Use of Cold-Preserved Allografts Seeded with Autologous Schwann Cells in the Treatment of a Long-Gap Peripheral Nerve Injury

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Background: Limitations in autogenous tissue have inspired the study of alternative materials for repair of complex peripheral nerve injuries. Cadaveric allografts are one potential reconstructive material, but their use requires systemic immunosuppression. Cold preservation (≥7 weeks) renders allografts devoid of antigens, but these acellular substrates generally fail in supporting regeneration beyond 3 cm. In this study, the authors evaluated the reconstruction of extensive nonhuman primate peripheral nerve defects using 7-week cold-preserved allografts repopulated with cultured autologous Schwann cells.

Methods: Ten outbred *Macaca fascicularis* primates were paired based on maximal genetic disparity as measured by similarity index assay. A total of 14 ulnar nerve defects measuring 6 cm were successfully reconstructed using autografts ($n = 5$), fresh allografts ($n = 2$), cold-preserved allografts ($n = 3$), or cold-preserved allografts seeded with autogenous Schwann cells ($n = 4$). Recipient immunoreactivity was evaluated by means of enzyme-linked immunosorbent spot assay, and nerves were harvested at 6 months for histologic and histomorphometric analysis.

Results: Cytokine production in response to cold-preserved allografts and cold-preserved allografts seeded with autologous Schwann cells was similar to that observed for autografts. Schwann cell–repopulated cold-preserved grafts demonstrated significantly enhanced fiber counts, nerve density, and percentage nerve ($p < 0.05$) compared with unseeded cold-preserved grafts at 6 months after reconstruction.

Conclusions: Cold-preserved allografts seeded with autologous Schwann cells were well-tolerated in unrelated recipients and supported significant regeneration across 6-cm peripheral nerve defects. Use of cold-preserved allogeneic nerve tissue supplemented with autogenous Schwann cells poses a potentially safe and effective alternative to the use of autologous tissue in the reconstruction of extensive nerve injuries. (*Plast. Reconstr. Surg.* 119: 246, 2007.)

In the setting of complex peripheral nerve injury, the ideal tension-free neurorrhaphy is frequently unattainable. Autografts are routinely used in the repair of surgically irreducible defects. However, autogenous donor tissue is limited in quantity, and its harvest risks additional morbidity. Allografts have been broadly studied as a potential alternative reconstructive material. This tissue has met with moderate success in its clinical application,[1] serving as a temporary scaffold for regenerating fibers. Cadaveric donor grafts, which represent a potentially unlimited supply of nerve tissue, are an attractive alternative to the use of autologous nerves[2]; however, significant potential side effects of necessary immunosuppression[3,4] and the risk of disease transmission[5] limit their application to only the most severe injuries.

To circumvent immunosuppressant-related toxicities, a host of techniques have been proposed to alter the immunogenicity of nerve allografts.[6–14] These methods rely on the destruction of donor alloantigens and/or the elimination of allogeneic cell lines. Unfortunately, pretreatment techniques reduce or eliminate graft

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Schwann cells, whose presence is integral to the maturation and survival of regenerating nerve fibers.[15] Without the trophic support of peripheral glia, acellular scaffolds are unable to support regeneration over long distances.[6,16,17]

Schwann cells produce a number of axonotrophic extracellular matrix molecules, including laminin, fibronectin, and collagen, that are instrumental in the guidance of regenerating neurites.[18] Moreover, these cells secrete a variety of neurotrophins such as nerve growth factor, brain-derived neurotrophic factor, insulin-like growth factor, and ciliary neurotrophic factor that are essential to neuronal survival and maturation.[19–23] Unfortunately, Schwann cells are highly antigenic, eliciting a potent inflammatory response when transplanted into unrelated recipients. Consequently, maximal regenerative potential may only be realized in the presence of autologous cells.

Nerve allografts are an attractive option for peripheral nerve reconstruction, but their use requires immunosuppression, which risks additional recipient morbidity. Prolonged cold preservation attenuates antigenicity by depleting allograft donor cells, thereby yielding nonantigenic substrates that are well tolerated in host animals.[24] This strategy may obviate the need for toxic immunosuppression. Previous studies using acellular guidance channels suggest, however, that the absence of Schwann cells limits neuroregenerative potential to a distance of approximately 3 cm.[15,17] To facilitate regeneration over greater distances, 7-week cold-preserved grafts would require reintroduction of autologous Schwann cells. In this study, the authors evaluated the capacity of Schwann cell–seeded cold-preserved allografts to support nerve regeneration across 6-cm ulnar nerve defects.

MATERIALS AND METHODS

Animal Model

Ten outbred female *Macaca fascicularis* primates were obtained from Covance Corp. (Alice, Texas). The study animals were screened and found to be herpes B–negative and tuberculosis-negative. After a 30-day quarantine period, the animals were transferred to a central animal care facility, where they were housed in pairs and maintained in a 12-hour light/dark cycle. Study primates were provided a standard balanced diet and water ad libitum. Animals were examined daily and were provided frequent, intermittent environmental enrichment. All protocols were approved by the Washington University Animal Studies Committee and were in accord with National Institutes of Health guidelines.

Experimental Design

Animals ranged in weight from 1.75 to 2.4 kg at the initiation of the study. Animals underwent reconstruction of 6 cm ulnar nerve defects with fresh autografts, fresh allografts, cold-preserved allografts, or cold-preserved allografts seeded with autogenous Schwann cells. Donor/recipient pairs were assigned on the basis of similarity index calculation using a DNA profile assay comparing various base pair combinations. Maximally disparate animals were paired so as to maximize the probability of reciprocal rejection in allograft exchanges. Reversed ipsilateral ulnar nerve grafts were used for autologous nerve grafting as a positive control.

Animals were operated on in pairs to synchronize timing of donor nerve harvests with recipient nerve grafting. Animal operations were scheduled such that all ulnar nerves, whether fresh allograft, fresh autograft, cold-preserved allograft, or cold-preserved allograft with Schwann cells, would be grafted into freshly created ulnar nerve defects. Sural nerve harvest and expansion for Schwann cell culture was timed so as to minimize differences in animal age at the time of ulnar nerve grafting. Because of the constraints of this experimental design, four monkeys that donated ulnar nerves for use as allografts could not receive allografts because of the lack of available donors. Therefore, 16 ulnar nerves were grafted in the 10 macaques, but two of these reconstructions later proved technically unsuccessful.

