Repair With Collagen Tubules Linked With Brain-Derived Neurotrophic Factor and Ciliary Neurotrophic Factor in a Rat Sciatic Nerve Injury Model

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Objective: To determine if brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) can be successfully delivered to transected and repaired peripheral nerves by cross-linking the factors to collagen tubules (CTs).

Methods: Forty-eight Sprague-Dawley rats underwent left sciatic nerve transection and repair. In the control group, CTs were implanted with no neurotrophic ligand (n = 13). There were 3 experimental groups: CT with BDNF covalently linked to the collagen matrix (CT/BDNF; n = 12), CT with CNTF covalently linked (CT/CNTF; n = 12), and CT with both BDNF and CNTF covalently linked (CT/BDNF/CNTF; n = 11). Functional outcome of neural regeneration was assessed every 10 days using walking track analysis, which was submitted to a sciatic functional index. Nerve morphometry, electrophysiologic studies, and molecular analysis for neural proteins were performed at the completion of the study at postoperative day 90.

Results: Animals in all 3 experimental groups achieved significantly superior maximal functional recovery, larger nerve cross-sectional areas, and a greater number of axons when compared with the control CT group (P<.001, P<.05, and P<.05, respectively). The animals in the CT/BDNF/CNTF group displayed the best functional recovery and had the largest axon diameters, greatest amplitude, and the fastest nerve conduction velocities. Molecular analysis revealed significant differences in the expression of neurofilament, neural cell adhesion molecule, myelin-associated glycoprotein, and myelin basic protein.

Conclusions: We present the first evidence that CNTF covalently linked to CTs can improve functional recovery compared with CTs alone. We also support the previous finding that BDNF covalently linked to CTs significantly increases the functional recovery of transected and repaired nerves. Finally, we found that cotreatment produced the best functional recovery in our model.


NERVE INJURY, due to either deliberate surgical resection or inadvertent nerve transection, will often leave significant cosmetic and functional deficits in the patient. However, because of the remarkable plasticity of the nervous system, neuronal cell bodies can be maintained, neurons can regenerate by axonal sprouting, and functional synapses can form, leaving the patient with some recovery of sensation or function. Manipulation of any of these regenerative steps can improve clinical outcome and serves as the focus of intense basic and clinical research.

Suture techniques, such as epineurial, perineurial, and interfascicular suture repair, tissue adhesives, and autogenous grafts, have been examined experimentally in an effort to achieve a technique that may improve outcomes after nerve transection. Early epineurial suture repair with 9-0 or 10-0 fine monofilament nylon remains the gold standard with which other innovative techniques must be compared. However, this traditional repair strategy appears to have reached a point of maximal benefit, often resulting in disappointing outcomes, and any further advances in peripheral nerve regeneration will need to incorporate more than just mechanical manipulations.

Neurotrophic factors, soluble substances produced by peripheral target structures, are taken up by axons and then transported through retrograde axonal flow to the nerve cell body, where they exert a trophic effect. Some of these factors, which have been investigated for their ability to enhance the regenerative capacity of nerve tissue, include members of the neurotrophin family (such as the nerve growth factor and the brain-derived neurotrophic factor [BDNF]) and the ciliary neurotrophic factor (CNTF).

Brain-derived neurotrophic factor promotes the survival and neurite outgrowth...
METHODOLOGY

Forty-eight Sprague-Dawley rats underwent functional evaluation of their sciatic nerves as measured by an index originally described by de Medinaceli et al.1 and modified by Bain et al.11 The animals were then randomized into the following 4 groups: (1) CTs (Otogen Corporation, Palo Alto, Calif) with no neurotrophic ligand (n = 13); (2) CTs with BDNF (Regeneron Pharmaceuticals, Tarrytown, NY) covalently linked to the collagen matrix (CT/BDNF; n = 12); (3) CTs with CNTF (Regeneron Pharmaceuticals) covalently linked (CT/CNTF; n = 12); and (4) CTs with both BDNF and CNTF covalently linked (CT/BDNF/CNTF; n = 11).

