● INVITED REVIEW

Transforming growth factor beta 1, a cytokine with regenerative functions

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Abstract

We review the biology and role of transforming growth factor beta 1 (TGF-β1) in peripheral nerve injury and regeneration, as it relates to injuries to large nerve trunks (*i.e.*, sciatic nerve, brachial plexus), which often leads to suboptimal functional recovery. Experimental studies have suggested that the reason for the lack of functional recovery resides in the lack of sufficient mature axons reaching their targets, which is a result of the loss of the growth-supportive environment provided by the Schwann cells in the distal stump of injured nerves. Using an established chronic nerve injury and delayed repair animal model that accurately mimics chronic nerve injuries in humans, we summarize our key findings as well as others to better understand the pathophysiology of poor functional recovery. We demonstrated that 6 month TGF-β1 treatment for chronic nerve injury significantly improved Schwann cell capacity to support axonal regeneration. When combined with forskolin, the effect was additive, as evidenced by a near doubling of regenerated axons proximal to the repair site. We showed that *in vivo* application of TGF-β1 and forskolin directly onto chronically injured nerves reactivated chronically denervated Schwann cells, induced their proliferation, and upregulated the expression of regeneration-associated proteins. The effect of TGF-β1 and forskolin on old nerve injuries is quite impressive and the treatment regiment appears to mediate a growth-supportive milieu in the injured peripheral nerves. In summary, TGF-β1 and forskolin treatment reactivates chronically denervated Schwann cells and could potentially be used to extend and prolong the regenerative responses to promote axonal regeneration.

Key Words: chronic nerve injuries; transforming growth factor; Schwann cells; axonal regeneration; regeneration-associated proteins; functional recovery

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Introduction

The peripheral nervous system (PNS) has the capacity to regenerate injured axons, in contrast to the central nervous system, in which axons do not regenerate after injury. Significant advancements in microsurgical repair of severed nerves further augment this regenerative potential of the PNS. However, not all patients with injured nerves regain appropriate functions, let alone full use of their extremities especially after injuries to large nerve trunks such as the brachial plexus. Injury to large nerve trunks requires the regenerated axons to traverse over long distances at the very slow rate of 1 mm/d. At this slow rate, reestablishment of innervation to the target organ may take months or even years, a condition referred to as chronic axotomy. At the distal nerve stump, the Schwann cells (SCs) and the target organs remain denervated, conditions known as chronic SC denervation and chronic muscle denervation, respectively. Under this chronic state, SCs exhibit a progressive decline in the capacity to support regenerating axons and the axotomized neuron fails to regenerate their axons. Further, misdirection of regeneration axons into wrong endoneurial tubes and end organs also contribute to poor functional recovery. These two effects result in poor axonal regeneration and subsequent suboptimal return of function.

Delayed repair of peripheral nerve injury occurs frequently in clinical practice because injured nerves remain in physical continuity and there is no clear clinical guide as to the likelihood of significant recovery. If indeed recovery occurs, this does not become evident for months. Furthermore, there appears to be a window of opportunity of 4–6 weeks during which there is maximal upregulation of regeneration-associated proteins and axonal regeneration and muscle reinnervation are fully supported. Beyond this time window the molecular response and regenerative capacity decline rapidly. Strategies to increase the rate of axonal growth and/or to prolong the growth permissive environment after injuries to large nerve trunk remain a significant challenge.

The provision of the growth-supportive environment by SCs is related to the loss and timely reestablishment of

