Morphological and neurochemical assessment of regenerated peripheral nerves in bridging conduits

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Abstract

This research evaluated the use of proteins associated with neurite outgrowth, synapse formation, and Schwann cell proliferation as surrogate measures for morphological measurement of rat sciatic nerve regeneration across a 10-mm gap in silicone rubber conduits (SRCs), genipin-crosslinked gelatin conduits (GGCs), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ N-hydroxysuccinimide (NHS)-crosslinked gelatin conduits (ENGs). After 8 weeks, axonal growth of regenerated nerves was determined using light microscopy and computer-based quantitative image analysis. Expression of the axonal growth associated protein 43 (GAP-43), the synaptic protein synapsin I, and the transforming growth factors type β (TGF-βs) in regenerated nerves were assessed simultaneously by Western blot. As a result, the nerve regenerates in the SRCs and the ENGs had a significantly larger endoneurial area containing more myelinated axons compared to those in the GGCs at $p<0.05$. The levels of GAP-43 and synapsin I, but not TGF-β, correlated well with axonal growth in the regenerated nerves. These data suggest that the combined techniques can be used to assess the status of regenerating axons in bridging conduits with different construction and physical properties.

Keywords: Polymer conduit; Peripheral nerve regeneration; Nerve injury

1. Introduction

As designing soft polymer scaffolds used for nerve regeneration, investigators must evaluate the effect of tube characteristics on the quality of regenerated nerves. Nondegradable tubes can provide an isolated and stable environment for the nerves to regenerate across the gap and toward the distal stump. However, a second surgery is necessary to remove the conduit which may cause possible damage to the nerve. To eliminate this disadvantage, a biodegradable nerve chamber seems to offer the greatest promise as an ideal tubulization material. In the past few years, my group has successfully developed several types of polymer chambers for peripheral nerve regeneration. However, the dynamic nature of the developing nervous system is so complex that it is not easy to assess the suitability of the implanted materials in the animal just based on a single criterion. We have tried to use electrophysiological methods accompanied with morphological observations to evaluate regenerated nerves in the bridging conduits.[1,2] However, large variations in the electrophysiological measurements could be seen which may result from serious gastrocnemius muscle atrophy even though the muscle fibers had been reinnervated.[3] Gait analysis is another popular way to evaluate the nerve regenerates. However, the missing toes caused by automutilation due to nerve injury could result in inconsistent walking patterns.[4,5] In the present study, we therefore tried to use neurochemical ways to assist in the morphological assessments to evaluate regenerated nerves repaired with polymer scaffolds.

Neurochemical measurements of specific proteins such as neurotypic and glialtypic proteins have been used to detect injury in the developing nervous system *in vivo*.[6] Although not widely applied to date, the use of neurochemical measures of nerve regeneration has the potential to add to data obtained from the use of the

abovementioned morphological measures. A number of neurotypic and glialtypic proteins have been associated with PC12 cell differentiation and Schwann cell proliferation, including growth associated protein 43 (GAP-43), presynaptic membrane-associated proteins, such as synapsin, and type β transforming growth factors (TGF-βs).[7-9]

The present study examined the use of proteins associated with neurite outgrowth, synapse formation, and Schwann cell proliferation and myelination as surrogate measures of rat sciatic nerve regeneration across a 10-mm gap in nondegradable silicone rubber conduits (SRCs) and degradable genipin-crosslinked gelatin conduits (GGCs) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ *N*-hydroxysuccinimide (NHS)-crosslinked gelatin conduits (ENGs). We used the three types of bridges here not to demonstrate that one has greater efficacy for a regenerative medium, but rather to test various types that investigators might employ and, thus, to test if the neurochemical markers, such as GAP-43, synapsin I, and TGF-β are sensitive to chemical disruption of regenerated nerves under differing conditions. These neurochemical markers were determined at the same period of time and compared with axonal growth in the regenerated nerves assessed using the morphological methods among the three types of polymer chambers.

