neuroReport

NeuroReport 5, 989–992 (1994)

Leukaemia inhibitory factor rescues motoneurones from axotomy-induced cell death

S. S. Cheema,[CA] L. J. Richards, M. Murphy and P. F. Bartlett

The Walter and Eliza Hall Institute of Medical Research, and The Co-operative Research Centre for Cellular Growth Factors, Royal Parade, Parkville, Victoria, Australia 3052

[CA] Corresponding Author

THE death of spinal motoneurones after axotomy provides a useful model for studying novel factors which prevent motoneurone loss *in vivo*. Peripheral nerves of newborn rats were unilaterally transected and treated with either a vehicle solution or leukaemia inhibitory factor (LIF). Compared with the vehicle controls, treatment with a gelfoam containing LIF significantly reduced motoneurone loss: from 38% to 22% after 3 days and from 55% to 38% after 7 days. The loss of motoneurones was further reduced by placing the LIF-containing gelfoam inside a silicone chamber: from 39% to 15% after 7 days, which represented a 62% rescue. Thus, LIF is a potential therapeutic agent for preventing the loss of injured or diseased motoneurones.

Key words: Leukaemia inhibitory factor; Motoneurone rescue; Spinal cord; Motoneurone disease; LIF

Introduction

The cell death which many neurones undergo during embryonic development appears to be attributable to the limiting amounts of neurotrophic factors secreted by their target tissues.[1] It now appears that injured neurones in the postnatal nervous system also undergo a similar apoptotic-like cell death,[2] and can also be rescued by various neurotrophic factors. While there is an expanding list of growth factors which promote the survival of different neuronal phenotypes *in vitro*, relatively few factors have been shown to prevent the loss of axotomized motoneurones *in vivo*. These include brain-derived neurotrophic factor (BDNF)[3] and ciliary neurotrophic factor (CNTF).[4] Recently, we showed that leukaemia inhibitory factor (LIF) supports the *in vivo* survival of sensory neurones in the dorsal root ganglia after a sciatic nerve lesion.[5] LIF's ability to rescue injured sensory neurones *in vivo* was not unexpected as previous studies had shown that LIF supported the survival of sensory neurones *in vitro* and sensory neurones express receptors for LIF[6,7] and that LIF is retrogradely transported by sensory axons *in vivo*.[8] Since LIF has also been shown to enhance the survival of cultured spinal cord cells,[9] including motoneurones,[10] and is found in target tissues innervated by motoneurones,[7,11] we have examined the role of LIF on the survival of axotomized motoneurones in the cervical spinal cord. In this paper, we show that LIF rescues up to 62% of the motoneurones which are normally lost after axotomy when placed at the site of nerve injury.

Materials and Methods

An *in vivo* model of axotomy-induced death of motoneurones has been employed in these studies.[12] The model involves the transection of the median and ulnar nerves in newborn rodents which causes a loss of approximately 50% of injured motoneurones in segment C8 of the spinal cord within 1 week of axotomy. In the present experiments, Wistar rat pups of both sexes were operated on within 24 h of birth under ice-induced anaesthesia. The nerves were exposed at the level of the clavicle, transected and treated with phosphate buffered saline (PBS) or LIF (details below). Upon recovery, the lesioned animals displayed an obvious dorsi-flexion of the right forepaw. After 3 or 7 days, the pups were transcardially perfused with 2% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.3, a laminectomy was performed, spinal cord segments containing C8 were removed and postfixed in the same fixative overnight. The cord segments were dehydrated, embedded in paraffin wax, serial 8 μm thick sections were cut and stained with 0.1% cresyl violet. Motoneurones displaying a prominent nucleolus were counted using an eye-piece graticule at a magnification of 400 ×. Counts were performed on every 10th section. The neuronal loss was expressed as a percentage of the unlesioned contralateral side. Appropriate steps were undertaken to obtain an unbiased estimate of neuronal numbers as previously described.[5,13] The means and standard errors for each group were calculated and the Student's *t*-test and analysis of variance (ANOVA) was used to determine if significant statistical differences existed between the various groups.

