Neuroscience 279 (2014) 23–32

MANNOSE-6-PHOSPHATE FACILITATES EARLY PERIPHERAL NERVE REGENERATION IN *THY-1-YFP-H* MICE

A. J. HARDING,[a] C. R. CHRISTMAS,[a] M. W. J. FERGUSON,[b†] A. R. LOESCHER,[a] P. P. ROBINSON[a] AND F. M. BOISSONADE[a*]

[a] *Unit of Oral and Maxillofacial Medicine and Surgery, School of Clinical Dentistry, University of Sheffield, Claremont Crescent, Sheffield S10 2TA, UK*

[b] *Renovo Group plc, Core Technology Facility, 48 Grafton Street, Manchester M13 9XX, UK*

Abstract—The formation of scar tissue following nerve injury has been shown to adversely affect nerve regeneration and evidence suggests that mannose-6-phosphate (M6P), a potential scar reducing agent that affects transforming growth factor (TGF)-β activation, may enhance nerve regeneration. In this study we utilized *thy-1-YFP-H* mice – a transgenic strain expressing yellow fluorescent protein (YFP) within a subset of axons – to enable visual analysis of axons regenerating through a nerve graft. Using this strain of mouse we have developed analysis techniques to visualize and quantify regeneration of individual axons across the injury site following the application of either M6P or vehicle to the site of nerve injury. No significant differences were found in the proportion of axons regenerating through the graft between M6P- and vehicle-treated grafts at any point along the graft length. Maximal sprouting occurred at 1.0 mm from the proximal graft ending in both groups. The maximum change in sprouting levels for both treatment groups occurred between the graft start and 0.5-mm interval for both treatment groups. The difference between repair groups was significant at this point with a greater increase seen in the vehicle group than the M6P group. The average length of axons regenerating across the initial graft entry was significantly shorter in M6P- than in vehicle-treated grafts, indicating that they encountered less impedance. Application of M6P appears to reduce the disruption of regenerating axons and may therefore facilitate quicker recovery; this is likely to result from altered scar tissue formation in M6P grafts in the early stages of recovery. This study also establishes the usefulness of our methods of analysis using the *thy-1-YFP-H* mouse strain to visualize and quantify regeneration at the level of the individual axon.

Key words: nerve regeneration, nerve repair, mannose-6-phosphate, *thy-1-YFP-H*, scarring.

INTRODUCTION

Peripheral nerve injuries are a common occurrence worldwide with over 300,000 cases annually in Europe alone (Ciardelli and Chiono, 2006). Most occur through domestic, workplace and traffic accidents but a significant number are caused by surgical procedures (Ciaramitaro et al., 2010). In one study Kretschmer et al. (2001) reported that 17.4% of traumatic nerve lesions treated over a 9-year period at one nerve-injury centre were iatrogenic in origin.

It has been well documented that peripheral nerves are able to undergo regeneration following injury; however functional recovery following surgery is unpredictable and is rarely complete (Robinson et al., 2000). The formation of intra-neural scarring following peripheral nerve injury has long been considered to adversely affect functional recovery in peripheral nerves, with Bora (1967) noting a negative correlation between the quantity of fibrous tissue at a nerve repair site and the overall level of functional recovery. More recently Atkins et al. (2006b) provided 'proof of concept' in a study that demonstrated that reducing intra-neural scarring improved nerve regeneration. In Atkins' study, regeneration of the sciatic nerve in wild-type (WT) mice was compared with that in two transgenic strains – one with an increased propensity for scarring (interleukin (IL)-4/IL-10 null mice) and the other with a decreased propensity for scarring (M6PR/IGF2 null mice) – with the results demonstrating that reducing the level of scarring at the injury site could improve nerve regeneration.

Alteration of the ratios of transforming growth factor (TGF)-β1, -β2 and -β3 at the site of injury has been shown to affect the level of scar formation in dermal wounds (Shah et al., 1994, 1995), with neutralisation of TGF-β1 and -β2 using antibodies, and exogenous addition of TGF-β3 reducing scarring in rat models. Neutralisation of TGF-β1 and -β2 at the site of a nerve injury has also been shown to significantly reduce the level of scarring (Atkins et al., 2006a). However the effect upon

*Corresponding author. Tel: +44-0-114-271-7964; fax: +44-0-114-271-7863.
E-mail addresses: a.harding@sheffield.ac.uk (A. J. Harding), c.christmas@sheffield.ac.uk (C. R. Christmas), mark.w.ferguson@manchester.ac.uk (M. W. J. Ferguson), a.loescher@sheffield.ac.uk (A. R. Loescher), p.robinson@sheffield.ac.uk (P. P. Robinson), f.boissonade@sheffield.ac.uk (F. M. Boissonade).
† Now at: Faculty of Life Sciences, University of Manchester, 3.239 Stopford Building, Oxford Road, Manchester M13 9PT, UK; and Science Foundation Ireland, Dublin, Ireland.

Abbreviations: ANOVA, analysis of variance; IL, interleukin; LAP, latency-associated peptide; LTBP, latent TGF-β-binding protein; M6P, mannose-6-phosphate; TGF, transforming growth factor; WT, wild-type; YFP, yellow fluorescent protein.

http://dx.doi.org/10.1016/j.neuroscience.2014.08.034

nerve regeneration is less clear, with Atkins et al. (2006a) reporting no improvement in regeneration and Davison et al. (1999), who neutralised only TGF-β1, reporting a significant improvement.