Macaque Genetic Testing

DNA profile analysis by Therion International (Saratoga Springs, N.Y.) was used to confirm unrelated genotype in study animals. This analytic method evaluates random noncoding sequences found across individuals within a species. Subjects are judged as more or less related on the basis of sequence homology or mismatch. A shared sequence, or lack thereof, is derived from the gel electrophoresis pattern of digested DNA fragments.

Initially, DNA was isolated from macaque peripheral blood using an organic extraction procedure. Sequences were cleaved with the restriction enzyme HinfI. Digested fragments were separated electrophoretically and transferred to a nylon me-

dium, where they were independently hybridized with radioactively labeled multilocus DNA probes OPT-03 and OPT-12. Banding patterns were quantified and compared to generate a similarity index, allowing interpretation of genetic congruence or disparity in the study population.

Surgical Procedures

All surgical procedures were performed in a negative-pressure sterile environment. Induction of anesthesia was achieved by means of intramuscular injection of ketamine (10 to 15 mg/kg), after which intravenous access was attained and endotracheal intubation was performed. One milligram of atropine sulfate and 60 to 75 mg/kg cefazolin were administered intravenously before incision. The surgical sites in all cases were shaved, prepared, and draped in standard sterile fashion. Heart rate, temperature, and pulse oximetry were monitored intraoperatively and followed in the immediate postoperative period. Incisions were uniformly closed in two layers with interrupted 5-0 Vicryl sutures (Ethicon, Inc., Somerville, N.J.) approximating the fascia and 4-0 nylon interrupted sutures closing the skin. All operative sites were infiltrated with 5 to 12 cc of 1% lidocaine before emergence from anesthesia.

Sural Nerve Harvests

Macaques assigned to receive cold-preserved allografts seeded with autologous Schwann cells underwent sural nerve harvest at the initiation of the study. These cables were dissociated to yield Schwann cells for culture and expansion as described later. After standard sterile preparation, a no. 15 blade was used to create a cutaneous incision just behind the right lateral malleolus. Tenotomy scissors were used to extend the incision 3 cm proximally, identifying the saphenous vein superficially and the sural nerve adjacent and deep to the vessel. The nerve was dissected carefully from surrounding tissue and a 3-cm segment was resected, rinsed with normal saline, and maintained at 4°C pending further processing.

Preparation of Autologous Schwann Cells

Macaque Schwann cells were processed for cell culture using previously described methods.[25] Briefly, autologous sural nerve fascicles were stripped of surrounding tissue and minced in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 2 μM Forskolin (GIBCO), 10 μM heregulin (Genetech, South San Fransisco, Calif.), and penicillin-streptomycin. The tissue was then incubated in 6% carbon dioxide at 37°C in the above media for 2 weeks.

Fascicles were further dissociated with 0.05 mg/ml collagenase type 1 (Worthington Biochemical Corp., Lakewood, N.J.), and 1 mg/ml Dispase (Invitrogen Corp., Carlsbad, Calif.). Cells were then plated on laminin-coated tissue cultures. Daughter cells were then washed with trypsin and ethylenediaminetetraacetic acid. The solution was centrifuged at 500 *g* and the supernatant drawn off. Cells were treated with 1 ml of Thy 1.1 antibody (American Type Culture Collection, Manassas, Va.) to eliminate fibroblasts, resuspended in DMEM with 20% fetal bovine serum and 10% dimethyl sulfoxide (Sigma, St. Louis, Mo.), and stored at –180°C.

Ulnar Nerve Harvest

A no. 15 blade was used to create a cutaneous incision between the medial epicondyle and the olecranon. Tenotomy scissors were used to divide the fascia overlying the ulnar nerve. The incision was extended 6 cm proximally, and the underlying nerve was meticulously neurolysed from surrounding tissue. An ulnar nerve segment measuring 6 cm was resected. Excised segments were then treated as allografts, cold-preserved allografts, cold-preserved allografts seeded with Schwann cells, or autografts as described later. The small finger of each upper extremity was amputated by means of sharp excision through the proximal interphalangeal joint to avoid subsequent autotomy of the insensate finger. This amputation site was closed in two layers. At 6 months, nerve grafts were harvested, including native ulnar nerve stumps measuring approximately 1 cm proximal and distal to the graft (Fig. 1).

Cold Preservation

Ulnar nerve allografts were obtained as described above. These segments were rinsed with 0.9% normal saline immediately after resection. Under sterile conditions, the tissue was transferred into University of Wisconsin solution (NPBI International BV, Weesp, The Netherlands) supplemented with 100 U/ml penicillin G, 40 units of regular insulin, and 16 mg/liter dexamethasone.[16] The cold-preserved grafts were maintained at 4°C for 7 weeks before transplantation.

Preparation of Schwann Cell–Seeded Grafts

Immediately before preparation of repopulated grafts, expanded autologous Schwann cells

