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Exogenous leukaemia inhibitory factor enhances nerve regeneration after late secondary repair using a bioartificial nerve conduit☆

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Summary The clinical outcome of peripheral nerve injuries remains disappointing, even in the ideal situation of a primary repair performed with optimal microsurgical techniques. Primary repair is appropriate for only about 85% of injuries, and outcome is worse following secondary nerve repair, partly owing to the reduced regenerative potential of chronically axotomised neurons. Leukaemia inhibitory factor (LIF) is a gp-130 neurocytokine that is thought to act as an 'injury factor', triggering the early-injury phenotype within neurons and potentially boosting their regenerative potential after secondary nerve repair. At 2–4 months after sciatic nerve axotomy in the rat, 1 cm gaps were repaired using either nerve isografts or poly-3-hydroxybutyrate conduits containing a calcium alginate and fibronectin hydrogel.

Regeneration was determined by quantitative immunohistochemistry 6 weeks after repair, and the effect of incorporating recombinant LIF (100 ng/ml) into the conduits was assessed. LIF increased the regeneration distance in repairs performed after both 2 months (69%, $P = 0.019$) and 4 months (123%, $P = 0.021$), and was statistically comparable to nerve graft. The total area of axonal immunostaining increased by 21% ($P > 0.05$) and 63% ($P > 0.05$), respectively. Percentage immunostaining area was not increased in the 2 months group, but increased by 93% in the repairs performed 4 months after axotomy. Exogenous LIF, therefore, has a potential role in promoting peripheral nerve regeneration after secondary repair, and can be effectively delivered within poly-3-hydroxybutyrate bioartificial conduits used for nerve repair.
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Peripheral nerve injuries are amongst the most common injuries encountered in trauma surgery, with an estimated annual incidence of 1/1000 population. Yet, despite major technical advances in surgical repair, outcome remains disappointingly poor,[1] particularly in the 11–16% of injuries unsuitable for primary neurorrhaphy.[2–4]

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Several factors explain why outcome is worst,[4-6] after late secondary nerve repair, many of which cannot be improved by further advances in microsurgical technique. The delay in repair prolongs the period of denervation atrophy in the target muscles and sensory organs, while endoneurial fibrosis[7,8] combines with Schwann cell atrophy and phenotypic changes[7,9,10] to impair the distal nerve stump's ability to support neurite ingrowth after repair.[10,11] Owing to nerve-stump retraction, the repair usually involves bridging a gap, yet fibres must still reach type-specific and topographically appropriate endoneurial tubes in order to have any chance of functionally useful regeneration.[1] Within the dorsal root ganglia, up to 40% of all primary sensory neurons will die,[13] massively reducing the pool of neurons available for regeneration, and leading to approximately a 70% loss of CNS endings.[14] Furthermore, both the axotomised motor neurons and the surviving sensory neurons begin to lose their early-injury phenotype, rendering them less able to regenerate after any subsequent repair.[5,15]

Although promising strategies are under development to address many of these problems, restoration of the early-injury, or regenerative, phenotype has not been described. Immediately after axotomy, induction of this neuronal phenotype may result from the action of retrogradely transported 'injury factors' that undergo receptor-mediated uptake and act synergistically with other neurotrophic factors. Such injury factors are expressed at low levels in normal peripheral nerves, but are rapidly upregulated after injury, thereby mimicking the time-course of the injury phenotype in axotomised neurons. Increasing evidence exists for the gp-130 neuroregulatory cytokines, principally leukaemia inhibitory factor (LIF), fulfilling this role after peripheral nerve injury.[16]

If the increased regenerative capacity of acutely injured neurons is due to injury factors, then local administration ought to enhance the regenerative capacity of chronically axotomised neurons. This study, therefore, sought to determine whether nerve regeneration after late secondary repair could be enhanced by exogenous LIF, delivered within a bioartificial nerve conduit used for gap repair.

Materials and methods

All work was conducted in keeping with the terms of the Animals (Scientific Procedures) Act 1986, and the experimental design recognised the need to optimise animal welfare.

