Labeled Schwann cell transplants versus sural nerve grafts in nerve repair

Daniel H. Kim, M.D., Sean E. Connolly, B.S., David G. Kline, M.D., Rand M. Voorhies, M.D., Andrea Smith, M.S., Mary Powell, M.S., Tracy Yoes, B.A., and Joanne K. Danilloff, Ph.D.

Departments of Neurosurgery, Louisiana State University Medical Center and Ochsner Clinic and Alton Ochsner Medical Foundation, New Orleans; and Department of Anatomy and Cell Biology, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana

This study evaluated the ability of Schwann cell transplants to enhance the recovery of function in injured nerves and compared the results to those produced by sural nerve grafts. Schwann cells were isolated from sciatic nerves, prelabeled with gold fluorescent dye admixed with collagen gel, and placed in resorbable collagen tubes. Twenty-four adult rats underwent severing of the bilateral sciatic nerves, with a 10-mm gap between the nerve stumps. The rats were then divided into two groups. A collagen tube with implanted Schwann cells was implanted in one leg of the Group I rats, and the contralateral leg served as a control and was repaired with a collagen tube filled with collagen gel only. The Group II animals received conduits packed with labeled Schwann cells in one leg to bridge the 10-mm gap; the contralateral leg was repaired with an autogenous sural nerve graft. Recovery of function was assessed physiologically and morphologically.

Nerve conduction velocity and nerve action potential amplitude measurements showed that the Schwann cell implants induced return of function comparable to that of the sural nerve grafts. Morphological assessments of myelination suggested a tendency toward greater numbers of myelinated axons in Schwann cell implants than in sural nerve grafts. Anatomical analyses of gold fluorescent dye showed both high viability of prelabeled Schwann cells at 120 days after transplantation and migration as far as 30 mm away from the implant site.

Key Words • nerve regeneration • nerve conduit • Schwann cell • cell implantation • nerve action potential • sural nerve graft • rat

Severe nerve injuries occur secondary to stretch and contusion injuries to the limbs, missile wounds, fractures, lacerations, and tumor excisions. Surgical intervention for these nerve injuries commonly involves implantation of autogenous sural nerve grafts and intubation with inert prostheses. Autografting has been found to be more successful than intubation in augmenting recovery in severely injured nerves. Extracellular substances including laminin, fibronectin, and collagen have been reported to stimulate nerve regeneration. These neurotrophic substances may direct axonal regrowth by targeting receptor sites on neurite growth cones.

The versatility of Schwann cell reactions to nerve injury suggests that these cells make significant and multifaceted contributions to restoration of function. One essential component in recovery after severe injury is the establishment of Schwann cell cords (bands of Büngner) to bridge nerve gaps and direct neurite regrowth. Schwann cells produce type IV collagen and undergo mitosis in response to nerve injury to form these cords. Therefore, we assessed the ability of Schwann cell transplants to enhance the recovery of function in injured nerves and compared these results with those produced by autografting sensory nerves.

Materials and Methods

Treatment Groups

Twenty-four Sprague-Dawley rats, each weighing between 300 and 320 gm, were used in this study. All of the animals underwent severing of the bilateral sciatic nerves. The animals were then divided into two groups of 12 rats each. The Group I (Schwann cell vs. collagen gel) animals were intubated on one side with resorbable collagen conduits containing cultured Schwann cells and contralaterally with collagen tubes filled with collagen gel. The Group II (Schwann cell vs. sural nerve graft) animals were intubated with Schwann cells on one side, matched with sural nerve grafts contralaterally. Results were evaluated for each group at both 60 and 120 days postoperatively. A total of 36 nerves were evaluated: six in each of the three treatment groups at two survival points. Six of the 12
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Schwann cell implant-treated nerves were randomly selected to represent the group in all assays.

Animal Preparation

The animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (Ketalar) and acepromazine maleate. With the animals under deep anesthesia, the sciatic complexes were exposed bilaterally by splitting the hamstring muscle via a posterior midline thigh incision from the gluteal region down to the popliteal fossa. Hemostasis was obtained by means of bipolar coagulation. Under a Zeiss operating microscope, each sciatic nerve was isolated at its emergence from the sciatic notch, and the tibial division was sharply separated from the peroneal division and mobilized from the gluteal region to the popliteal fossa.