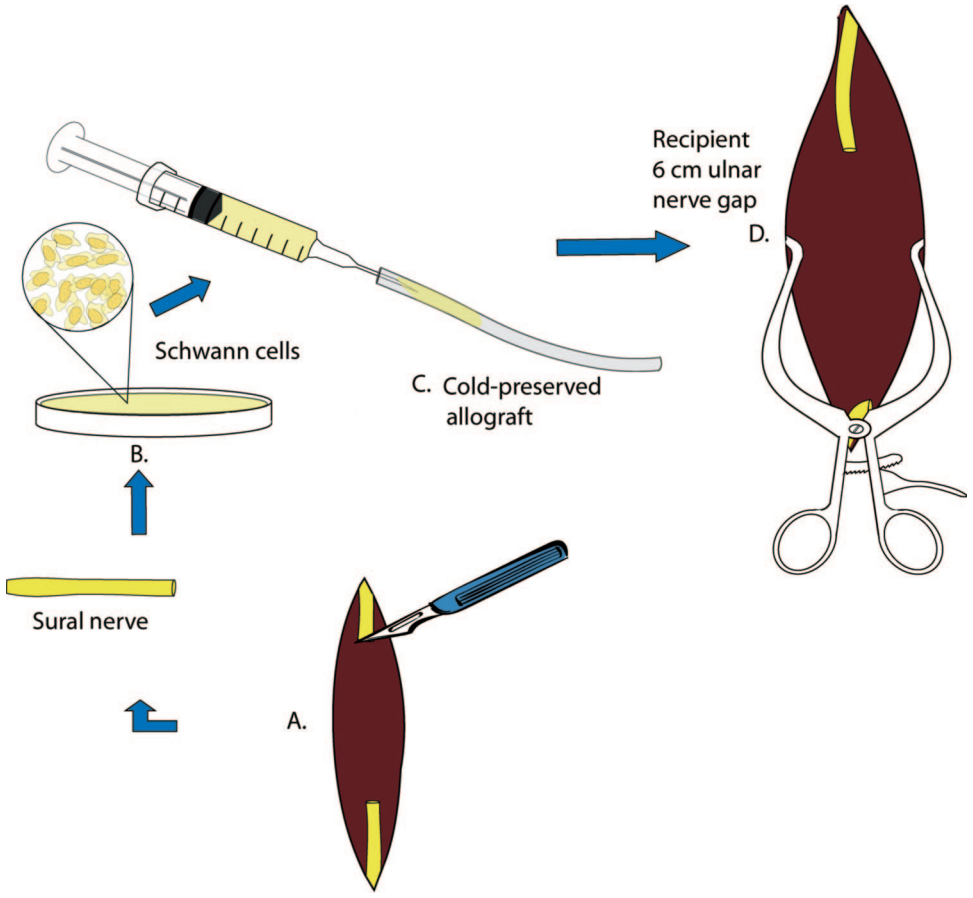

Fig. 1. Schematic representation of the experimental model. Animals grouped to receive cold-preserved allografts seeded with autologous Schwann cells underwent initial (*A*) sural nerve harvests and (*B*) autologous Schwann cell expansion. (*C*) A volume of 100 μl (1.0×10^6 Schwann cells) was introduced into cold-preserved allografts. All other grafts were placebo-injected with 100 μl of 0.9% normal saline. (*D*) Recipient ulnar nerve defects measuring 6 cm were repaired with an equivalent length of allograft, autograft, cold-preserved allograft, or cold-preserved allograft plus Schwann cells. At 6 months, grafts were harvested for histologic and histomorphometric evaluation.

were rapidly thawed, centrifuged, and resuspended in phosphate-buffered saline. Trypan blue exclusion was used to assess cell viability, which was judged greater than 95 percent in all cases. One hundred microliters of the cell line suspension was drawn into a Hamilton syringe with a 25-gauge, 5-cm blunt needle (1.0×10^6 cells). The needle was inserted into the end of the graft substance and 5.0×10^5 cells were introduced throughout half of the graft's substance. The remaining cells were seeded throughout the opposing graft end. The repopulated segments were then coapted to ulnar nerve stumps by means of epineural suture repair.

Ulnar Nerve Graft Placement

All grafts, regardless of pretreatment method (autograft, allograft, cold-preserved allograft plus Schwann cells, or cold-preserved allograft), were coapted in the standard end-to-end fashion, repairing 6-cm right or left ulnar nerve defects with the aid of 4.5× loupe magnification. Interrupted epineural 9-0 nylon sutures were used in the repair. Surgically manipulated extremities were splinted in 90 degrees flexion for approximately 2 weeks postoperatively. To standardize treatment, untreated allografts, cold-preserved allografts, and autografts were all placebo-injected with 100 μl of sterile 0.9% normal saline before placement.

Histologic Evaluation and Quantitative Histomorphometry

Study nerves were harvested at 6 months and placed in cold phosphate-buffered 3% glutaraldehyde for a minimum of 24 hours. The tissue was then postfixed in 1% osmium tetroxide and de-

hydrated in graded ethanol solutions. Specimens were embedded in epoxy resin (Araldite 502; Polysciences, Inc., Warrington, Pa.), and 1-μm cross-sections were taken from the middle portion of the graft and 5 mm distal to the nerve graft using an ultramicrotome (LKB-III; LKB Produkter, Broma, Sweden).

These sections were stained with toluene blue and examined under light microscopy to evaluate myelin thickness, fiber diameter, evidence of Wallerian degeneration, and overall nerve architecture. Ten randomly selected fields per region were evaluated under 1000× oil immersion magnification with the assistance of linked image-analysis morphometric software (Leco Instruments, St. John, Mich.).[26] Captured digital images were displayed on a video monitor calibrated to 0.125 μm/pixel, and further analysis was based on gray/white-scale data. Total fiber numbers were derived from measures of total fiber area and fiber count per millimeter squared. Percentage neural tissue was calculated from the total cross-sectional area containing myelin, axons, and Schwann cells and subtracting debris.

Interferon-γ Enzyme-Linked Immunosorbent Spot Assay

Using previously described methods,[27] cytokine-producing cells isolated from macaque peripheral blood were quantified. Briefly, multiscreen 96-well filtration plates (Millipore, Bedford, Me.) were coated with the capture antibody anti-monkey interferon-γ and diluted in phosphate-buffered saline. Plates were incubated overnight at 4°C. After 12 hours, wells were blocked with 10% bovine serum albumin and washed with phosphate-buffered saline. Cell-free culture media (RPMI-1640 medium supplemented with HEPES buffer, sodium pyruvate, penicillin-streptomycin, L-glutamine, 2-mercaptoethanol, fetal bovine serum, and nonessential amino acids) (negative control) and phytohemagglutinin (positive control) were plated for each animal. Irradiated (500,000 rads) stimulator allogenic and isogenic lymphocytes at 500,000 cells/well were also plated for each animal. An equal amount of responder (transplant recipient monkey) lymphocytes was added to each well. The plates were then incubated at 37°C in 5% carbon dioxide for 24 hours and then washed with phosphate-buffered saline before the addition of the secondary biotinylated interferon-γ antibody (Endogen, Woburn, Mass.). After a 12-hour incubation period at 4°C, plates were washed with phosphate-buffered saline–Tween, and then horseradish peroxidase–labeled streptavidin (BD Biosciences, San Diego, Calif.) was added to each well. After 2 hours, wells were washed with phosphate-buffered saline and developed by means of addition of 3-amino-9-ethylcarbazole substrate (Sigma, St. Louis, Mo.). Plates were left to air-dry at room temperature. The resulting spots correlated with cytokine-producing cells and were counted using the ImmunoSpot Analyzer (Cellular-Technology, Cleveland, Ohio). The resulting data are expressed as the number of interferon-γ spots (spot-producing cells) per 1×10^6 lymphocytes plated. The baseline mean (culture media–negative control well mean) is subtracted from the given data.