2. Materials and Methods

2.1. Fabrication of GGCs and ENGs

A 10% (w/w) solution of gelatin (300 bloom number, Sigma #G2500) in distilled water was prepared by magnetic stirring. A silicone rubber tube (1.96 mm OD; Helix Medical, Inc., Carpinteria, CA) was used as a mandrel vertically dipped into the gelatin solution at a constant speed where it remained for 5 min. The mandrel was

then withdrawn slowly and allowed to stand for 30 min for air-drying. The mandrel was rotated horizontally consistently to reduce variations in the wall thickness along the axis of the tube. Eight coating steps were used to obtain a gelatin tube with wall thickness of about 470 μm. The gelatin-coated mandrel was then immersed in 1% (w/w) solution of genipin (Challenge Bioproducts Co., Taichung, Taiwan) for 1.5 hr for cross-linking. The coated mandrel was rinsed twice with distilled water, dehydrated for 10 min with 95% of ethanol, and air-drying for 1 week. The GGCs were slipped off the silicone rubber mandrel and cut to 12 mm length. Similarly, ENGs were made by immersing the gelatin-coated mandrel in a solution containing 0.032 M of EDC and 0.0128 M of NHS (Sigma Chemical Co., St. Louis, MO) for 28 hours for cross-linking. The subsequent treatments for the ENGs were the same as abovementioned procedures for the GGCs. Finally, the GGCs and the ENGs were sterilized with 25 kGy of γ-ray for subsequent implantation. To allow fixation of the nerve tissue to the conduit, two small holes were drilled at both ends of the GGCs and the ENGs.

2.2. Cross-linking Degree of GGCs and ENGs

Ninhydrin assay was used to evaluate the cross-linking degree of GGCs and ENGs. Ninhydrin (2,2-dihydroxy-1,3-indanedione) was used to determine the amount of amino groups of each test sample. The test GGCs and ENGs were heated with a ninhydrin solution at 100°C for 20 min. After heating with ninhydrin, the optical absorbance of the solution was recorded using a spectrophotometer (Model Genesys™ 10, Spectronic Unicam, New York, NY) at 570 nm (wavelength of the blue-purple color) using glycine at various known concentrations as standard. The amount of free amino groups in the residual gelatin, after heating with ninhydrin, is

proportional to the optical absorbance of the solution. Six samples were tested to determine the cross-linking degree of GGCs and ENGs.

2.3. Bridging Conduit Implantation

A total of 36 adult Sprague-Dawley rats underwent placement of SRCs (1.47 mm ID, 1.96 mm OD; Helix Medical, Inc., Carpinteria, CA), GGCs, and ENGs (12 rats per each tube group), which were removed upon sacrifice at 8 weeks. The animals were anesthetized with an inhalational anesthetic technique (AErrane®, Baxter, USA). Following the skin incision, fascia and muscle groups were separated using blunt dissection, and the right sciatic nerve was severed into proximal and distal segments. The proximal stump was then secured with a single 9-0 nylon suture through the epineurium and the outer wall of the nerve conduits. The distal stump was secured similarly into the other end of the chamber. Both the proximal and distal stumps were secured to a depth of 1 mm into the chamber, leaving a 10-mm gap between the stumps. The muscle layer was re-approximated with 4-0 chromic gut sutures, and the skin was closed with 2-0 silk sutures. All animals were housed in temperature (22°C) and humidity (45%) controlled rooms with 12-hour light cycles, and they had access to food and water ad libitum. All animals were maintained in facilities approved by the China Medical University for Accreditation of Laboratory Animal Care, according to the regulations and standards of the National Science Council of Health of the Republic of China.