Under inhalational anaesthesia (Halothane, May and Baker Ltd, UK), six groups of young adult male Sprague–Dawley rats ($n = 5$ in each group) underwent unilateral sciatic nerve division at the upper border of the quadratus femoris muscle in the mid-thigh. The nerve stumps were ligated with 6/0 prolene and placed within silicone rubber caps to prevent spontaneous regeneration. Either 2 or 4 months later, the animals were anaesthetised, and 1 cm gap repairs were performed using either freshly harvested 1 cm syngeneic reversed sciatic nerve isografts or bioartificial nerve conduits. The conduits were tubes 1.4 cm long made from compressed sheets of longitudinally orientated poly-3-hydroxybutyrate (PHB) fibres (Astra Tech, Mölndal, Sweden), filled with approximately 75 μl of a hydrogel comprising 2% ultra-pure low-molecular-weight high-mannuronic-acid-content calcium alginate (Pronova, Sweden) and 0.05% bovine fibronectin (Sigma Pharmaceuticals, UK). This gel served as a matrix substitute, and as a delivery vehicle for recombinant murine LIF (rhLIF 100 ng/ml, Autogen Bioclear, UK) in the two treatment groups. The nerve stumps were trimmed back to 'healthy' nerve and inset 2 mm into either end of the conduit using paired horizontal mattress sutures, to leave a 1 cm gap across which the nerve had to regenerate.

The repairs were harvested 6 weeks later in continuity with approximately 3 mm of both the proximal and distal nerves, fixed in Zamboni's solution at 4 °C and equilibrated in cryoprotectant solution (15% sucrose in phosphate buffered saline). Blocks were then prepared by rapid freezing into OCT™ mounting medium, from which a systematic random sample of longitudinal 15 μm cryosections was collected. These were stained by fluorescence immunohistochemistry to show nerve fibres by using primary antisera against pan-axonal marker of neurofilament (PAMNF) (monoclonal antibody against PAMNF; Affiniti Research Products Ltd), and a secondary horse anti-mouse antisera conjugated to Cy-3 (Amersham Pharmacia Biotech). The Schwann cell marker S-100 (polyclonal antibody, DAKO, Denmark) was also used, in combination with a goat anti-rabbit antisera conjugated to fluorescein isothyocyanate (FITC; Vector Laboratories Inc., USA). PAMNF staining, therefore, appeared as red fluorescence, and S-100 appeared as green. Nonspecific tissue binding of the secondary antibodies was limited by pre-incubation of the tissue sections with normal goat (Sigma-Aldrich UK) or normal horse (Sigma-Aldrich UK) sera, and nonspecific hydrophobic attachment was minimised by co-incubation with normal rat serum (DAKO, Denmark).

Two outcome measures were quantified. The 'regeneration distance' was measured from the end of the proximal nerve stump to the tip of the most distal nerve fibre in each repair, by examining all sections under a 10 × objective lens with a 0.1 mm graticulated eyepiece. Next, using a high-resolution fluid-cooled digital camera (Diagnostic Instruments, USA) and a 20 × objective lens, a band of images was captured across the full width of the two nonconsecutive sections that lay most central to the growth cone in each repair. The area of immunostaining within this band of images was then quantified by image analysis (Image-Pro-Plus™ Version 4.0 software), a process reliant upon intensity thresholding for unichromatic light, deselection of occasional areas of background staining and digital quantification of the area stained. The total area of immunostaining was calculated for each section, as was the total area of the nerve repair present in the captured images, in order to express immunostaining as a percentage of the area of the nerve repair examined, thereby facilitating comparison between conduits of different diameters. The 'total area of immunostaining' was expressed in μm^2, and the 'percentage area' was calculated directly. Ten measurements of the area of immunostaining (both absolute and percentage) were, therefore, recorded for each antiserum in each experimental group, and the mean value was used for statistical examination of the effect of treatment.

Statistical analysis of the results was performed by paired t-test, using SigmaStat™ (Version 2.0) software, after confirming the normality of the data distribution by the Kolmogorov-Smirnov test.

Results

The nerve conduits exhibited minimal fibrosis and good neovascularisation along their full length 6 weeks after implantation; the nerve grafts were equally healthy in appearance. At the microscopic level, there was a qualitative improvement in nerve-fibre morphology within the conduits that contained rhLIF, as compared to the plain conduits.

In the nerves repaired 2 months after axotomy, fibres were found to have regenerated into the distal nerve stump when nerve grafts were used (Fig. 1), but they had proceeded a significantly shorter distance through the plain PHB conduits ($P < 0.001$). The addition of rhLIF resulted in a 69% increase in regeneration distance ($P = 0.019$), which was comparable to that found within nerve grafts (Fig. 1). A similar pattern was found when the