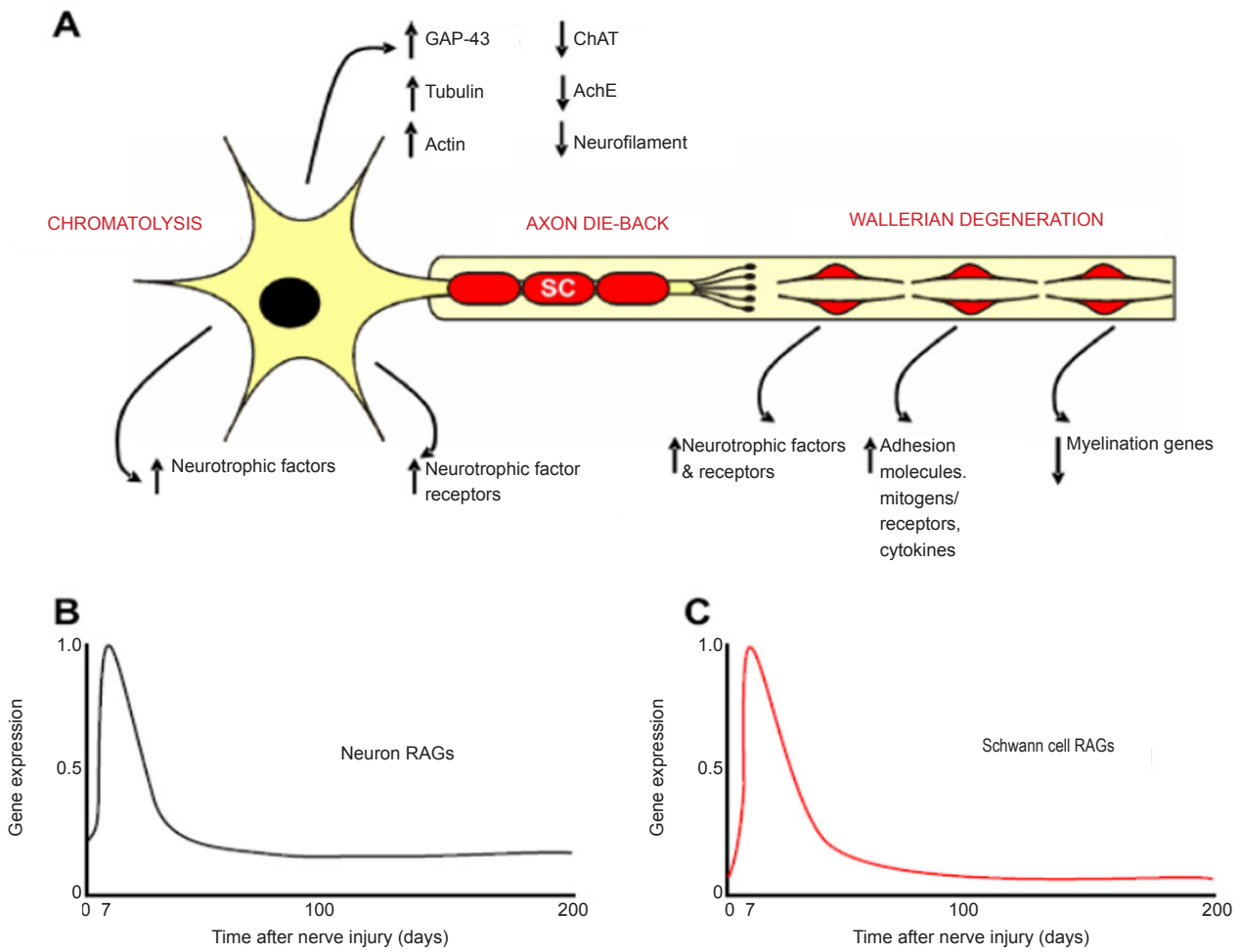**Figure 1 Expressions of regeneration-associated genes (RAGs) are short-lived in injured neurons and Schwann cells and support for regeneration diminishes over time.**
(A) After nerve injury, RAGs are upregulated in the neurons while genes associated with normal synaptic transmission are downregulated. In the distal nerve stump Schwann cells (SCs) undergo proliferation during Wallerian degeneration and express many RAGs; (B, C) Expressions of RAGs progressively decline and are not maintained in chronically axotomized neurons, and denervated Schwann cells. The mRNA levels for tubulin in neurons and for glial cell-derived neurotrophic factor (GDNF) in Schwann cells after injury are shown in the graphs. AchE: Acetylcholinesterase; ChAT: choline acetyltransferase; GAP-43: growth associated protein 43.
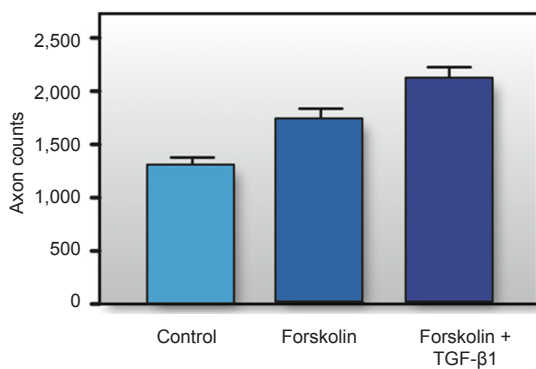
Figure 2 Transforming growth factor beta 1 (TGF-β1) and forskolin treatment lead to an increase in the number of axons that had regenerated into the nerve stump.
Axon counts of transverse sections of tibial distal nerve stump after 8 weeks of chronic denervation followed by 6 weeks of regeneration in Control, Forskolin, and Forskolin with TGF-β1.

axonal contact with the cells. We found good concordance between the proportion of motor neurons that regenerated their axons into the chronically denervated nerve stump and the proportion of freshly axotomized motor neurons that regenerated and reinnervated the denervated muscle after 4 to 6 months (Gordon et al., 2003). In short, the longer it takes for the regenerating axons to reinnervate SCs in the distal nerve stump, the greater likelihood that SCs capacity to support regenerating axons is diminished. These results demonstrated that the progressive decline of the growth supportive capacity of SCs in the distal nerve stumps as the main attribute to poor functional recovery and muscle atrophy is a secondary effect. We also found the number of motor neurons that regenerated their axons decreased, as a result of prolonged time of chronic axotomy and chronic denervation of the SCs. The deleterious consequences of chronic axotomy

and chronic denervation are significant when considering injuries to large nerve trunks.