Electrophysiology

Electrophysiological studies of 48 posterior tibial nerves provided baseline nerve conduction velocities. The nerves were suspended proximally and distally on bipolar stimulating and recording electrodes, with the distance between the two electrodes averaging 25 mm. The stimulus, which was supplied via a stimulus isolation unit, was supramaximal so that the largest and most complete nerve action potential (NAP) was recorded. Stimuli were very brief (0.02 to 0.04 msec) to minimize shock artifact and its interference with the evoked NAP. A monopolar electromyographic electrode was placed in the gastrocnemius muscleature to record the presence or absence of muscle action potentials. Recordings were made with a Tektronix oscilloscope with differential amplifiers. Measurements of amplitude and conduction velocity were calculated using baseline recordings of NAP's.

Schwann Cell Cultures

Schwann cells were isolated from the sciatic nerves of young adult Sprague-Dawley rats. The nerves were removed, minced, placed in sterile flasks, covered with saline containing 0.25% trypsin plus 0.03% collagenase, and incubated at 37°C for 1 hour. The trypsin-cell mixture was centrifuged for 8 minutes at 1000 rpm to form pellets of cells. The pellets were resuspended in 20 ml minimum essential medium supplemented with 10% human placental serum, 0.6% glucose, 0.7% glutamine, 0.05% nerve growth factor, and 2 × 10⁻⁵ M fluorodeoxyuridine. The Schwann cells were divided into 1-ml aliquots, which were incubated at 37°C in 24-well plates. During the first 7 days, the antimetabolite drug fluorodeoxyuridine was added to the culture medium to eliminate fibroblasts. Cultures were examined through an inverted photomicroscope equipped with fluorescent capabilities to determine fibroblast contamination. Random wells were fixed for 1 hour in a solution containing 2.5% formaldehyde and 0.02% glutaraldehyde in phosphate-buffered saline.

Polyclonal S-100 antibodies, an established marker for Schwann cells, and rhodamine-conjugated second antibodies were applied and visualized with fluorescent microscopy. Horseradish peroxidase-conjugated second antibody was also applied to observe double labeling fluorescent and indacandescent light sources in the same sections. Since all cells were positive for S-100, the probability of fibroblast contamination was considered low.

Fluorescent Labeling Before Surgery

The unique tag Fluoro-gold was used to label cultured Schwann cells 24 hours before surgery. Its properties include intense staining, wide-band excitation (emission maximum 408 nm), resistance to fading, purity, and lack of interference with normal growth. Its most distinctive capacity lies in its ability to double-label cells directly and simultaneously for both light and electron microscopy. In light microscopic assays, Fluoro-gold is a direct fluorescent label, and at the ultrastructural level its electron-dense particles are apparent. Ultimately, Fluoro-gold was chosen to label Schwann cells because it does not leak from labeled cells. Fluoro-gold was mixed with 0.85% sterile saline at a concentration of 5 mg/ml and filter-sterilized; 100 μl of this solution was added to each well and incubated at 37°C for 24 hours. The cells were pooled, washed in 20 ml sterile saline, and centrifuged for 10 minutes at 400 rpm. The cells were pooled, washed in 20 ml sterile saline collagen gel and 20-μl aliquots were added to fill each of 24 sterile collagen tubes (length 1.2 cm, inner diameter 1 mm, and wall thickness 0.2 mm). Care was taken to count Schwann cells with a hemocytometer before they were implanted; approximately 4 × 10⁵ cells were loaded into each tube.

Surgical Lesion and Repair

All animals underwent bilateral resection of a 10-mm segment of the sciatic nerves via a fine dissecting microsissor. To repair these nerves, the Group I (Schwann cell vs. collagen gel) rats were implanted with a 12-mm collagen tube filled with the labeled Schwann cells in a collagen gel in one leg (Subgroup A), while the other leg (Subgroup B) received a 12-mm collagen tube filled with the collagen gel only. The Group II (Schwann cell vs. sural nerve graft) animals were implanted with a 12-mm collagen tube filled with the labeled Schwann cells in a collagen gel in one leg (Subgroup C), while the other leg (Subgroup D) received a 2 × 10⁵-mm sural nerve graft (Fig. 1).