Care of the animals was administered according to guidelines of the Institutional Animal Care and Use Committee of Stanford University, Stanford, Calif.

SURGICAL TECHNIQUE

Anesthesia consisted of an intraperitoneal injection of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, Ill) solution (30-80 mg/kg). Each animal then underwent unilateral exposure and transection of the sciatic nerve. Repair of the nerve was performed by tubulization using purified bovine CTs. The tubules measured 7 mm in length with an inner diameter of 1.5 mm. The tubules were first hydrated in lactated Ringer solution before implantation into the animals. The tubules were then cut longitudinally, allowing placement of the proximal and distal portions of the cut nerve into the tubule. The memory of the collagen material allowed the return of the tubed configuration to wrap the material around the nerve with 0.5 mm of overlap. A single tacking suture was placed (roughly 3 mm proximal to and distal to the site of transection) through the tubule and the epineurium to secure the tubule. The surgeon was blinded to the type of tubule used.

FUNCTIONAL ASSESSMENT

Functional analysis of neural regeneration was assessed at 10-day intervals from postoperative day (POD) 10 to 90 using walking track analysis. Pawprints were obtained by moistening the hindpaws of each animal and having them walk unassisted along a corridor lined with water-sensitive bromphenol blue–impregnated paper. Measurements were then made of paw length, distance between the first and fifth toes, and the distance between the second and fourth toes. These values, which were measured for both the operated, experimental, and unoperated normal sides, were then submitted to a sciatic functional index (SFI) as described by Bain et al:11

\[ SFI = -38.3 \frac{EPL-NPL}{NPL} + 109.5 \frac{ETS-NTS}{NTS} + 13.3 \frac{EIT-NIT}{NIT} - 8.8. \]

where EPL indicates the operated experimental pawlength; NPL, unoperated normal pawlength; ETS, operated experimental toe spread; the distance between the first and fifth toes; NTS, the normal toe spread, ie, the distance between the first and fifth toes; EIT, the experimental intermediary toe spread, ie, the distance between the second and fourth toes; and NIT, normal intermediary toe spread, ie, the distance between the second and fourth toes.

During this testing, the observer remained blinded to ensure unbiased functional and anatomical assessment.

ELECTROPHYSIOLOGIC ASSESSMENT

Electrophysiologic studies were performed to assess the integrity of the regenerated nerve on POD 90. Under anesthesia, the animals were placed in a prone position to provide exposure of both limbs. The nerve from the experimental side was exposed for access to electrophysiologic testing. The contralateral gluteal musculature was exposed to accommodate the ground electrode.

Bipolar stimulating electrodes were inserted into the plantar surface of the experimental foot in the receptive area of the sciatic nerve (Figure 1). Active recording electrodes were placed around the sciatic nerve 5 mm proximal from and 3 mm distal to the original site of nerve repair. Ground electrodes were placed in the contralateral exposed gluteal musculature. Stimulation consisting of 500-mV square waves of 200-microsecond duration was provided by a generator (Tektronix pulse

of sensory neurons in the spinal ganglion and has been shown to influence motor neuron survival during development.5 Ciliary neurotrophic factor, a neurocytokine, is known to be an important trophic protein for nerve development and regeneration.4 It has recently been shown that BDNF, and to a lesser extent CNTF, prevent lesion-induced axonal degeneration in rat optic nerves.7 Furthermore, Mitsumoto et al11 reported in 1994 that while administration of either BDNF or CNTF slows progression of motor neuron dysfunction in wobbler mice, cotreatment with these factors arrested disease progression, strongly suggesting the synergism of BDNF and CNTF in vivo in neuronal rescue. Although these factors and their effects have been extensively described in the literature, investigation of their use in peripheral nerve regeneration remains insufficient to warrant clinical implementation.4 The purpose of this study was to investigate further the synergism between BDNF and CNTF and to determine its effect on functional recovery in a regenerating nerve.