Statistical Analysis

All results are reported as mean ± SD unless otherwise indicated. Statistica version 6 (StatSoft, Tulsa, Okla.) was used in the analysis of the histomorphometric data. If significant differences were discovered in one-tailed analysis of variance, the Student-Newman-Keuls post hoc test was used to derive the specific p values for comparisons between individual groups. Unequal animal enrollment for the four experimental groups required the use of an approximated sample size in comparisons using the Student-Newman-Keuls test. The alpha level was set at $p < 0.05$.

RESULTS

General

All subjects completed the study without significant handicap or other morbidity. Macaques retained the use of bilateral upper extremities throughout the course of the study, with the exception of a 10-day postoperative period, during which manipulated extremities were splinted in lightweight casts to immobilize nerve repairs.

Interferon-γ Enzyme-Linked Immunosorbent Spot Assay

Enzyme-linked immunosorbent spot assay analysis yields a value relative to the number of interferon-γ–producing lymphocytes per study animal. In the present work, assay controls demonstrated minimal production of interferon-γ in wells with no cells or with isogenic stimulator cells. This confirmed the expected need for stimulation with in vitro alloantigen. Vigorous cytokine production was observed with concanavalin A stimulation (data not shown).

Lymphocytes demonstrated moderately elevated interferon-γ production in response to one

Table 1. Enzyme-Linked Immunosorbent Spot Assay Data*

	No.	Baseline	1 Month	2 Months	4 Months	6 Months
Autograft	5	255	205	290	13	40
CP allograft	2	305	35	313	13	30
CP allograft + SCs	4	360	285	343	32	89
Allograft	3	558	540	455	48	8

CP, cold-preserved; SCs, Schwann cells.
* Measures recipient lymphocyte interferon-γ production in response to transplanted graft tissue, demonstrated here in spot-forming cells per 1.0×10^6 peripheral blood mononuclear cells. As expected, autograft placement resulted in minimal recipient reactive cytokine production. Cold-preserved allograft recipients responded similarly, with minimal interferon-γ production, consistent with graft tolerance. Recipient response to cold-preserved allograft plus Schwann cell tissue was variable, but seeded grafts were largely well-tolerated. Cytokine production in response to allogeneic tissue was elevated near baseline and declined over the following 6 months, indicative of host inflammatory response.

of two donor allografts immediately after placement. This production gradually tapered to near baseline over the ensuing 6 months (Table 1). The second untreated allograft recipient induced only slightly elevated interferon-γ production in recipient lymphocytes (data not shown). This response peaked at 2 months and gradually declined. Interferon-γ production varied over the 6-month course in animals receiving autologous Schwann cell–seeded grafts. Overall, cytokine-producing cells were minimal across all groups at 6 months, with the exception of cold-preserved allografts plus Schwann cell recipients, where interferon-γ production remained minimally elevated. Cold-preserved allografts, both with and without autologous Schwann cells, prompted minimal inflammatory cytokine production that was not markedly different from autologous graft placement.

Macaque Genetic Testing

A total of 114 radioactively labeled bands were detected for the DNA of animals following digestion and labeling with the two-probe assay. The average number of markers per individual study animal was 19 for the OPT-03 probe and 24 for OPT-12. Band sharing was determined on the basis of the DNA fragment migration pattern that was transferred to a nylon membrane and radioactively labeled. Similarity indices were calculated using the following formula: Similarity index = (No. of genetic markers shared)/(Total no. of genetic markers identified).

On the basis of this calculation, study animal indices ranged from 0.39 to 0.50. For reference, the similarity index of first-order relatives is approximately 0.69 and that of unrelated animals is 0.47.

Gross Appearance

At the time of harvest, all ulnar nerves, regardless of treatment method, were relatively free of surrounding scar tissue. Technical failure occurred in two nerve grafts (one nerve autograft and one cold-preserved allograft), with avulsion of the suture line resulting in discontinuity between graft and the host nerve. In the remaining 14 nerve grafts, there was no obvious disruption in the suture line, and neuroma formation was not observed. Autografts had larger diameters and more significant vascular ingrowth than cold-preserved grafts or grafts seeded with Schwann cells. Schwann cell–seeded grafts were larger in caliber than their unseeded counterparts (Fig. 2). Unseeded cold-preserved grafts were grossly smaller in diameter than grafts in all other treatment groups.

Histology

Autografts supported mature, large-diameter fibers, as demonstrated in Figure 3, *above*, *left*. Untreated allografts exhibited similar histologic features, because of unexpected consanguinity (Fig. 3, *above*, *right*). Myelin thickness and underlying nerve architecture were also similar. Cross-sections from Schwann cell–seeded groups (Fig. 3, *below*, *left*) demonstrated large-diameter fibers, with lesser surrounding debris when compared with unseeded cold-preserved grafts in nearly all cross-sections analyzed (Fig. 3, *below*, *right*). The epineurial and perineurial tissues were more adherent to underlying axoplasm in the Schwann cell–seeded groups than in the cold-preserved allografts and were similar to autografts in this regard. There were no obvious tumors or needle tract injuries in the seeded nerve cross-sections. Examination of nerve tissue distal to nerve grafts showed analogous findings (Fig. 4). Fresh nerve autografts and allografts exhibited significant regeneration across the distal suture line, with several large, mature nerve fibers present. In contrast, the cold-preserved nerve grafts supported significant nerve regeneration only when supplemented with autogenous Schwann cells.