The proximal nerve stumps were treated with PBS or LIF using two methods (Fig. 1). In method A, a 1 mm^3 piece of gelfoam was soaked in 20 μl PBS or in 20 μl PBS containing 18 μg LIF and placed directly at the site of the lesion (Fig. 1A). In method B, the gelfoam was

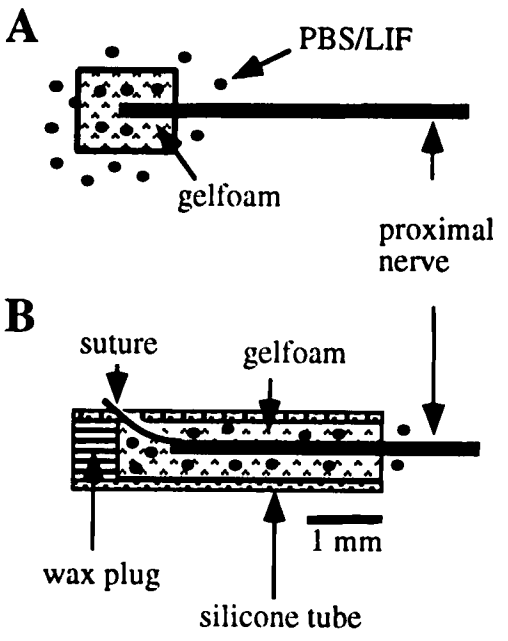

FIG. 1. A schematic illustration of the two methods employed to treat injured nerves with PBS or LIF. In the 'open' method (**A**), a 1 mm³ piece of gelfoam was soaked in 20 μl of the above solutions. In the 'closed' method (**B**), gelfoam pieces soaked with the same solutions were placed inside a silicone chamber into which the proximal nerve stump was secured.

first soaked in 10 μl of PBS alone or in 10 μl of PBS containing 9 μg of LIF and then inserted into a chamber constructed from medical grade silicon tubing sealed at one end (Fig. 1B). The proximal nerve stump was drawn into the chamber and held within it for up to 7 days by means of a 9-0 silk suture. Recombinant murine LIF (Amrad, Australia) produced in *Escherichia coli*, was used.

Results

Identification of median and ulnar motoneurones: Previous studies on rodents have shown that motoneurones which project via the median and ulnar nerves are located within cervical segments C7 and C8 of the spinal cord. This localization was confirmed in this study using the retrograde transport of horseradish peroxidase and the fluorescent dye, fast blue (unpublished observations). The enumeration of these motoneurones was quite straightforward as they were clustered in the medio-lateral part of spinal cord segment and they were clearly delineated from the ventral motoneurones as shown in Figs 2A and B.

