adult dorsal root ganglion neurons that were implanted into the spinal cord of adult rats can robustly regenerate their axons along the myelin-rich white matter tracts (Davies et al., 1999) may cast a doubt on the idea of myelin-inhibition *in vivo*. Furthermore, a glial scar takes several days to form after CNS injuries (Silver and Miller, 2004), while severed axons start regeneration within 6 h (Ramon y Cajal, 1928; Kerschensteiner et al., 2005; Nishio et al., 2008). If regenerative axons reach the lesion site before a scar formation, a structure other than a glial scar would be associated with the growth inhibition of such rapidly regenerating axons (regenerative pioneering axons).

We have previously reported a successful regeneration of the corticospinal tract after a sharp transection of the cord in young rats (Iseda et al., 2003; Iseda et al., 2004). In those animals, regenerative axons rapidly traversed the lesion site within 12–18 h of a sharp section and later a glial scar did not take place at the lesion site, while regeneration failed after a more traumatic injury and a glial scar was later formed. Therefore, the spatiotemporal correlation of these events suggests that a glial scar would follow, rather than cause, the failure of regeneration and that a local environment at the lesion site during the first several hours of injury would determine the fate of regenerative pioneering axons in young rats.

In cordotomized adult rats, we previously found an early access, within 6 h, of the regenerative pioneering axons to the lesion site,

Abbreviations: AGC, axon-glial complex; BSA, bovine serum albumin; CNS, central nervous system; DxRh, dextrans conjugated with tetramethylrhodamine; Dx488, dextrans conjugated with Alexa Fluor 488; IR, immunoreactivity; GM, gray matter; NFH, high-molecular-weight neurofilament subunit; GFAP, glial fibrillary acidic protein; PBS, phosphate buffered saline; WM, white matter; YG, yellow green.

* Corresponding author.
E-mail address: nishio.takeshi.5m@kyoto-u.ac.jp (T. Nishio).

https://doi.org/10.1016/j.neures.2017.10.011

where abnormal zipper-like axon segments were formed within a few hours of severance with a fine scalpel (Nishio et al., 2008). By extension of the view in young rats, we hypothesized that the axon segments would be a barrier for the regenerative pioneering axons in adult rats. In the present study, we further characterized the regenerative pioneering axons and the abnormal axon segments using axonal tracings, immunofluorescence, and electron microscopy. To verify this hypothesis, we developed a novel surgical treatment to eliminate the axon segments and examined whether a successful regeneration would occur under the barrier-eliminating surgery or not. Fortunately, we achieved a successful regeneration under the barrier-eliminating surgery.

However, in studies showing *in vivo* regeneration of CNS axons, the "spared axon problem", in which axons that survive a lesion are mistakenly identified as having regenerated, comes often to an issue (Steward et al., 2003). Therefore, we adopted several methods according to the criteria for identifying regenerated axons in injured spinal cord (Steward et al., 2003). To be specific, according to the criterion (II: the axon extends from the host CNS into a non-host graft or transplant), we inserted an artificial sheet or injected immobile microspheres into the lesion site to allow regeneration across the nonhost sheet or microspheres. According to the criteria (IV: the axon takes an unusual course through the tissue environment of the CNS, VI: the axon is tipped with a growth cone, VII: the axon has a morphology that is not characteristic of normal axons of its type), we characterized morphological features of regenerative pioneering axons, which could distinguish regenerative axons from normal ones.

2. Materials and methods

2.1. Animals

All procedures were in compliance with NIH guidelines and were approved by the Animal Care and Use Committee of Graduate School of Medicine, Kyoto University (No. 12036), and all efforts were made to minimize the number of animals used and their suffering. Adult female rats of Sprague-Dawley strain (60–70 days of age, N = 31) were used. Animals receiving a single transection of the cord were subjected to a quantitative analysis (N = 7, survival time was 2 and 4 h), to an immunofluorescence (N = 6, survival time was 2 and 6 h), and to an electron microscopy (N = 3, survival time was 2 h). Animals receiving cord sections with a local tissue removal (N = 6, survival time was 1 and 4 h), receiving cord sections with a local tissue removal plus a microsphere injection (N = 3, survival time was 4 h), or receiving cord sections with a local tissue removal plus a sheet insertion (N = 6, survival time was 24 h) were subjected to histologic processing described below.