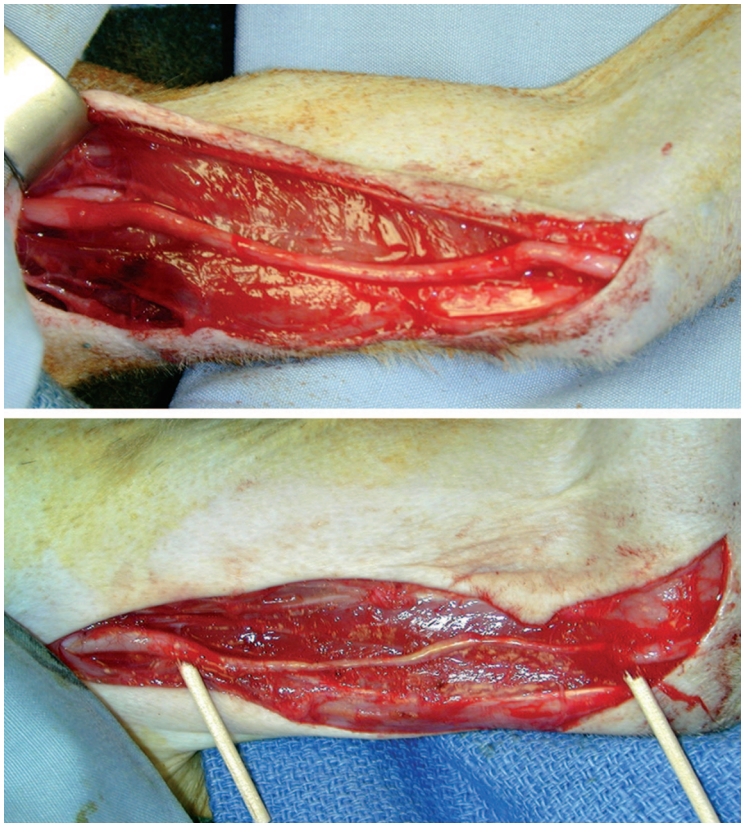

Fig. 2. Representative photographs of ulnar nerve grafts in cold-preserved allografts plus Schwann cells and cold-preserved allografts groups, respectively, 6 months after graft placement. (*Above*) Cold-preserved allografts plus Schwann cells grafts were more robust in diameter and vascularity than (*below*) cold-preserved allografts at harvest.

Histomorphometric Analysis

Morphometric analysis results are as depicted in Table 2 and Figure 5. The data shown are from the 14 intact nerves; the two avulsed nerves were excluded from analysis. Six months after transplantation, the cold-preserved allograft plus Schwann cell grafts supported significantly enhanced regeneration beyond that observed in cold-preserved allografts, as reflected in the parameters below. Cold-preserved allograft plus Schwann cell distal graft cross-sections demonstrated mean total fiber counts of 3525 ± 2352 as compared with 1488 ± 2549 fibers in unseeded acellular grafts ($p < 0.05$). This improved performance was also depicted in fiber density measures of 5956 ± 4562 versus 2550 ± 4365 in Schwann cell versus sham-injected cold-preserved allografts, respectively ($p < 0.05$). Percentage nerve measures of 4.49 ± 3.70 percent in Schwann cell-seeded grafts were also significantly enhanced beyond those of unseeded cold-preserved allografts (2.24 ± 3.85 percent) ($p < 0.05$). There was no significant difference in fiber width as measured across all groups.

Distal allograft fiber counts (6115 ± 3611) were not significantly different from autografts (8059 ± 5557). This similarity was also present in comparison of percentage nerve means from distal allografts (12.15 ± 5.33 percent) to those of autografts (9.19 ± 4.24 percent). Untreated distal allograft fiber density (14,243 ± 3618) was significantly greater than both cold-preserved allografts plus Schwann cells (5957 ± 4562) and cold-preserved allografts (2550 ± 4365) ($p < 0.05$), and was similar to untreated autografts (10,696 ± 4405).

DISCUSSION

The presence of viable Schwann cells is a prerequisite for axonal regeneration across long peripheral nerve defects.[28,29] After prolonged cold preservation (≥7 weeks), allografts retain the axonotrophic basal lamina scaffolding native to untreated donor nerves[16] but lack peripheral glia. As a

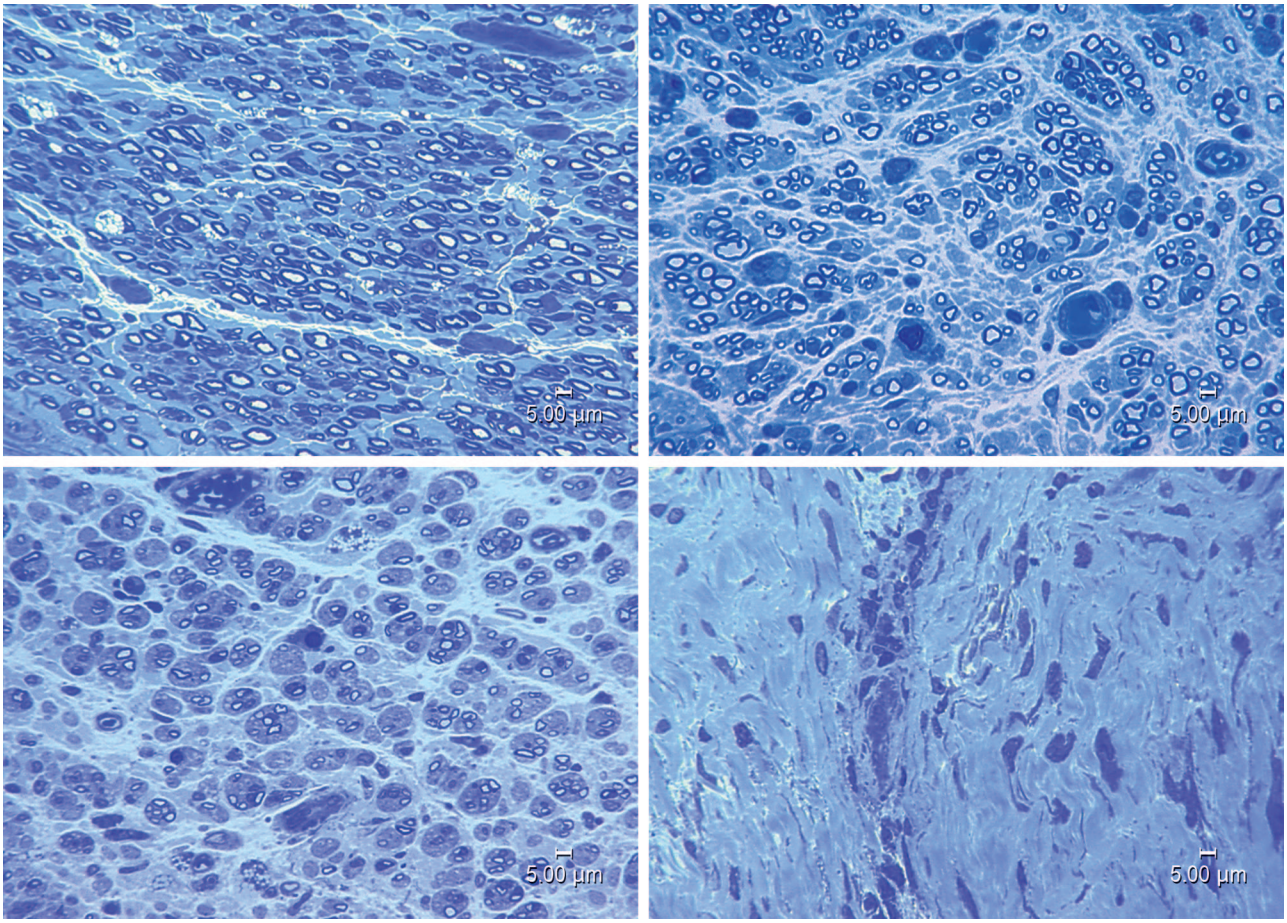

Fig. 3. Representative histology of nerve grafts evaluated at 6 months sectioned through the midgrafts (toluidine blue stain; original magnification, ×660). (*Above*, *left*) Robust regeneration is observed in midgraft sections through autografts. Autografts show organized neural architecture and multiple mature nerve fibers. (*Above*, *right*) Midgraft sections through fresh allografts are similar in appearance to autografts because of unexpected donor consanguinity. (*Below*, *left*) Cross-sections distal to cold-preserved allografts plus Schwann cell grafts reveal significant regeneration of fibers, although it is less than that observed with fresh nerve grafts. (*Below*, *right*) Midgraft sections of cold-preserved allografts without Schwann cells failed to support significant nerve regeneration.