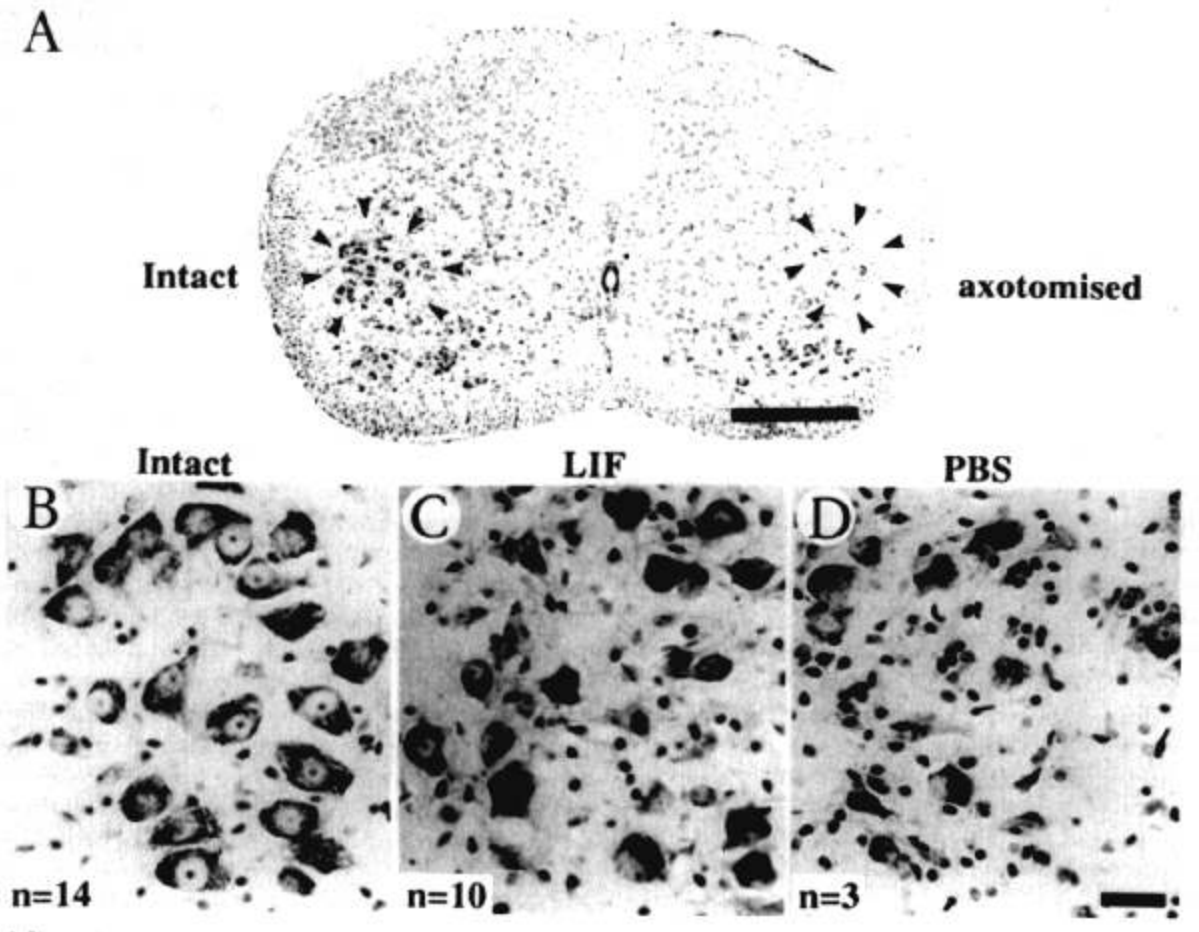

FIG. 2. Photomicrographs of histological sections stained with cresyl violet of the spinal cord at the level of C8 demonstrating the changes which occur 7 days after axotomy and treatment with PBS (right side in **A** and **D**), LIF (**C**) and the intact side (left side in A and **B**). The lateral region where ulnar and median motoneurones normally reside, or are lost after axotomy, is outlined by the arrowheads (A), and this area is shown at higher magnification in panels B, C and D. The numbers indicated at the bottom left corner of panels B–D represent the number of neurones in the photograph, which display nucleoli. The scale bar in A is 250 μm and for B–D it is 25 μm and is shown in panel D.