All nerves were repaired with 10-0 monofilament Ethilon sutures. The tubes were secured with two epineurial stitches placed 180° from each other, while the interpositional sural nerve grafts were secured to the

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* Stimulator, Model 44, and stimulus isolation unit, Model STU-5, manufactured by Grass Instrument Co., Cambridge, Massachusetts.

† Fluoro-gold supplied by Fluorochrome, Inc., Englewood, California.

‡ Collagen gel provided by Collagen Corp., Palo Alto, California; collagen tubes manufactured by American Biomaterials Corp., Plainsboro, New Jersey.

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proximal and distal stumps of the posterior tibial nerve by applying one suture to each stump.

Postrepair Invasive Evaluation

At 60 or 120 days postoperatively, the animals were anesthetized and their repair sites explored. Following gross inspection and debridement of scar tissue, measurements of compound NAP amplitudes were recorded.

Each animal was sacrificed while under deep anesthesia. The sciatic nerves were removed and processed histologically for quantitative and qualitative morphometric analysis. Each sciatic nerve was dissected into three segments for light microscopic analysis: the graft tissue itself, the tissue immediately proximal to the graft, and the tissue immediately distal to the graft. Before the tissue was placed into fixative, small specimens were cut from each nerve for electron microscopic analysis. The light microscopy specimens were fixed for 1 hour in 0.02% glutaraldehyde, 2.0% formalin/0.1 M sodium phosphate buffer (pH 7.2). The fixed specimens were immersed for 45 minutes in 0.1 M glycine to quench aldehydes, and then placed in a serially concentrated cryoprotectant solution (30% to 80% sucrose in 0.1 M sodium phosphate buffer) for 8 hours. Cryosections 10 μm in length were cut from each specimen and examined by fluorescent microscopy for Fluoro-gold.

The tissue sections were stained with Sudan black in standard preparation for localizing myelin. Slides were postfixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C. Osmium tetroxide (0.1%) was applied for 1 hour at room temperature after the slides were rinsed with 0.1 M sodium phosphate buffer. The slides were rinsed serially with 30%, 50%, and 70% alcohol at 5-minute intervals, then incubated in 0.5% Sudan black stain for 1 hour at room temperature. The slides were again rinsed in 70% alcohol, serially rehydrated with distilled water, placed in xylene, and coverslipped. The number of myelinated fibers in distal sections of recovered nerves was estimated by averaging four independent counts.

The specimens for electron microscopy were placed in fixative consisting of: 10% formaldehyde stock solution (40%), 2% glutaraldehyde stock solution (50%), and 88% Millonig buffer (17.0 ml 2.25% NaOH, 83.0 ml 2.25% NaH2PO4, and 33.0 ml H2O). The specimens were processed for electron microscopy by serial alcohol dehydration, infiltration with Epon/Araldite resin, and polymerization at 60°C. These sections were placed on copper grids, stained with uranium acetate and lead citrate, viewed, and photographed under the electron microscope.

Results

At the time of exploration 60 or 120 days postrepair, there were no signs of infection or autophagia in the legs in any of the 24 animals. Pressure ulcers were observed on the heels of three animals, but no serious complications were noted. By 2 weeks after nerve injury, atrophy of the gastrocnemius muscle became evi-
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Fig. 3. Graphs showing nerve conduction velocity and nerve action potential amplitude recovery for Group I (Schwann cell vs. collagen gel) animals (upper) and for Group II (Schwann cell vs. sural nerve graft) animals (lower) at both 60 and 120 days.

Electrophysiology

The baseline conduction velocities measured before transection of the posterior tibial nerves in 24 rats averaged 60 m/sec. Nerve action potential amplitudes and nerve conduction velocities were recorded at a postoperative interval of either 60 or 120 days. Statistical analyses of variance were performed to compare NAP amplitude and conduction velocities for all subgroups at 60 and 120 days postrepair (Figs. 2 and 3). Comparison of Schwann cell implants (Subgroups A and C) with collagen gel intubation (Subgroup B) at 60 days postrepair showed that the Schwann cell group had greater average values for NAP amplitude and conduction velocity than did the collagen gel group, but these differences were not statistically significant (NAP amplitude: $p = 0.0611$; conduction velocity: $p = 0.1030$). Comparison of Schwann cell implants (Subgroups A and C) with sural nerve grafts (Subgroup D) showed that the average value for NAP amplitude and conduction velocity of the Schwann cell group was greater than that of the sural nerve graft group; however, these differences were not statistically significant (NAP amplitude: $p = 0.3843$; conduction velocity: $p = 0.0556$).