A second goal of this investigation was to evaluate the use of tubulization techniques that have been investigated for their ability to promote and improve regeneration across nerve gaps. Tubulization repairs have included decalcified bone, rubber, artery, and vein. The recent focus has been on absorbable materials, such as polyglycolic acid, polyglactin, and collagen as tubules. With the use of these materials, there is prevention of scar and neuroma formation, lack of an inflammatory response to suture material, increased neoangiogenesis, and encasement of regenerating nerve fibers. Collagen tubulization has been reported by Rosen and colleagues6 to be comparable histologically and physiologically with epineurial suture repair. We investigated the use of collagen tubules (CTs) primarily to repair nerves after transection. Brain-derived neurotrophic factor and CNTF were covalently linked to these tubules to optimize the neural microenvironment for enhancement of nerve regeneration after nerve transection.
RESULTS

Forty-four of the 48 animals were evaluable. One animal from the CT/BDNF group had a foot infection, and 3 animals (2 from the CT/BDNF group and 1 from the CT/BDNF/CNTF group) exhibited automutilation of the operated limb. To prevent further pain or distress, each of them was killed when this discovery was made.

SFI ANALYSIS

The SFI values decreased precipitously after nerve transaction with repair and dropped to their nadir at POD 10 or 20 and rose their maximal value at POD 80 or 90 (Figure 2). Examination of the maximal SFI values reached in the experimental groups revealed the following: animals in all 3 experimental groups (CT/BDNF, CT/CNTF, and CT/BDNF/CNTF) achieved superior maximal functional recovery compared with the control CT group (P < .001). Furthermore, the rate of recovery of the SFI for all 3 experimental groups was superior to the control CT group (P < .01, P < .005, and P < .005, respectively). Animals whose nerves were repaired by CT/BDNF/CNTF displayed the best functional recovery of all groups, although it was not statistically significantly better than the other 2 experimental groups (P > .10).

Because the functional recovery of some of the animal groups plateaued and actually decreased slightly at POD 90, a second analysis of function at POD 90 was performed. Subsequent examination of the SFI values revealed the same observations noted when comparing maximal function; animals in all 3 experimental groups (CT/BDNF, CT/CNTF, and CT/BDNF/CNTF) achieved superior recovery compared with the control CT group (P < .001, P < .005, and P < .001, respectively). At POD 90, the animals’ nerves repaired by CT/BDNF/CNTF achieved the best functional recovery of all groups; the function

MOLECULAR ASSESSMENT

Finally, 5-mm segments of nerve were obtained: 5 mm proximal and 5 mm distal to the sciatic nerve repair site at the time of killing. These segments were frozen immediately at −80°C and grouped according to the repair technique to allow for blinded molecular analysis. Each group of nerves was then homogenized in phosphate-buffered saline solution containing 2% sodium dodecyl sulfate. Protein concentrations were determined by the bicinchoninic acid method according to the manufacturer’s instructions (Pierce Chemical Co, Rockford, Ill). Fifty micrograms of denatured protein obtained from each of the groups was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electroblotted to pyroxylin paper, and assessed using standard Western blot analysis. The following antibodies were used: monoclonal antineurofilament, anti-neural cell adhesion molecule, polyclonal antiamyelin-associated glycoprotein (MAG), polyclonal antimyelin basic protein, anti-pan-cadherin, and anti-β-catenin. Differences in the level of protein expression were determined by visual inspection of band intensity.

STATISTICAL ANALYSIS

A paired 2-tailed Student t test was then performed to determine the significance of differences between the treatment and control group means. Specifically, the differences in maximal functional recovery between groups were examined. Also, the rate of functional recovery was evaluated by comparing analysis of linear regression between groups from POD 10 to 90. In addition, the axon counts and the nerve conduction amplitude for the control and treatment groups were compared using a paired 2-tailed Student t test. The control and experimental codes were maintained until after the experiment was completed and the data were analyzed.
was significantly better than the CT/BDNF group (P = .008). Animals’ nerves repaired by CT/BDNF and by CT/CNTF had essentially the same sciatic functional recovery (P = .52).