Our research objective in the past several years has focused on ways to reactivate SCs and to prolong the growth-supportive environment for axonal regeneration in chronically denervated nerve stump. Specifically, we have focused on the role of transforming growth factor beta 1 (TGF-β1) in modulating SC reactivation and axonal regeneration. TGF-β1 is a cytokine and a member of a TGF-β superfamily of signaling proteins that are important for development and tissue homeostasis. TGF-β1 signaling is mediated through binding of the TGF-β type I and type II serine/threonine kinase receptor and activation of a family of signaling proteins called Smad (Li et al., 2015). TGF-β1 is produced by SCs and macrophages, two major participants in nerve injury and repair. Likewise, several lines of *in vitro* evidence have implicated TGF-β1 in the maintenance of the nonmyelinating, growth-promoting SC phenotype and the essential role it plays in the neurotrophic effects of several neurotrophins (Einheber et al., 1995; Sulaiman and Gordon, 2002).

After injury, the distal nerve stumps undergo a degenerative process termed Wallerian degeneration. The SCs play a major role in the clearance of axonal and myelin debris that is necessary for axonal regeneration to proceed. Loss of axonal contact immediately triggers SC responses and a switch from myelinating phenotype to a growth-supportive, non-myelinating phenotype (Gordon et al., 2003). Expressions of myelin-associated proteins such as P0 are downregulated; expressions of growth supportive molecules such neuroregulin and its receptors (erbBs) are upregulated. These upregulated genes are collectively called regeneration-associated genes (RAGs). Furthermore, the SCs secrete cytokines including TGF-β that mediate the recruitment of macrophages into the denervated distal nerve stump to enhance debris clearance and suppress fibroblast proliferation. We and other investigators found that intricate cell-molecular interactions between the SCs and infiltrated macrophages *via* cytokines and growth factors release (Hoke et al., 2002; Gordon et al., 2003; Li et al., 2015). It has been demonstrated that lack of macrophage infiltration impeded axonal regeneration and there is a strong correlation between loss of SC growth supportive phenotype and decline in the number of infiltrated macrophages (Beuche and Friede, 1986).

As we and others have established, there is a 4-week window where injured axons can maximally regenerate, which corresponds to the time when active Wallerian degeneration and TGF-β secreting macrophages are present. Delayed nerve repair beyond 4 weeks results in progressive decline in numbers of injured neurons that regenerated their axons and reinnervated targets. Expression of RAGs is short-lived in the injured neurons and SCs (**Figure 1**). By 6 months most of the RAGs are downregulated, thereby losing the growth supportive milieu for regenerating axons. The reason for the loss of

growth-supportive environment of the distal nerve stump is not known. Jonnson et al. (2013) showed that expressions of erbB3 and erbB4 in the distal nerve stump of a 3 and 6 month delayed repair model are reduced, as are the numbers of SCs in the distal nerve stump. Saito et al. (2009) showed that decrease in regenerative capacity is related to impaired SC activation and increase in caspase-3 expression, consequently SCs death. Axons regenerating over long distance will not be supported by SCs in the distal nerve stump due to premature loss of RAGs. However, SCs isolated from chronically denervated distal region have similar proliferation and response to neurotrophic stimulation as SCs isolated from proximal region (Jonnson et al., 2013). Reactivation of SCs is important to restore the regenerative milieu in the distal region.