result, both the sprouting and elongation of developing neuritis is impaired.[30,31] Previous authors have supported the use of acellular biogenic substrates in the reconstruction of short gaps.[32–34] However, these channels limit both the rate[35] and extent[15,36–38] of overall axonal regeneration and maturation.

The results of the present study are in accord with those of previous research in that Schwann cell–repopulated grafts significantly enhanced regeneration beyond that observed in acellular substrates. This neuroregenerative effect of Schwann cells is evident in measures of nerve density, percentage nerve, and total fiber counts. All of these indices were significantly elevated in Schwann cell–supplemented versus sham-injected cold-preserved grafts. Cold-preserved allografts, both with and without autologous Schwann cells, prompted minimal inflammatory cytokine production that was comparable to autologous grafts. The unusually low interferon-γ production in the cold-preserved allograft group at 1 month likely reflected the variability inherent in the assay, as the macaque enzyme-linked immunosorbent spot assay data have a wider standard deviation than observed in murine studies.[39] Overall, the muted immunoreactivity between allografted animals was indicative of likely consanguinity and does make it more difficult to establish the nonantigenic nature of cold-preserved grafts.

Building on previous work,[25] we have improved the regenerative capacity of 7-week cold-preserved allografts by means of introduction of autologous cells within acellular scaffolding. Prolonged cold preservation of allografts yields an optimal substrate for axonal regeneration because of muted graft immunogenicity[16,24] and abundant laminin content.[16] Autologous

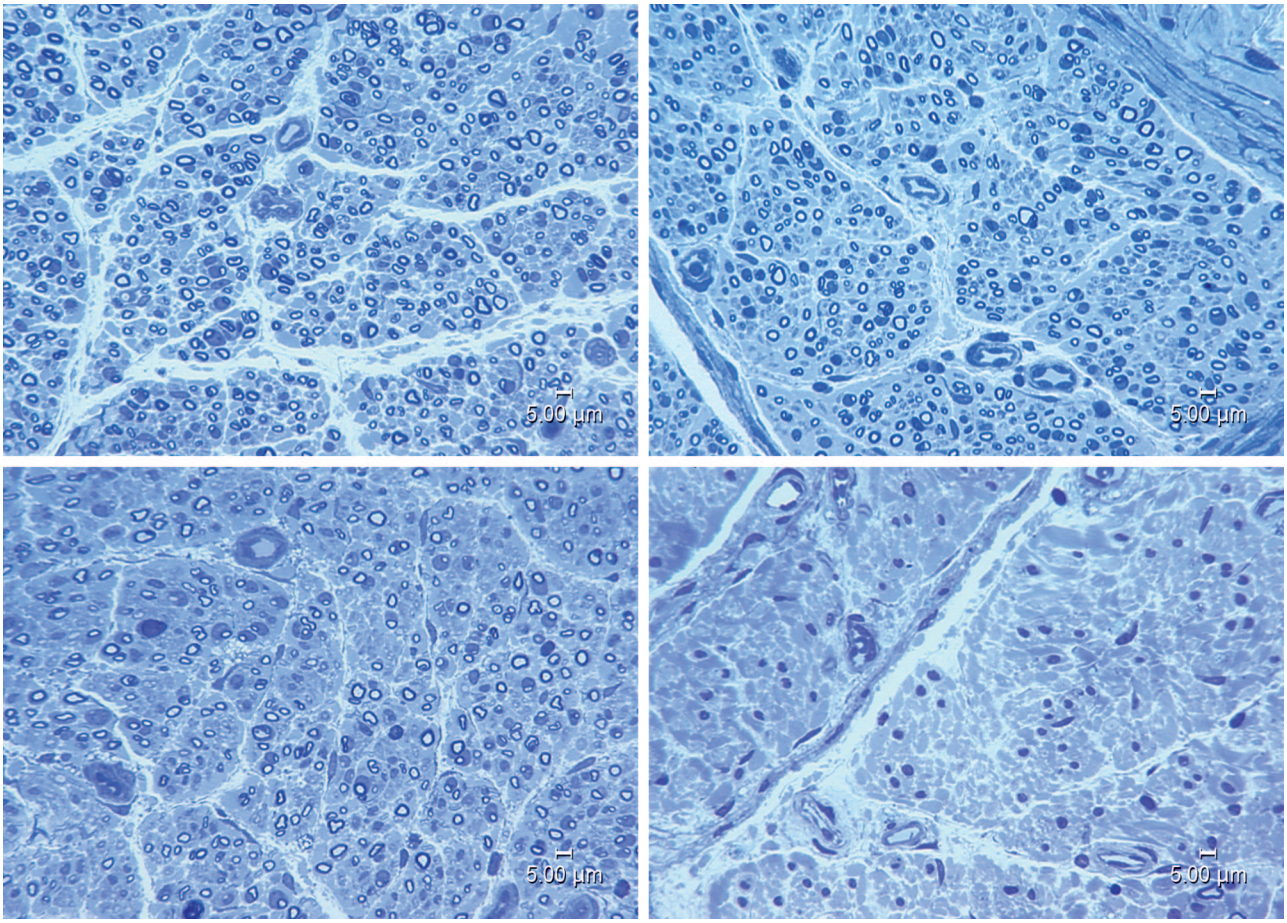

Fig. 4. Representative histology of host nerve segments evaluated at 6 months sectioned distal to nerve grafts (toluidine blue stain; original magnification, ×660). (*Above*, *left*) Maximal regeneration and maturation is demonstrated in cross-sections distal to autografts, reflected in fiber density, diameter, and myelin thickness. (*Above*, *right*) Regeneration distal to allografts is comparable to that of autografts because of unexpected donor consanguinity. (*Below*, *left*) Cross-sections distal to cold-preserved allografts plus Schwann cell grafts reveal significant regeneration of fibers and varying degrees of maturation. (*Below*, *right*) Cross-sections distal to cold-preserved allografts demonstrate minimal regeneration, with only scant, poorly myelinated fibers.