Comparison of Subgroups A and C with Subgroup B at 120 days postrepair showed that the Schwann cell group had a significantly greater NAP amplitude and conduction velocity than did the collagen gel group (NAP amplitude: $p = 0.0483$; conduction velocity: $p = 0.0277$). Comparison of Subgroups A and C with Subgroup D showed that the Schwann cell group had significantly greater NAP amplitude values than did the sural nerve graft group; however, no statistically significant difference was demonstrated in conduction velocity when comparing the two groups (NAP amplitude: $p = 0.045$; conduction velocity: $p = 0.6381$).

Morphological Analyses

Four normal and four experimental sciatic nerves were removed 10 days after implantation. From these, S100-positive Schwann cells were isolated and counted. The mean number (± standard deviation) of Schwann cells isolated from 30-mm sections of the four normal sciatic nerves was $1 \times 10^6 \pm 3 \times 10^5$. We assumed that each 10-mm resection removed approximately $3.34 \times 10^6$ Schwann cells from the nerve. The number of implanted Schwann cells (approximately $4.0 \times 10^6$) was determined to compensate for this loss. The average number of Schwann cells isolated from 15-mm segments of the four experimental nerves was $9 \times 10^5 \pm 1 \times 10$, or 90% of that expected.
Fig. 4. Photomicrographs of nerve cross-sections obtained 60 days after Schwann cell transplantation. A: Schwann cells (arrows) were identified throughout the nerves. Bar = 100 μm. B: Phase contrast revealed reorganization of nerve morphology. Schwann cells (arrows) were identified with S-100 and rhodamine-conjugated fluorescent antibodies. C: The same pattern was demonstrated using the fluorescent gold label to mark transplanted cells (arrows).

Fig. 5. Photomicrographs of nerve cross-sections obtained 120 days after repair with Schwann cell transplants. A: Immunoperoxidase-stained S100-positive Schwann cells (arrows) were identified throughout the nerves. Bar = 100 μm. B: Most Schwann cells in this section were also positive for the fluorescent gold label used to mark transplanted cells (arrows).

in a normal segment. Therefore, the 33% loss of Schwann cells due to the excision was compensated for at 10 days after implantation with the 23% gain from the Schwann cell transplants.

Fluoro-gold-labeled Schwann cells were identified throughout the nerves after 60 and 120 days of survival (Figs. 4 and 5). In distal nerves, individually labeled Schwann cells were observed to have traveled as far as 10 mm from the transplant site after 60 days and as far as 30 mm from the transplant site after 120 days. Because uninjured fibers of passage reportedly do not absorb Fluoro-gold, presumably sprouted fibers were labeled in some distal nerve sections. Implanted cells double-labeled with Fluoro-gold and S-100 antibodies visualized with horseradish peroxidase-conjugated antibodies were also observed (Fig. 5).

Schwann cells were identified ultrastructurally with prominent, blotchy nuclei. On electron photomicrographs, labeled Schwann cells contained cytoplasmic fluorescent gold dye particles (Fig. 6).

The number of myelinated fibers (Table 1) were counted by four examiners unaware of the treatment group they were counting. Four different, randomly selected nerves from each group were analyzed. Analysis of variance (Tukey's test) indicated that significant differences existed between the nerve count groups (p < 0.05). Therefore, post hoc comparisons were analyzed with Student t-tests to compare myelinated fiber counts. At 60 days postoperatively, the Schwann cell
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Fig. 6. Electron micrographs of experimental sciatic nerves showing multiple dense particles (arrows) in the cytoplasm of Schwann cells at 60 days postrepair (A) and at 120 days after transplantation (B). The fluorescent gold label did not appear to interrupt reformation of myelin (B). Bars = 1 μm (A), 0.3 μm (B).

transplant group had a significantly greater number of myelinated fibers than did the sural nerve graft group (p < 0.001), which in turn had a significantly greater number of myelinated fibers than the collagen gel group (Table 2). At 120 days postoperatively, there was no significant difference in the number of myelinated fibers between the Schwann cell and sural nerve graft groups. A statistically significant difference was found between the number of myelinated axons found in the Schwann cell group as compared with the collagen gel group (Table 2).