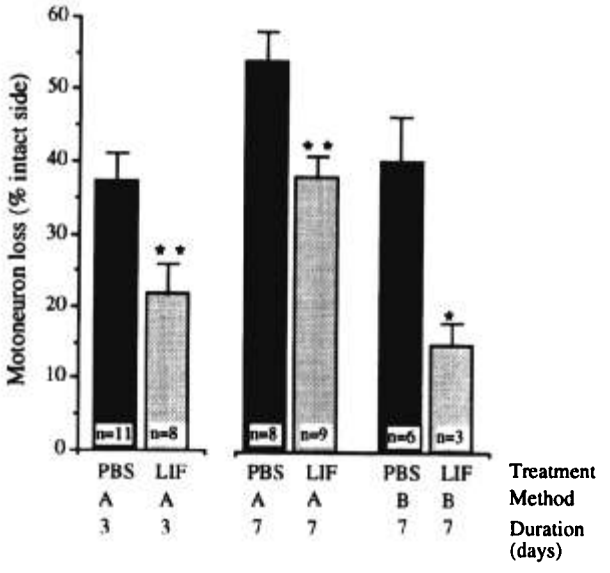

FIG. 3. Loss of motoneurones 3 and 7 days subsequent to axotomy and exposure to either PBS or LIF. The loss which results from using the 'open' gelfoam (method A) or a chamber (method B) is shown. Data points represent mean values ± s.e.m. All the LIF-treated groups showed significant reductions in motoneurone losses compared with PBS-treated controls as determined using Student's *t*-test (*$p < 0.05$, **$p < 0.01$). LIF treatment by method A was significantly ($p < 0.001$, ANOVA) less effective at 7 days than at 3 days; and LIF administered by method B was significantly ($p < 0.001$) more effective than when delivered by method A.

Effects of LIF treatment on motoneurone survival: Median and ulnar nerve transection followed by exposure to gelfoam containing the vehicle, PBS, resulted in the loss of 38% of motoneurones after 3 days, and 55% after 7 days, in the medio-lateral region of the cervical spinal cord segment C8 (Figs 2A, D and 3). When the axotomized nerves were treated with LIF the motoneurone loss was significantly reduced to 22% after 3 days and 38% after 7 days (Figs 2C and 3). This represents a rescue of 42% and 31% of axotomized motoneurones respectively. This difference between the two groups was found to be significant using analysis of variance ($p < 0.001$, ANOVA), suggesting that the effect of LIF may be short-lived. In an attempt to improve the effectiveness of LIF *in vivo*, LIF containing gelfoam was placed in a silicone chamber, into which the proximal nerve stump was drawn (Fig. 1B). After 7 days it was found that LIF administered by this method was significantly ($p < 0.001$, ANOVA) more active in preventing neuronal loss than when administered in gelfoam alone (Fig. 3).

Discussion

The ability of LIF to rescue motoneurones from axotomy-induced cell death again demonstrates the *in vivo* efficacy of factors which possess neurotrophic properties *in vitro*. This also suggests that apoptosis of these neurones in survival assays *in vitro* and, following axotomy *in vivo*, occur via similar mechanisms. It is not surprising that LIF[10] and CNTF[4] both support motoneurone survival as CNTF transmits its biological signal via a tripartite receptor comprising the two LIF receptor subunits and a CNTF specific subunit.[14] However, apoptosis in neurones may proceed through a number of pathways, one of which involves *bcl-2* and the neurotrophins, BDNF and NGF, and a second pathway which is independent of *bcl-2* and is related to CNTF, and presumably LIF.[15] Why different pathways are required to induce neuronal cell death is unclear but it may be related to different developmental phases of these cells, or alternatively to distinct apoptotic pathways within neuronal sub-populations.

The ability of LIF to rescue motoneurones when placed at the lesion site suggests that retrograde transport may be important in mediating this effect. However, LIF's capacity to prevent cell death was evident in axotomized sensory neurones[5] and motoneurones even though previously we had shown it to be only transported retrogradely by sensory neurones after injecting target tissues.[8] However, this does not preclude retrograde transport as a necessary step in the survival mechanism as recent experiments have shown that both CNTF and LIF can be retrogradely transported by injured motoneurones after direct injection into the nerve.[16,17]

One of the problems in preventing motoneurone death *in vivo* is that it may require continual treatment of the neurones with factor until normal connections are re-established. Recent studies have shown that the relative expression patterns of the low affinity nerve growth factor receptor $p75^{NGFR}$ and a motoneurone marker, MO-1, resemble that of an immature neurone following axotomy in the adult rat.[18] In both situations these neurones show elevated $p75^{NGFR}$ and reduced MO-1 levels. This immature state is maintained until the isolated motoneurones re-innervate their target tissue. Since it has been demonstrated that $p75^{NGFR}$ mediates a death signal in some neurones,[19,20] the continued expression of this receptor may reflect a state of reduced viability in axotomized mature neurones.[21] Neurones may remain in this state unless they re-connect with target tissues even though they receive an exogenous supply of appropriate factors which override the apoptotic signal.