Table 2. Summary Histomorphometric Analysis of Nerve Regeneration

Graft Type	No.	Mean Fiber Count	Nerve Density	Percentage Nerve	Mean Fiber Width (µm)
Autograft	5	8059 ± 5557	10,696 ± 4405	9.19 ± 4.25	2.60 ± 0.32
Allograft	2	6115 ± 3611	14,243 ± 3619	12.15 ± 5.34	2.74 ± 0.13
CP allograft plus SCs	4	3525 ± 2352	5957 ± 4563	4.49 ± 3.70	1.90 ± 1.27
CP allograft	3	1488 ± 2549	2550 ± 4365	2.24 ± 3.85	1.52 ± 1.39

CP, cold-preserved; SCs, Schwann cells.

Schwann cells expanded in vitro[40–42] are known to maintain essential neurotrophin production following transplantation.[43] These neurotrophins likely act in synergism with neurite-promoting acellular allograft adhesion molecules to enhance axonal elongation. Unseeded cold-preserved grafts supported only very modest regeneration across a 6-cm defect, whereas the addition of cultured autologous Schwann cells was clearly beneficial, with fiber counts approaching those of autologous tissue.

The essential neuroenhancing properties of Schwann cells are likely rooted in the ligand-receptor interactions between axons and the myelin sheath.[44–46] After transplantation, Schwann cells have been shown to stimulate the regeneration, elongation, and maturation of peripheral fibers by means of up-regulation of axonotrophic cellular

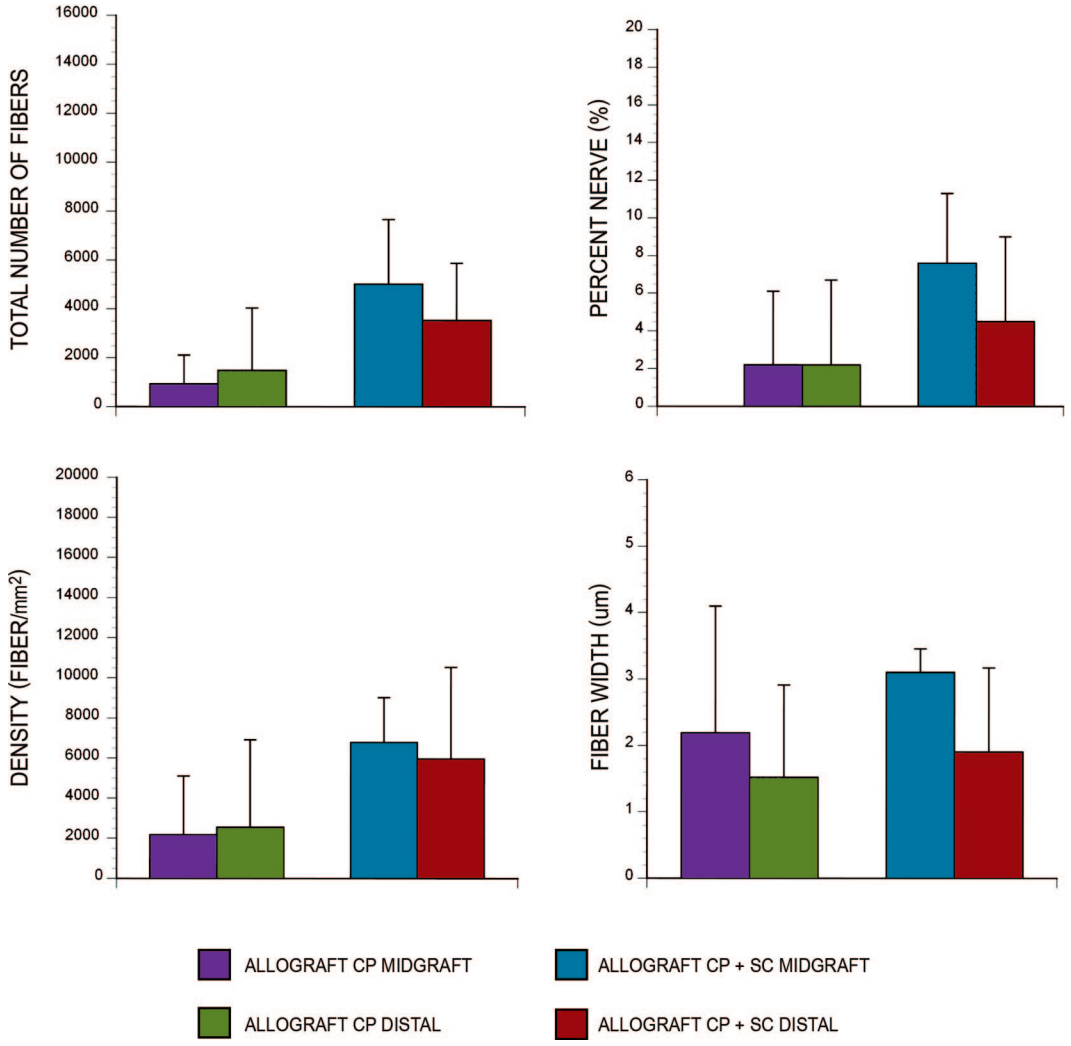

Fig. 5. Histomorphometric analysis of nerve regeneration across autografts, allografts, cold-preserved allografts, and cold-preserved allografts seeded with autologous Schwann cells. Schwann cell–seeded grafts demonstrated significantly enhanced regeneration compared with cold-preserved allografts, as demonstrated in total fiber counts, nerve density, and percentage nerve ($p < 0.05$).

adhesion molecules.[47–50] In the injured or degenerating peripheral nerve, these cells also amplify production of nerve growth factor, brain-derived neurotrophic factor, and glial-derived neurotrophic factor, stimulating axonal outgrowth and providing trophic support to regenerating growth cones.[51–54]

After axotomy, Schwann cells undergo a complex phenotypic transformation up-regulating the expression of multiple adhesion molecules, including N-CAM, L1, P30, and myelin-associated glycoprotein.[55,56] These cells proliferate, dedifferentiate, and migrate within the injured territory, phagocytizing debris and forming long columns that are instrumental in the guidance of regenerating fibers.[57] Axonal elongation is partially dependent on the presence of viable Schwann cells. The migratory capacity of these cells is limited to approximately 40 mm,[55,56] a length that mirrors the maximal extent of fiber regeneration observed in acellular guidance channels. In the setting of lengthy defects, Schwann cells residing within recipient nerve ends proliferate and repopulate bridging substrates, eventually transforming the graft phenotype to one resembling the native peripheral nerve.[58] This gradual transformation is limited in complex injuries because of the rate and distance limitations in recipient Schwann cell migration.