Discussion

In transected peripheral nerves, degeneration of axons and myelin occurs distal to the lesion. In the distal segment, the Schwann cells begin to undergo mitotic division to form columns of Schwann cells known as bands of Büngner which, during axonal regeneration, are thought to channel axons to distal target sites. Over a period of time, the myelin and axonal degenerated fragments are phagocytosed by macrophages to prepare a future course for regenerating axons. During nerve regeneration, regenerating axons stimulate Schwann cells to proliferate and migrate from both proximal and distal nerve stumps toward these axons. Regenerating axons encoded to be myelinated send signals to Schwann cells to initiate the myelination process. During development, however, a direct contact between axon tip and Schwann cell is necessary to activate Schwann cell division for the subsequent formation of the myelin sheath. \(^2,4,20\) Similar mechanisms may exist in regenerating nerves. \(^6,7,11,17,21\)

### TABLE 1

Myelinated axon counts in regenerated distal sciatic nerves

<table>
<thead>
<tr>
<th>Measurement*</th>
<th>60 Days After Repair</th>
<th>120 Days After Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schwann Cell Implant</td>
<td>Collagen Gel</td>
</tr>
<tr>
<td>count 1</td>
<td>4995 ± 942</td>
<td>3825 ± 1166</td>
</tr>
<tr>
<td>count 2</td>
<td>4329 ± 400</td>
<td>3900 ± 3500</td>
</tr>
<tr>
<td>count 3</td>
<td>6327 ± 2500</td>
<td>5700 ± 3900</td>
</tr>
<tr>
<td>count 4</td>
<td>4329 ± 3000</td>
<td>3900 ± 3500</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>4995 ± 942</td>
<td>3415 ± 1166</td>
</tr>
</tbody>
</table>

* SD = standard deviation.
TABLE 2
Statistical comparison of pre- and postoperative myelinated fiber counts in distal sciatic nerves

<table>
<thead>
<tr>
<th>Days After Repair</th>
<th>Comparison</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>Schwann cell implant vs. sural nerve graft</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>60</td>
<td>sural nerve graft vs. collagen gel</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>120</td>
<td>Schwann cell implant vs. sural nerve graft</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>120</td>
<td>sural nerve graft vs. collagen gel</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

As the regenerating axons propagate distally, orientation of axonal regrowth is thought to be influenced by molecules such as laminin and fibronectin, which are found within the extracellular matrix of the basal lamina of the Schwann cell plasmalemma. Laminin and fibronectin are believed to direct axonal regrowth by targeting receptor sites on the growth cone of regenerating axons, promoting neurite growth. In vitro studies have shown that interaction between Schwann cells and neurons is necessary for their survival and that trophic factors produced by Schwann cells are necessary for the growth and maturation of neurites.

Morphological aspects of this study involved Schwann cells labeled with gold fluorescent dye before transplantation. These labeled Schwann cells were observed in both proximal and distal regions of regenerating nerves. The viability of transplanted Schwann cells was demonstrated by postsurgical isolation from regenerating nerves. Morphological assays, including myelinated fiber counts, revealed a greater number of myelinated axons present in the Schwann cell transplant repairs than in the sural nerve graft repair. Labeling did not obstruct development of myelin in recovered nerves. When combined, the multidimensional results of this study support the long-standing presumption that Schwann cells make significant contributions to the complex process of nerve regeneration.20 In fact, the similarity in nerve autografts and Schwann cell treatment suggests that Schwann cells may be vital for recovery. More refined studies are underway that will help to define these contributions; eventually this knowledge may contribute to the development of more comprehensive treatment.

References

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Address for Mr. Connolly: Department of Neurosurgery, Ochsner Clinic and Alton Ochsner Medical Foundation, New Orleans, Louisiana.

Address for Mr. Smith, Powell, and Yoes, and Dr. Daniloff: Department of Anatomy and Cell Biology, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana.

Address reprint requests to: Daniel H. Kim, M.D., Department of Neurosurgery, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112.