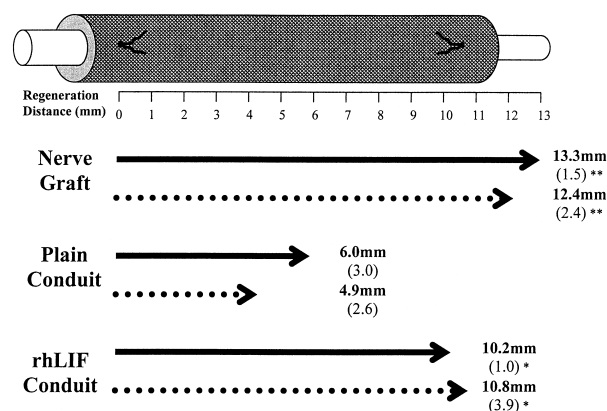

Fig. 1 Nerve regeneration distances 6 weeks after sciatic nerve repair. Sciatic nerve repairs were performed either 2 months (solid arrows) or 4 months (dashed arrows) after nerve transection. Regeneration distance was quantified by measuring the distance (mm) from the end of the proximal nerve stump to the tip of the most distal nerve fibre in longitudinal sections stained by fluorescence immunohistochemistry with primary antiserum to PAMNF. Figures given are the means (s.d.) for each experimental group ($n = 5$); $^{*}P < 0.05$; $^{**}P \leq 0.001$ (compared to repair using plain conduits).

area of immunostaining for PAMNF was examined (Table 1). Nerve-graft repairs contained the highest total area of staining, and although the area within plain PHB conduits was 56% less than this the addition of rhLIF was associated with a 21% increase. The highest percentage area of immunostaining for PAMNF was also found within the nerve grafts, reflecting their comparatively small diameter, while plain PHB conduits showed an 87% lower percentage area ($P < 0.001$). Conduits containing rhLIF had a similar percentage area of staining to plain PHB conduits.

When nerve repair was delayed until 4 months after axotomy, fibres still reached the distal nerve stump when nerve graft was used, but the regeneration distance within the plain PHB conduits was even worse (Fig. 1). Despite this, the effect of rhLIF was more pronounced than after a 2 month delay in nerve repair and resulted in a 123% increase ($P = 0.021$) in regeneration distance, to a figure that was not significantly different ($P > 0.05$) from that achieved with nerve graft. Similarly, the total area of immunostaining for PAMNF within nerve grafts was 71% greater (Table 1) than in plain PHB conduits, although the addition of rhLIF resulted in a 63% increase. Again, the percentage area of immunostaining for PAMNF was greatest when nerve graft was used, the figure for plain PHB conduits being considerably lower. The percentage area of staining for PAMNF increased by 93% with

Table 1 Areas of immunostaining against PAMNF within sciatic nerve repairs. Sciatic nerve repairs were performed either 2 months or 4 months after nerve transection

Type of repair	Repair after 2 month delay		Repair after 4 month delay	
	Total area of PAMNF staining	% Area of PAMNF staining	Total area of PAMNF staining	% Area of PAMNF staining
Nerve graft	58 259 ± 25 405	7.3 ± 2.3**	56 890 ± 24 546*	7.1 ± 1.9**
Plain conduit	25 471 ± 22 634	0.93 ± 0.81	16 488 ± 12 957	0.52 ± 0.36
rhLIF Conduit	30 744 ± 13 047	0.82 ± 0.49	26 802 ± 21 594	1.0 ± 0.78

The area of immunostaining against PAMNF was quantified 6 weeks after repair by digital image analysis of a band of images across the full width of the repair at a position 1 mm distal to the end of the proximal nerve stump. This was expressed as the mean ± s.d. for either the total area of immunostaining (μm^2) for each experimental group or the percentage of the entire area of the band of images that was captured ('percentage area of staining'). * $P < 0.05$, ** $P \leq 0.001$, compared to repair using plain conduits.

the addition of rhLIF to the conduits when repair was delayed for 4 months rather than 2 months.

Quantification of immunostaining for S-100 revealed that nerve-graft repairs contained a greater total area than plain PHB conduits (Table 2), which had 75 and 68% lower values in the groups repaired 2 months and 4 months after axotomy, respectively. The percentage area of immunostaining was 93% lower in the plain conduits than in the equivalent nerve-graft repairs in both these groups. In the group, where the sciatic nerve was repaired after a 2 month delay the area of S-100 staining in the plain PHB conduits was not increased by the addition of rhLIF. However, in the group repaired after a 4 month delay the rhLIF conduits contained somewhat more staining than the plain conduits (18% increase in percentage area of immunostaining).