There exists a well-established correlation between prolonged axotomy and poor functional outcome following repair.[59–62] As reinnervation is delayed, target organs atrophy and the growth-

permissive state of denervated Schwann cells progressively deteriorates. This gradual decline in regenerative capacity is partially mediated by a reduction in the Schwann cell expression of both the p75 receptor[63] and the neuregulin receptors c-erb2 and c-erb4.[64] In addition, prolonged axotomy results in decreased production of glial cell line–derived neurotrophic factor, a substance with particularly potent influence on developing motor neurites.[65] In the interest of improving functional recovery, it is desirable to minimize the time interval necessary for distant Schwann cell migration and thus axonal elongation.

By using repopulated grafts in the reconstruction of a long gap, regenerating units are provided immediate contact with viable Schwann cells and their essential neurotrophic support. The delay in native Schwann cell migration and phenotypic transformation is likely partially supplanted by the presence of expanded autologous cells. We suggest that the additive influence of both existing and reintroduced cell populations may enhance the quality and quantity of overall fiber regeneration. It is difficult to ascertain whether axonal regenerative rate and functional reinnervation are affected by this treatment, but future study in these areas will likely prove invaluable.

The current findings reaffirm a potential role for clinical nerve banking and support the use of Schwann cell culture and transplantation in the reconstruction of complex injuries. Although previous studies have reported encouraging results using autologous cultured cells in the repair of short nerve gaps,[28,66–69] few have examined their utility in the repair of extensive injuries. In a study by Strauch et al.,[70] a gluteal vein graft seeded with autologous Schwann cells was used to bridge a 6-cm peroneal nerve gap in rabbits. The authors reported large- and small-caliber axons mixed throughout the length of the conduit, which was sectioned at 1, 3, and 6 cm from the proximal end. The histologic appearance on light microscopy was somewhat similar to that observed in the present study. Direct comparison of the studies is difficult, however, because of use of a different animal model and different endpoints, use of qualitative assessments in the earlier experiment, and use of sections at the distal end of the nerve conduit rather than from the distal nerve stump. Although our findings demonstrate that regeneration across Schwann cell–seeded acellular grafts was superior to unseeded acellular grafts, further work is needed for comparison against vein grafts of similar length containing Schwann cells.

Schwann cells have been successfully isolated and exponentially expanded from human pediatric and adult nerve biopsy specimens.[41,71] In the clinical setting, these cells could feasibly be isolated and cultured from tissue acquired during the initial exploration of an acute nerve injury. Repopulated graft constructs could then be manufactured in the interim to definitive repair. This modality would obviate the need for the traditional harvest of donor grafts but would preserve the neurotrophic benefit of autologous cells.

The use of cultured Schwann cells represents a promising alternative to the standard treatment of complex nerve injuries, but there are limitations inherent in the primate model that necessitate further consideration. Although the performance of our repopulated grafts was consistent with expectations, it is interesting to note that untreated allografts supported regeneration beyond that which would be expected in an unrelated recipient. These findings may be partially explained by unexpected consanguinity in our study population.

An allograft group with no regeneration would have been helpful in comparisons against the cold preservation–alone group. The cold preservation–alone group not seeded with Schwann cells had minimal nerve regeneration, suggesting that long acellular scaffolds do not support regeneration across long nerve gaps. However, in the absence of an independent no-regeneration control, it could be argued that acellular scaffolds only *retard* regeneration and simply require more time for Schwann cell migration. The relatively late endpoint used in this study (6 months) and the concordance of histologic findings in midgraft and distal nerve histologic specimens argue fairly strongly against such an interpretation. Furthermore, retarded nerve regeneration would still have negative implications for functional recovery.

Although similarity indices here demonstrated values consistent with outbred progeny, this assay's use of nuclear restriction fragment length polymorphisms is only partially indicative of absolute genetic variation and is not an index of major histocompatibility complex mismatch. The interpretation of this test is based on the amount of genetic polymorphism available for analysis (i.e., the number of polymorphic loci and the number of alleles per locus). In this regard, genetic disparity may be more appropriately evaluated by means of analysis of animal microsatellite DNA,[72,73] as there are more alleles per locus than in that of the nuclear genetic material evaluated here.

In this study, a total of 14 ulnar nerves were analyzed. Our results may have been more compelling if more animals had been included in each treatment group, although research involving large primate populations raises ethical concerns. The small number of animals enrolled in this study was sufficient to demonstrate meaningful benefit with introduction of autologous Schwann cells despite technical failure of two nerves. Macaques are available through a limited number of vendors and, in the present study, were likely affected by inbreeding depression and consanguinity, which influenced immunologic findings. Despite these limitations, future investigations involving Schwann cell transplantation are essential in seeking improved functional outcome after repair. Establishing functional outcomes are an important long-term goal for research on nerve regeneration, with collection of electrophysiologic, electromyographic, and behavioral data.

Investigators have recently demonstrated the ability to induce potent overexpression of the neurotrophins brain-derived neurotrophic factor and glial-derived neurotrophic factor in rodent Schwann cell cultures.[74] These cells have proven to be well tolerated in recipients and are known to maintain their heightened neurotrophic output in vivo. Transplantation of virally transduced Schwann cells has gained attention in animal studies involving central lesions,[75] but their utility in the peripheral nervous system requires further evaluation.

CONCLUSIONS

Autologous Schwann cells appear to enhance regeneration when seeded into 7-week cold-preserved allografts. These constructs present a potentially safe and feasible alternative to the use of autologous tissue, particularly in the setting of complex injuries. The use of these grafts would obviate the need for traditional autograft harvest, thereby avoiding donor-site morbidity and potentially abbreviating recovery time. Although the performance of repopulated grafts lags slightly behind that of autogenous tissue, further work with virally manipulated Schwann cells may prove beneficial in enhancing functional outcome.

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DISCLOSURE

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.

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