2.2. Surgical procedures

After an intra-peritoneal anesthesia with sodium pentobarbital (25 mg/kg), laminectomies, pediculectomies and a dural incision were done to expose a dorsolateral surface of the lower thoracic cord (T8-12) under a surgical microscope (OPMI CS-NC, Contraves, Carl Zeiss Germany). The exposure of many segments of cord was required for a local axonal labeling as described below. The lateral funiculus was cut at T10 level on the left side with a disposable ophthalmic scalpel (microfeather P-715, Feather Safety Razor Co. Ltd, Osaka, JAPAN). The wound was gently closed in animals of a single transection.

We developed a novel surgical procedure to remove the abnormal axon fragments or axon-glial complex at the lesion site. Immediately after the primary section (lateral funiculotomy), an oblique section of the left lateral funiculus was added at the site 300–500 μm caudal to the primary lesion (cutting angle was about 30° to the primary section). Then, the intervening tissue between the 2 sections was removed. In addition, the surface of both stumps was carefully debrided with a pair of tweezers under a visual guidance (surgical debridement). The widely open cut stumps were closed by drawing a dura mater or nerve roots and held stable for 10 min to induce immediate tissue adhesion (see Supplementary Video online). To effectively perform the above surgery, we precoated the pial surface with a surgical adhesive [mixture of bovine serum albumin (BSA) and glutaraldehyde].

2.3. Fluorescent microspheres

Fluorescent microspheres (Fluoresbrite YG microsphere 3.0 μm #17155, Polysciences, Inc. 400 Valley Road, Warrington, PA) were coated with BSA by soaking them overnight in a 20% BSA solution. The BSA-coated microspheres (0.3 μl of 2.5% aqueous suspension) were manually injected into the tissue-removed space through a glass micropipette at a rate of 0.05 μl per a minute.

2.4. Epoxy-based sheets with pores

SU-8 photoresist coated on Al-coated glasses was exposed to UV light through a photo-mask of dot pattern of 125 μm in diameter. After curing them at 350 °C, 19.6 μm-thick SU-8 layer with 125 μm pores and 150 μm pitch was prepared on the glass substrates. Finally, the epoxy-based SU-8 sheets were peeled off from the glass substrates. The 19.6 μm-thick epoxy-based sheet ($575 \times 475\ \mu m^2$) was inserted into the tissue-removed space with a pair of tweezers under a visual guidance. The epoxy-based sheet was left at the lesion site until the histological processing, during which a frozen cord with the sheet was cut at 50-μm width by a freezing microtome for immunohistology, or a fixed cord with the sheet was cut at 70 nm width by an ultra-microtome for electron microscopy.

2.5. Local axonal tracing

Axons in the left lateral funiculus were labeled with an aldehyde-fixable fluorescent tracer (0.1 μl of 20% solution, dextrans conjugated with tetramethylrhodamine, DxRh, D-3308, or dextrans conjugated with Alexa Fluor 488, Dx488, D22910, Molecular Probes, Inc. Thermo Fisher Scientific, USA). Immediately after the surgery, rats were manually injected with the tracer solution into the lateral funiculus through a glass micropipette at a rate of 0.05 μl per a minute. In a preliminary study, a maximal distance of axonal labeling from an injection point was 4.5 mm at an hour, 5.6 mm at 2 h, 7.8 mm at 4 h and 9.6 mm at 6 h post-injection. Thus, the tracer was injected 4 mm rostral (or caudal) to the lesion site in animals who would survive 1 or 2 h of surgery, and 5 mm rostral (or caudal) to the lesion site in animals who would survive 4 h or more of surgery.

2.6. Histological processing and immunofluorescence

The immunohistological procedures were described elsewhere (Kawasaki et al., 2003; Nishio et al., 2005; Nishio et al., 2008). Briefly, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and were fixed by transcardiac purfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The spinal cords were removed and subjected to postfixation with the same fixative overnight at 4° C. They were cryoprotected with 20% sucrose in 0.1 M PBS at 4° C. Frozen thoracic cord was cut at 50-μm width in a horizontal plane or in a parasagittal plane by a freezing microtome. All serial 50-μm sections were preserved in 0.1 M PBS at 4° C in 96-well plate. For the antigen exposure,

Please cite this article in press as: Nishio, T., et al., Immediate elimination of injured white matter tissue achieves a rapid axonal growth across the severed spinal cord in adult rats. Neurosci. Res. (2017), https://doi.org/10.1016/j.neures.2017.10.011