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Adjuvant neurotrophic factors in peripheral nerve repair with chondroitin sulfate proteoglycan-reduced acellular nerve allografts

Richard B. Boyer, BS,[a,b,*] *Kevin W. Sexton, MD,*[b] *Charles L. Rodriguez-Feo, BS,*[b] *Ratnam Nookala, MBBS,*[b] *Alonda C. Pollins, MLI,*[b] *Nancy L. Cardwell, BS,*[b] *Keonna Y. Tisdale,*[c] *Lillian B. Nanney, PhD,*[b] *R. Bruce Shack, MD,*[b] *and Wesley P. Thayer, MD, PhD*[a,b]

[a] *Department of Biomedical Engineering, Vanderbilt University, Nashville, Tennessee*
[b] *Department of Plastic Surgery, Vanderbilt University Medical Center, Nashville, Tennessee*
[c] *Vanderbilt Center for Science Outreach, Vanderbilt University, Nashville, Tennessee*

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ABSTRACT

Background: Acellular nerve allografts are now standard tools in peripheral nerve repair because of decreased donor site morbidity and operative time savings. Preparation of nerve allografts involves several steps of decellularization and modification of extracellular matrix to remove chondroitin sulfate proteoglycans (CSPGs), which have been shown to inhibit neurite outgrowth through a poorly understood mechanism involving RhoA and extracellular matrix-integrin interactions. Chondroitinase ABC (ChABC) is an enzyme that degrades CSPG molecules and has been shown to promote neurite outgrowth after injury of the central and peripheral nervous systems. Variable results after ChABC treatment make it difficult to predict the effects of this drug in human nerve allografts, especially in the presence of native extracellular signaling molecules. Several studies have shown cross-talk between neurotrophic factor and CSPG signaling pathways, but their interaction remains poorly understood. In this study, we examined the adjuvant effects of nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) on neurite outgrowth post-injury in CSPG-reduced substrates and acellular nerve allografts.

Materials and methods: E12 chicken DRG explants were cultured in medium containing ChABC, ChABC + NGF, ChABC + GDNF, or control media. Explants were imaged at 3 d and neurite outgrowths measured. The rat sciatic nerve injury model involved a 1-cm sciatic nerve gap that was microsurgically repaired with ChABC-pretreated acellular nerve allografts. Before implantation, nerve allografts were incubated in NGF, GDNF, or sterile water. Nerve histology was evaluated at 5 d and 8 wk postinjury.

Results: The addition of GDNF *in vitro* produced significant increase in sensory neurite length at 3 d compared with ChABC alone ($P < 0.01$), whereas NGF was not significantly different from control. *In vivo* adjuvant NGF produced increases in total myelinated axon

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* *Corresponding author*. Department of Plastic Surgery, Medical Center North S-2221, Vanderbilt University Medical Center, Nashville, TN 37232. Tel.: +1 615 343 1873; fax: +1 615 343 2050.
E-mail address: Richard.b.boyer@vanderbilt.edu (R.B. Boyer).

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count ($P < 0.005$) and motor axon count ($P < 0.01$), whereas significantly reducing IB4+ nociceptor axon count ($P < 0.01$). There were no significant differences produced by *in vivo* adjuvant GDNF.

Conclusions: This study provides initial evidence that CSPG-reduced nerve grafts may disinhibit the prosurvival effects of NGF *in vivo*, promoting motor axon outgrowth and reducing regeneration of specific nociceptive neurons. Our results support further investigation of adjuvant NGF therapy in CSPG-reduced acellular nerve grafts.

1. Introduction

The peripheral nervous system has an impressive resilience to injury, in part, because of the ability of peripheral neurons to regenerate axons. After transection, proximal segments of injured axons form growth cones that respond to neurotrophic and chemotropic extracellular signals to direct neurite outgrowth. Supportive endoneurial cells, such as Schwann cells and fibroblasts, secrete growth factors that participate in complex signaling pathways with regenerating nerve fibers, and ultimately promote either neuron survival or cell death. Extracellular matrix (ECM) components also provide growth cues, either stimulatory or inhibitory, in addition to serving as a guiding substrate for nerve regeneration. For example, chondroitin sulfate proteoglycans (CSPGs) are well-known inhibitors of neurite elongation and their degradation by the bacterial enzyme chondroitinase ABC (ChABC) has been studied extensively as a potential treatment for spinal cord injury [1].