Our results support this concept and it appears that the action of LIF in gelfoam has its most profound effect within the first 3 days after axotomy, after which neuronal loss proceeds at the same rate as in the PBS controls. It appears that this acute effect may be related to levels of LIF maintained at the injured site since our results show a small, but significant, improvement in neurone survival, which can be attributed to the action of LIF, when it is restricted to the site by means of a silicone chamber. However, from our results we cannot determine whether the improvement is due to

maintenance of higher levels initially or is due to prolonged exposure to LIF over the entire 7 days. It will be important to determine if better biological delivery systems,[22,23] which provide a continuous exogenous supply of appropriate survival factor/s, are able to keep the apoptotic signal switched off in injured neurones and prevent severe cell atrophy and death of motoneurones prior to re-innervation.

Conclusion

The present findings show that the loss of axotomized motoneurones in the spinal cord is greatly reduced by the application of LIF at the site of nerve injury. The efficacy of the factor at the site of the lesion suggests that the signal which delays the death of these neurones is mediated by a retrograde axonal process. LIF, and other similar molecules, therefore represent potential therapeutic agents in the prevention of motoneurone loss due to injury or disease.

References

1. Oppenheim R. *Ann Rev Neurosci* **14**, 453–501 (1991).
2. Wilcox B, Clatterbuck R, Price D *et al. Soc Neurosci Abstr (USA)* **19**, 441 (1993).
3. Yan Q, Elliott J and Snider WD. *Nature* **360**, 753–755 (1992).
4. Sendtner M, Kreutzberg GW and Thoenen H. *Nature* **345**, 440–441 (1990).
5. Cheema S, Richards L, Murphy M *et al. J Neurosci Res.* **37**, 213–218 (1994).
6. Murphy M, Reid K, Hilton D *et al. Proc Natl Acad Sci (USA)* **88**, 3498–3501 (1990).
7. Murphy M, Reid K, Brown M *et al. Development* **117**, 1173–1182 (1993).
8. Hendry IA, Murphy M, Hilton DJ *et al. J Neurosci* **12**, 3427–3434 (1992).
9. Richards L, Kilpatrick TJ, Bartlett PF *et al. J Neurosci Res* **33**, 476–84 (1992).
10. Martinou JC, Martinou I and Kato AC. *Neuron* **8**, 737–744 (1992).
11. Patterson P and Fann MJ. *Ciba Found Symp* **167**, 125–135 (1992).
12. Pollin MM, McHanwell S and Slater CR. *J Neurocytol* **19**, 29–38 (1990).
13. Clarke P. *Trends in Neurosci* **15**, 211–212 (1992).
14. Ip N *et al. Cell* **69**, 1121–1132 (1992).
15. Allsopp TE, Wyatt S, Paterson HF *et al. Cell* **73**, 295–307 (1993).
16. Curtis R, Zhu Y, Lindsay R *et al. Soc Neurosci Abstr* **18**, 617 (1992).
17. Curtis R *et al. Nature* **365**, 253–255 (1993).
18. Chiu AY, Chen EW and Loera S. *J Comp Neurol* **328**, 351–363 (1993).
19. Rabizadeh S *et al. Science* **261**, 345–348 (1993).
20. Barrett G and Bartlett P. *Proc Natl Acad Sci (USA).* In press (1994).
21. Chiu AY, Chen EW and Loera S. *J Comp Neurol* **328**, 351–363 (1993).
22. Sahenk Z, Seharaseyon J, Mendell JR *et al. Brain Res* **606**, 126–129 (1993).
23. Friedmann T and Jinnah H. *Trends in Biotech* **11**, 192–197 (1993).

ACKNOWLEDGEMENTS: We gratefully acknowledge the histological assistance provided by Mr Steven Mihajlovic and Mr Adrian Warmington. This work was kindly supported by the National Health and Medical Council; The Co-operative Research Centre for Cellular Growth Factors; The AMRAD corporation and the A.L.S.-Motoneuron Disease Research Institute Incorporated, Australia.

Received 25 November 1993; resubmitted 21 January 1994; accepted 29 January 1994