Discussion

Further major advances in the treatment of peripheral nerve injuries, are more likely to result from modulating the response of neurons and their supporting cells to injury and regeneration than from further enhancement of microsurgical technique. This approach may involve novel adjuvant therapies to prevent neuronal death,[17] techniques such as short gap repair to facilitate the selection of appropriate endoneurial tubes by regenerating neurites[18] and modulation of the normal regenerative responses of both neurons and their supporting cells.[19] In experimental models of primary nerve repair, promising results have been found with the use of exogenous growth factors,[19] and bioengineered conduits, some of which incorporate cultured Schwann cells, are under development to replace autologous nerve grafts.[20,21] Although a significant minority of nerve injuries are not suitable for primary neurorrhaphy,[4] and their outcome is compromised accordingly,[3] the specific therapeutic problems inherent in secondary repair have rarely been clearly addressed experimentally.

Endoneurial fibrosis causes a mechanical impedance to neurite ingrowth within chronically denervated distal nerve stumps and begins within 28–35 days of nerve transection.[8] It is significant by 3–6 months after injury;[7,22] endoneurial tube diameter is reduced to 26% of control one year after transection and to only 1% of control after 2 years,[8,22] and yet there are no therapies available to prevent it. Further, functional inhibition of regeneration results from the profound atrophy and loss of neuregulin responsiveness that occurs within the distal-stump Schwann cell population,[9,10]

Table 2 Area of immunostaining against S-100 within sciatic nerve repairs. Sciatic nerve repairs were performed either 2 months or 4 months after nerve transection

Type of repair	Repair after 2 month delay		Repair after 4 month delay	
	Total area of S-100 staining	% Area of S-100 staining	Total area of S-100 staining	% Area of S-100 staining
Nerve graft	112 295 ± 29 902*	14 ± 2.1**	72 955 ± 21 562*	9.7 ± 3.7**
Plain conduit	28 290 ± 26 305	1.1 ± 1.1	23 593 ± 14 755	0.75 ± 0.39
rhLIF Conduit	24 586 ± 7084	0.64 ± 0.30	23 745 ± 15 873	0.89 ± 0.55

The area of immunostaining against S-100 was quantified 6 weeks after repair by digital image analysis of a band of images across the full width of the repair at a position 1 mm distal to the end of the proximal nerve stump. This was expressed as the mean ± s.d. for either the total area of immunostaining (μm^2) for each experimental group or the percentage of the entire area of the band of images that was captured ('percentage area of staining'). * $P < 0.05$, ** $P \leq 0.001$, compared to repair using plain conduits.

although the use of cultured autologous Schwann cells and exogenous glial growth factor holds promise in addressing these changes.[23]

However, irrespective of the state of the distal nerve stump, there is evidence to suggest that chronically axotomised neurons display decreased regenerative capacity. Although, early studies suggested only a minor loss of regenerative capacity,[24] this has been questioned more recently in studies where the distal-stump effect was obviated by suturing onto fresh distal stumps or by using conduits. The capacity of motor neurons to regenerate into fresh distal stumps decreases with prolonged axotomy,[25] and expression by sensory neurons of the regenerative neuropeptide GAP-43 is lost 2 months after axotomy.[10] Furthermore, the complete loss of regenerative capability through denatured muscle grafts after more than 56 days delay has been demonstrated, with any delay impairing myelination.[5] Thus, in the rat, it is evident that regenerative capacity diminishes with delays of greater than around 60 days. Hence, in this study nerve repair was delayed for a minimum of 2 months, and, as expected, the regeneration distance and the area of staining within plain PHB conduits and nerve grafts were reduced when, there was a 4 month delay prior to repair, as compared with a 2 months delay. A 1 cm gap repair was used, since the purpose of this study was not to examine the effect of rhLIF upon the length of gap across which a nerve can regenerate, but to examine its effects upon regeneration across a standard bridgeable distance with the extra biological hurdle of a significant delay prior to nerve repair.

The reduction in regenerative potential may be due to loss of the early-injury, or regenerative, phenotype, which is characterised by chromatolysis and upregulation of markers such as CGRP, cytoskeletal proteins, GAP-43 and galanin.[15] Such phenotypic changes may result from the action of a range of retrogradely transported 'injury' factors, such as LIF.

LIF is a neurotrophic gp-130 neurocytokine[15,16,26] that is expressed at extremely low levels in normal peripheral nerve and muscle, but which undergoes rapid upregulation and increased specific receptor-mediated retrograde transport after injury.[16,27–29] Although very few intact adult motor neurones and only around 24% of uninjured primary sensory neurons (predominantly small nociceptive neurons) exhibit the LIF receptor, peripheral nerve injury induces marked upregulation by a majority of neurons,[16,30] rendering them sensitive to LIF. Exogenous LIF stimulates phenotypic changes akin to axotomy in intact primary sensory neurons[26,31–33] and, after axotomy, is neuroprotective for both sensory and motor neurons when applied to the proximal stump in neonates.[34,35] It meets the criteria for an injury factor: its time-course of expression is intimately related to injury, it acts by specific receptor-mediated uptake and retrograde transport, and it has trophic and tropic actions.[16,28] Furthermore, LIF enhances neurite outgrowth in vitro, and pre-treatment with antibodies against the gp-130 neurocytokine receptor moiety inhibits the response of sensory neurons to axotomy.[26]