As TGF-β1 is linked to promoting the transition of Schwann cells into their more active, proliferating, non-myelinating phenotype following nerve injury (Einheber et al., 1995), the complete removal of TGF-β1 from the injury site may negatively affect some aspects of regeneration. An alternative approach to using TGF-β antibodies to reduce intra-neural scarring is the use of mannose-6-phosphate (M6P); which has been shown to reduce scarring in dermal wounds (McCallion and Ferguson, 1996). TGF-β is secreted from cells in a latent form bound to a latency-associated peptide (LAP) and latent TGF-β-binding protein (LTBP), and only becomes active once LAP and LTBP are removed; M6P occupies receptors used to trigger the removal of LAP and LTBP and as such reduces local TGF-β activation (McCallion and Ferguson, 1996).

A previous study carried out in our laboratory, looking at the effects of M6P on nerve regeneration, found that the application of M6P to a nerve repair did produce a significant improvement in nerve regeneration compared to controls at 6 weeks post repair (Ngeow et al., 2011b). However, this was not accompanied by a reduction in scarring, and at 12 weeks post repair there was no significant difference between M6P-treated and control repairs. The analytical methods used by the authors to assess nerve regeneration (walking gait analysis and electrophysiology) are well-established methods; however, they do have limitations in that they only indicate whether a particular treatment has improved regeneration and give no indication of why some axons fail to cross the repair site.

In this study we utilized *thy-1-YFP-H* mice – a transgenic strain expressing yellow fluorescent protein (YFP) within a subset of axons (Feng et al., 2000) – to enable visual analysis of axons regenerating through a nerve graft. This strain of mouse has been used in previous studies of peripheral nerve regeneration (English et al., 2005, 2007; Groves et al., 2005) as it allows the path of individual axons to be traced across the injury site. Using this strain of mouse we have developed new methods of analysis to visualize and quantify regeneration of individual axons following the application of either M6P or vehicle to the site of nerve injury.

EXPERIMENTAL PROCEDURES

Surgical procedure

Fifty-three mice aged between 9 and 12 weeks (at the time of the initial surgery) were used in this study: 33 *thy-1-YFP-H* (YFP+) mice and 20 C57B/6J (WT) mice. The WT mice used were littermates of the YFP+ mice. The experiments were carried out under appropriate UK Home Office approval, in accordance with the Animals (Scientific Procedures) Act 1986. The experimental model involved unilateral repair of the common fibular nerve with a nerve graft treated with either M6P (600 mM) or vehicle (phosphate-buffered saline). The experimental groups were: M6P-treated grafts (n = 10 YFP+ mice and n = 10 WT mice), vehicle-treated grafts (n = 10 YFP+ mice and 10 WT mice), and uninjured controls (n = 10 YFP+ mice). A further group (n = 3 YFP+ mice) was used to assess the level of residual fluorescence in the nerve distal to the injury site when graft repair was not carried out.

Under general anesthesia (2–3% isoflurane; Abbott Laboratories, Maidenhead, UK), the common fibular nerve of a WT mouse was exposed and freed from the surrounding tissue. A section of nerve (5 mm minimum length) was then removed and placed in a numbered vial containing either M6P or vehicle (randomized with the investigator blind to the contents). The nerve tissue remained immersed in the solution for 30 min. Following the removal of the nerve, WT animals were euthanized by cervical dislocation while under deep anesthesia.

During the period of immersion a YFP+ littermate was prepared to receive the graft. Under anesthesia (2–3% isoflurane), the right common fibular nerve was exposed and carefully freed from the surrounding tissue. A 5-mm silicone trough was then inserted beneath the nerve, and the nerve was sectioned. A gap of 2.5–3 mm was created between the proximal and distal ends by careful trimming and the treated WT graft (as described above) was trimmed to fit the gap. The graft and nerve ends were then aligned and the graft was secured in place with fibrin glue, consisting of fibrinogen (10 mg/ml; Sigma–Aldrich, Gillingham, Dorset, UK) and thrombin (40 units/ml; Sigma–Aldrich, UK) in a 1:1 ratio, and allowed to set for 5 min. Once the repair was secure the silicone trough was carefully removed and the wound closed. A single dose of analgesic was administered subcutaneously (0.01 ml buprenorphine hydrochloride 0.3 mg/ml; Vetergesic®, Alstoe Animal Health, Sheriff Hutton, UK) and the mice were then allowed to recover for 2 weeks.

Following the recovery period, mice were re-anaesthetized (fentanyl/fluanisone, 0.8 ml/kg [Hypnorm, Janssen Animal Health, High Wycombe, UK] and midazolam, 4 mg/kg [Hypnovel, Roche Products Ltd, Welwyn Garden City, UK]; ip) and the common fibular nerve exposed and freed from surrounding tissue. The skin was sutured to a brass ring to form a pool, which was filled with 4% paraformaldehyde for 30 min to fix the nerve. Following fixation the nerve was excised and mounted on a microscope slide using Vectashield®, and the mouse culled under deep anesthesia by cervical dislocation. The uninjured control nerves were also obtained using this procedure.

To assess residual fluorescence in the portion of the nerve distal to the injury, the common fibular nerve in three YFP+ mice was exposed and sectioned as described above. Following sectioning the proximal end was tied off with a silk suture to prevent regeneration of axons. Following a 2-week recovery period the proximal and distal portions of the injured nerves were fixed and excised as described above.

Image acquisition and processing

Images of nerves were acquired with fluorescent microscopy (Zeiss Axioplan 2 imaging microscope with QImaging Retiga 1300R camera) using the Optigrid Structured Illumination system and Image Pro-Plus