ELECTROPHYSIOLOGIC ANALYSIS

Although electrophysiologic testing was carried out for each of the 44 animals, interpretable compound action potentials were recorded for 5 animals in the CT group, 3 in the CT/BDNF group, 10 in the CT/CNTF group, and 8 in the CT/BDNF/CNTF group. The mean nerve conduction velocity was 42.6 m/s for the CT group, 46.9 m/s for the CT/BDNF group, 50.5 m/s for the CT/CNTF group, and 52.2 m/s for the CT/BDNF/CNTF group (Table). There were no statistically significant differences in the mean nerve conduction velocity between groups (P > .10). Ratios of AUC were greatest for animals in the CT/BDNF/CNTF group and lowest for the control CT group (Table). The CT/CNTF and CT/BDNF/CNFT groups had significantly larger AUC ratios than the control group (P = .01 and P = .006, respectively).

AXON MORPHOMETRIC AND HISTOLOGICAL ANALYSIS

Nerve morphometry revealed that nerves repaired by CT/BDNF/CNTF had the largest mean distal nerve cross-sectional area among all groups (Table). When compared with the CT/CNTF and control CT groups, the CT/BDNF/CNTF group also had a significantly larger area (P < .05 for both).

Nerves repaired by CT/BDNF/CNTF had the highest mean distal axon count (Table), followed by the CT/BDNF, CT/CNTF, and CT groups. Animals in the CT/BDNF/CNTF group had a significantly higher mean axon count than those in the CT/CNTF group; this count was more than 2 times greater than that of the control CT group (P < .05 and P < .001, respectively). In addition, animals in the CT/CNTF group had a significantly higher mean axon count than the control CT group (P = .04).

Nerves repaired by CT/BDNF/CNTF had the largest distal axon diameter, followed by the CT/CNTF, CT/BDNF, and CT groups (Table). Animals in the CT/BDNF/CNTF group had significantly larger distal axon diameters than the CT group (P = .05).

Gross examination of the repaired nerve segments at the time of harvest revealed a moderate degree of fibrosis around the repair site. There was no discernible difference in the amount of fibrosis around the nerve repair sites between groups. Histological cross-sections of the repaired nerves in all groups showed axonal sprouting, identified by the small, poorly organized myelinated fibers. Longitudinal sections of experimental group nerves revealed that the orientation of the nerve fibers was largely parallel to the long axis of the nerve. There were no significant intergroup differences.

MOLECULAR ANALYSIS OF NEURAL PROTEINS

Expression of neurofilament, neural cell adhesion molecule, MAG, and myelin basic protein in the distal nerve segments of each of the 4 groups was decreased when compared with the corresponding proximal nerve segments (Figure 3). Expression of neurofilament was greatest in the control CT group and decreased in the distal segments of all groups. Expression of neural cell adhesion molecule was greatest in the proximal segment of the CT/BDNF/CNTF group with similar levels among the rest of the groups. Expression of myelin basic protein was greatest in the proximal segment of the CT/BDNF group compared with the other groups. Finally, MAG protein expression was decreased in the distal segments of those groups treated with trophic factors, with the lowest levels in the CT/BDNF/CNTF group. There were no signifi-
Neurotrophic factors are thought to mediate chemotaxis, required for successful reestablishment of functional synaptic connections. Brain-derived neurotrophic factor has a distinct role in supporting the survival of neurons of various stages of development and to prevent axotomy-induced, retrograde cell death of neurons in the adult rat's central nervous system. Only recently has the use of either BDNF or CNTF in the study of peripheral nerve regeneration with evaluation by a functional analysis been studied. In one study, BDNF, when covalently cross-linked to CT, demonstrated the most favorable functional recovery as measured by SFI compared with BDNF simply being pumped to the repair site. Furthermore, the systemic administration of the same neurotrophic factor provided only a modest improvement in function. The effects of CNTF were studied by Newman et al, who showed that CNTF significantly improves the rate of recovery of sciatic nerve function compared with nerves bathed in nerve growth factor after neurorhhaphy.

The concept of using multiple neurotrophic factors simultaneously was tested originally by Mitsumoto et al, who showed that BDNF and CNTF displayed a synergistic relationship when used together to completely arrest motor disease progression in wobbler mice compared with a control group that received no treatment. That this synergism exists in vivo for nerve function was suggested by a study in which cotreatment with both BDNF and CNTF significantly improved nerve function compared with treatment with BDNF alone. These results, along with prior data confirming the advantages of collagen tubulization repair for transected nerves, led to the hypothesis that improved results could be obtained using cotreatment with BDNF and CNTF, linked to CTs.