As a therapeutic agent, LIF also has the potential benefit over traditional neurotrophic factors, such as nerve growth factor, that it acts upon both motor and sensory neurons after injury, and is synergistic with other neurotrophic factors, such as nerve growth factor.[16] In primary nerve repair, LIF resulted in significant increases in nerve regeneration through a silicone tube and in subsequent motor function.[36,37] LIF is also myotrophic and enhances motor reinnervation by axon sprouts.[16,29]

Administration of rhLIF within a nerve conduit has several advantages over the systemic route. Although LIF acts on the neuronal cell body, receptor-mediated uptake and retrograde transport ensure that rhLIF administered at the site of injury will reach the cell body in a physiological manner. The tropic role of LIF is used to advantage by delivery at the repair site, and systemic complications can be avoided because lower doses can be used.

Furthermore, nerve conduits are useful in themselves, given the potential donor-site morbidity associated with using an autologous nerve graft, and are showing promise in clinical trials.[38] This may reflect the greater freedom that regenerating neurites have in determining which endoneurial tubes they enter, thereby potentially improving type or topographical specificity. PHB conduits have been shown to be effective in supporting nerve regeneration over gaps of 1–4 cm,[20,39] whilst calcium alginate has been shown to give sustained release of bioavailable rhLIF for several months in vivo.[40] A similar concentration of rhLIF was, therefore, employed in this study, and fibronectin was added since it enhances neurite outgrowth and has been found to be advantageous when combined with rhLIF.[37,41]

It is clear that, in common with other nonbioactive synthetic conduits, the plain PHB conduits did not adequately support peripheral nerve regeneration when compared with the clinical gold standard of a nerve graft. This presumably reflects the lack of a Schwann cell element to the conduit, in contrast to the endogenous population within a nerve graft, and this difference is heightened in

delayed repairs, given the deleterious changes occurring within the recipient nerve's Schwann cell population. This was confirmed by the significantly lower areas of staining for S-100 found within the conduits compared with the nerve grafts.

Despite this, the addition of rhLIF resulted in a significant improvement in the regenerative profile of the conduits. Although the area of staining for nerve fibres increased only modestly after the addition of rhLIF, regeneration distance increased significantly and was not significantly worse than that obtained with nerve graft. The regeneration distance, particularly when combined with the observed morphological improvement, reflects not merely the extension of multiple neurites but the stimulation of directed axonal growth into the distal nerve stump. It has been postulated that this is a more sensitive measure of functional regeneration than the area of staining, which may be increased by the extension of multiple random neurites rather than functional axonal elongation.[36] In keeping with this, rhLIF has been found to improve axonal morphometrics and function after primary repair, but with reduced axonal counts.[36,37] The effect of rhLIF upon regeneration distance and area of staining for nerve fibres was relatively greater when the nerve repair was delayed by 4 months (distance increased by 123%, total area increased by 63% and percentage area increased by 93%) than by 2 months (distance increased by 69%, total area increased by 21% and percentage area not increased). This is in keeping with the concepts that the regenerative capacity of the neurons deteriorates as the period of axotomy increases and that rhLIF tends to restore that regenerative potential by triggering the early-injury phenotype.

Although conduits containing rhLIF did not support regeneration quite as well as nerve grafts, it is possible that using a higher concentration of rhLIF might lead to a greater effect upon regeneration, and other studies using saline as a delivery vehicle have employed concentrations in the order of 0.5-0.9 $\mu g/\mu l$, to a total dose of approximately 10 μg.[34,36,37] It is also possible that regeneration could be further enhanced by varying the chemical or physical characteristics of the alginate. However, rhLIF need not be the sole additive in a bioartificial conduit, and further changes in conduit design may be determined by the need to incorporate features that enhance Schwann cell function.

This study, therefore, demonstrates that PHB conduits filled with a calcium alginate and fibronectin hydrogel matrix are an effective delivery system for rhLIF, which appears to have more effect on regeneration distance than on the area of staining for nerve fibres. LIF becomes relatively more effective as the delay between injury and repair increases, and it is evident that rhLIF has considerable potential as one component in the development of a bioartificial nerve conduit to replace autologous nerve grafts for late secondary nerve repairs.

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