Theoretically, all peripheral nerve injuries with intact spinal roots and ganglia have the potential for near-complete functional recovery. However, in high-grade nerve injuries this is rarely the case because of several impeding conditions—aberrant neurite growth and neuroma formation, physical barriers to innervation, anterograde degeneration, and insufficient rates of neurite outgrowth. The rate of neurite outgrowth at only 1–2 mm/d is an important determinant of functional outcomes because proximal injuries have an extended period of target muscle denervation and increased muscle atrophy [2].

There are numerous reports on the topic of enhanced peripheral nerve regeneration using various experimental therapeutics, nerve grafts, and nerve conduits. Experimental pharmacotherapeutics have mainly targeted the neurotrophic signaling pathways, using recombinant human neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor (GDNF) [3,4]. Additionally, significant progress has been made toward the development of biomimetic nerve scaffolds, using materials such as poly-L-lactic acid, electrospun nanofibers, and Schwann cell-seeded poly-ε-caprolactone [5–7]. Total regenerating axon counts have been the primary outcome for most of these studies, reflecting changes in the regenerative potential of all neurons in the injured nerve. Less effort has been made to understand the specific characteristics of the nerve fibers that are targeted by these regenerative therapeutics, or the molecular events involved in promoting axon elongation and survival *in vivo*.

Neurotrophic factors, such as NGF and GDNF, have well-characterized individual signaling pathways *in vitro*. NGF has two primary receptors—the p75 low-affinity neurotrophic factor receptor and TrkA high-affinity tyrosine kinase receptor. $P75^{NTR}$ binds a wide range of neurotrophins and is known to play a major role in apoptotic signaling through activation of c-Jun N-terminal kinase and caspase pathways [8]. Although TrkA is the primary NGF prosurvival receptor, $p75^{NTR}$ has also been found to activate the mitogen-activated protein kinase pathway and the NF-κB transcription factor, resulting in antiapoptotic effects [9,10]. In addition to promoting survival of sensory neurons *in vitro*, NGF has been shown to protect injured neurons from apoptosis *in vivo* in transected rat sciatic nerves [11,12]. In contrast, GDNF binds to GFRα1 and GFRα2, which both interact with the *RET* tyrosine kinase receptor to activate multiple prosurvival, intracellular signaling pathways [13,14]. In addition to promoting sensory and motor neuron survival, GDNF has been found to be protective against injury-induced degeneration of motor neurons *in vivo* [15–17].

CSPGs are commonly discussed in the context of spinal cord injury and central nervous system plasticity, but they are also highly expressed in the ECM of peripheral nerves and have been found to be upregulated after peripheral nerve injury [18]. In the peripheral nervous system, Schwann cells lining the basal lamina of endoneurial compartments produce CSPGs that are believed to interfere with the neurite-promoting activity of ECM-integrin interactions [19]. ChABC, an enzyme that degrades CSPG, has been reported to promote elongation of neurites when injected directly into acellular peripheral nerve grafts in rodent nerve injury models [20]. In fact, CSPG degradation is now a standard practice during the decellularization of cadaveric nerve in commercially available nerve allografts [21].

Interestingly, neurotrophic factors have been reported to overcome the inhibitory signaling of CSPGs and restore neurite outgrowth [22]. However, this effect is likely reduced *in vivo* as shown in previous reports of CSPG-induced RhoA activation causing downregulation of NGF-induced neurite outgrowth [23,24]. Because of this complex regulatory cascade of neurite growth mediated by neurotrophic factors and ECM interactions, it is difficult to estimate the effects of specific neurotrophic factors after CSPG inhibition *in vivo*.

In this study, we attempt to measure the effects of neurotrophic factors, specifically NGF and GDNF, on neurites regenerating into ChABC-pretreated substrates. We began by evaluating the effects of each neurotrophic factor independently in the presence of ChABC in a dorsal root ganglion (DRG) explant model. DRG neurite outgrowth is well known to