The results of our study suggest that the use of 2 neurotrophic factors is better than 1 for the enhancement of functional recovery in a peripheral nerve injury model. Furthermore, the use of BDNF and CNTF together was significantly superior to BDNF alone, but not significantly better than CNTF alone. This observation is similar to the findings of Mitsumoto et al, who concluded that CNTF was a more effective neurotrophic factor than BDNF in the arrest of disease progression. The data from this study lend further evidence to the synergism of the roles played by BDNF (preventing retrograde degeneration) and CNTF (a survival growth factor released under pathological conditions).

The equipment used in this study was unable to detect most of the electrical signals in the animals from the CT and CT/BDNF groups since these signals, as measured by the AUC and peak amplitudes, were significantly smaller than those signals in the animals from the CT/CNTF and CT/BDNF/CNTF groups. Because of our small sample size, the only statistically significant difference was for the AUC of the CT/CNTF and CT/BDNF/CNTF groups relative to...
the control group. Had our equipment been more sensitive to the lower signals from the animals in the CT and CT/BDNF groups, it is possible that a larger number of animals could have been recorded to make more statistically significant comparisons between groups. The electrophysiological observation that the CT/CNTF and CT/BDNF/CNTF groups had significantly larger AUC ratios than the control group correlated well with the functional SFI findings. As more functional axons innervate a muscle, better preservation of muscle tone and strength should result in better walking patterns, which will be reflected by the SFI; cotreatment may therefore enhance functional recovery by increasing axon myelination.

The animals in the CT/BDNF/CNTF group had the largest nerve cross-sectional areas and axon counts, suggesting that neurotrophic factors reduce the loss of myelinated nerve fibers, a conclusion reached by Mitsu¬moto et al. The finding that the animals’ nerves repaired by CT/BDNF/CNTF had the largest mean axon diameter while the control animals had the smallest axon diameter is consistent with the functional outcome. Larger distal axons have an increased chance of forming con¬nections with a target organ and have a greater influence on functional recovery. Since axon diameter is directly correlated with nerve conduction velocity, the best functional recovery would be expected in the groups with the largest mean axon diameters. The trophic effects of BDNF and CNTF combined with the CT technique may preserve or increase axon diameter after nerve transaction and repair. Furthermore, the fact that the combined treatment group had the largest axon diameter may result from synergism achieved by activation of distinct receptors to increase axon diameter or myelination.

The combined trophic effect of BDNF and CNTF on axonal regeneration was assessed using Western blot analysis with several related markers. As previously reported, neurofilament levels 5 mm distal to the repair site may not correlate with the degree of reinnervation occurring more distally at the motor endplates. Therefore, diminished neurofilament levels were seen in all 4 groups despite differences in functional recovery. As a neurite outgrowth-promoting factor, neural cell adhesion molecule displayed the highest level in the proximal segment treated with CT/BDNF/CNTF and similar levels among the rest of the groups, suggesting that it may play a role at an early stage of the regeneration process. Myelin basic protein is a Schwann cell–specific protein that is regulated by axon–Schwann cell contact. The increased levels of myelin basic protein seen in the proximal segment treated with CT/BDNF compared with the other groups suggest that BDNF plays a role in local axon–Schwann cell interaction.

Myelin-associated glycoprotein has been recently shown to inhibit neurite outgrowth of the adult spinal ganglion in vitro. In our studies, MAG displayed decreased levels in the distal segments treated with trophic factors, therefore suggesting a possible increase in axonal regeneration on trophic factor treatment. In particular, MAG levels in the distal segment were lowest in animals treated with both BDNF and CNTF, indicating the most axonal growth in this area. These results are consistent with the data on axon morphometric and histological analysis in which nerves repaired by CT/BDNF/CNTF had the highest mean distal axon count and the largest axon diameter among all the groups.

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