TGF-β1 Increases Axonal Regeneration

The effects of TGF-β1 on SCs are complex and outcomes are dependent on the metabolic state as well as the presence or absence of other mitogenic factors. Using our *in vitro* reactivation and chronic nerve injury and repair rat model, the number of regenerated motor neurons (as measured by retrograde labeling) was assessed (Sulaiman and Gordon, 2002). In this set of experiments, nerve repair in the 0 to 4 week optimal window resulted in 825 regenerated axons. A six month delay in repair resulted in 111 regenerated axons, a decrease to 13%. Jonnson et al. (2013), using a delay repair rat model, found that by 6 months, the number of regenerating motor neurons were reduced to 24% to that seen with one month delay. We found that treatment of chronically denervated SCs (6-month delay repair) with TGF-β1 and forskolin resulted in regeneration of 442 axons which was roughly the number that regenerated after a 3 month delayed repair, an increase in the number of motor neurons regenerated by almost 4-fold. *In vitro* experiments had established that 1 ng/mL TGF-β1 and 0.5 μM forskolin, a phosphodiesterase inhibitor known to potentiate the action of endogenous cAMP, were optimal for maximal *in vitro* SC proliferation (Sulaiman and Gordon, 2002). In an 8-week axotomized rat tibial nerve, treatment with TGF-β1 plus forskolin leads to a near doubling ($1,288 \pm 151$ *vs.* $2,108 \pm 200$, $P < 0.05$) of regenerated axons proximal to the repair site (**Figure 2**). Interestingly, it has been firmly established by others that after 8 weeks, the numbers of regenerating axons are decreased at least 50% compared to an immediate repair. This doubling of regenerated axons would suggest that TGF-β1 plus forskolin had the effect of returning chronic axotomized and denervated nerves back to the state comparable to immediate repair. Furthermore, the increase in the number of regenerating axons is also evident around the repair site as well as up to 25 mm proximal and distal to the suture site.

TGF-β1 and Regeneration-Associated Genes

Under chronic denervation state, the decline in growth supporting proteins contributes to a significant mismatch in the presence of growth-supportive milieu in the distal nerve

stump and the slow rate of axonal regeneration. Application of TGF-β1 directly onto 2 months chronically injured tibial nerves at the time of delayed nerve repair induced SC proliferation and extended expression of pertinent RAGs (Sulaiman and Dreesen, 2014). After 6 weeks, expressions of S100B and Ki-67, markers of SC activation and proliferation respectively, remained elevated with TGF-β1 treatment. Expression of CD-68, a marker of macrophage infiltration, was found elevated at the site of repair. These results suggest TGF-β1 promotes the continued expression of the growth supporting phase at the site of repair. These results lend credence to the SCs being "refreshed" to a more receptive fully activated state.

Furthermore, a combined treatment of TGF-β1 and forskolin, significantly improved axonal regeneration far greater than TGF-β1 or forskolin alone (Sulaiman and Dreesen, 2014). These findings are consistent with the hypothesis that while forskolin is sufficient to activate cAMP functions in SCs, and hence mimicking the action of various neurotrophic factors, the modulatory action of TGF-β1 is important for optimal effects. TGF-β1 plus forskolin treatment resulted in fewer cells undergoing apoptosis, as assessed using activated caspase-3, compared to forskolin only treatment, and this difference was more pronounced at the repair site and distal regions (Saito et al., 2009; Sulaiman and Dreesen, 2014). However, the molecular mechanism underlying the dramatic effect of TGF-β1 and forskolin on chronically denervated SCs is unknown.

TGF-β Modulates Axonal Repair

The release of TGF-β1 by SCs and partly by macrophages at the site of injury serve in part, responsible for SCs phenotypic switch during Wallerian degeneration. Inhibition of TGF-β1 expression leads to a decrease in SC proliferation during Wallerian degeneration (Li et al., 2015). This suggests the presence of TGF-β1 is indispensable for axonal repair to proceed. Furthermore, the increase of TGF-β1 expression after transection and delayed repair was not accompanied by a concomitant increase in collagen expression, which impedes regeneration through the formation of epineurium scar (Jonnson et al., 2013). Under a condition of chronic denervation and chronic axotomy, levels of TGF-β1 may be substantially reduced, hence exogenous addition of TGF-β1 may "refresh" and reactivate the denervated SCs. The addition of TGF-β1 may promote the survival of greater numbers of axons and/or axonal sprouts, as evident by increase of axon counts at the proximal stump (Sulaiman and Gordon, 2002). However, we found that the combination of TGF-β1 plus forskolin seems to promote regeneration of axons across the suture site more effectively. What remains to be elucidated is SCs response and integration of TGF-β1 binding and signaling from neurotrophic factors binding to cell surface receptors and to modulate the activity of other cells to maintain axonal regeneration.

Conclusions

Progress and advancements in microsurgical repair and management of injured nerves have improved the quality of care to patients inflicted with nerve injuries. Despite this progress, full recovery is often suboptimal and this lack of full recovery after axonal injuries can be attributed primarily to chronic neuronal axotomy, chronic Schwann cell denervation, and misdirection of regenerating axons into wrong endoneurial tubes. The window of opportunity for intervention is short-lived as neurotrophic support for regenerating axons and maintenance of SC activation progressively decline with time. As we have shown that TGF-β1 and forskolin treatment modality positively enhance SC reactivation, we are exploring their use in combination with stem cell transplantation. We anticipate that the treatment combination will provide a growth-supportive milieu for stem cell to transform into growth sustaining SCs. Additionally we are using new conduits that will allow regenerating axons to traverse the repair site to the distal nerve stumps.

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