2.4. Histological Measures

After an 8-week survival period, rats were euthanized with 0.4 g of chloral hydrate (IP). Sciatic nerve sections were then extracted from the middle of the

regenerated nerve (3.5 mm – 7 mm region) in the chamber. The rest of the nerve tissues were used for later neurochemical measurements. Following fixation, the nerve tissue was post-fixed in 0.5% osmium tetroxide, dehydrated, and embedded in spurs. The tissue was then cut to a thickness of 5 μm using a microtome with a dry glass knife, stained with Toluidine Blue. All tissue samples were observed under a light microscope (Olympus IX70, Olympus Optical Co., Ltd., Japan). An image analyzer system (Image-Pro Lite, Media Cybernetics, USA), coupled to the microscope then counted the blood vessels and calculated the cross-sectional area of each the nerve section at magnifications of between 40x and 400x. At least 30 to 50% of the nerve section area was randomly selected from each nerve specimen at a magnification of 400x to count the axons. The axon counts were extrapolated by using the area algorithm to estimate the total number of axons in each nerve. All data are expressed as mean ± standard deviation. Statistical comparisons between groups were made using the one-way ANOVA. A P value of < 0.05 was considered statistically significant.

2.5. Neurochemical Measures

Nerves segments were washed with ice-cold PBS and sonicated under 50-100 μl of lysis buffer (50 mM Tris (pH 7.5), 0.5M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1 mM BME, 1% IGEPAL-630 and proteinase inhibitor cocktail). The tissue was incubated at 4°C for 15 min and centrifuged at 12,000 rpm for 15 min at 4°C. GAP-43, synapsin I, and TGF-β were then quantified using electrophoresis and Western blot analysis. Protein concentration of nerve extracts was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA). Protein samples (50 μg/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with

a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a transfer apparatus (Bio-Rad Protein Assay, Hercules, CA). PVDF membranes were incubated in 5% milk in TBS buffer. Primary antibodies including GAP-43 (Abcam, Cambridge, MA), synapsin I (Millipore, Bedford, MA), and TGF-β(Millipore, Bedford, MA) and α-tubulin (Neo Markers, Fremont, CA) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP for 1 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. For repeated blotting, nitrocellulose membranes were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL) at room temperature for 30 min. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL Western blotting luminal reagent (Santa Cruz, CA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan). Statistical comparisons of Western blot data between groups were made using the one-way ANOVA. A P value of < 0.05 was considered statistically significant.

3. Results

Figure 1 shows macroscopic observations of the bridging conduits. The SRCs had a semitransparent chamber lumen whereas the GGCs were dark blue in appearance caused by the reaction between genipin and amino acids or proteins. The GGCs were concentric and round with rough outer surface and the inner lumen was smooth. By comparison, the ENGs were brownish in appearance which were

concentric and round with smooth outer surface and inner lumen. The cross-linking index of GGCs and ENGs, expressed as a percentage of free amino groups lost during cross-linking, was 39±2% and 30±2%, respectively. It means about one-third of the amino groups in these bridging conduits have been fixed by 1.0 wt.% of their cross-linking agents.

Light microscopy of the regenerated nerves retrieved from the bridging conduits is shown in Figure 2. After 8 weeks of implantation, all the SRCs were intact with no swelling or deformation. Brownish fibrous tissue encapsulation covered all over the chamber and the parts of the nerve stumps in the chamber openings. However, the regenerated nerve could be seen through the chamber lumen after trimming the fibrous tissue. No nerve dislocation was noted and the regenerated nerve, which was surrounded by fluid, occupied a central location within the chamber. By comparison, the GGCs featured a partially fenestrated outer layer, however, they still remained circular with a round lumen. As for the ENGs, their process of swelling and degradation was more obvious. Gross examination of the SRCs, GGCs, and ENGs revealed successful regeneration exhibiting a nerve cable across the 10 mm gap in 50%, 100%, and 100% of the animals, respectively.

Figure 3 shows representative cross sections of nerve specimen retrieved from each group. The epineurial and perineurial regions of the regenerated nerves in the SRCs consisted mainly of a collagenous connective tissue matrix in which circumferential cells resembling perineurial cells and fibroblasts were seen. In addition, the nerve fibers were packed in the nerve bundles with fluted appearances. Nuclei of Schwann cells were interspersed among these nerve fibers. Axons in the endoneurium were easily defined by their surrounding myelin sheaths stained dark blue by the toluidine blue. Blood vessels were numerous in the epineurium as well as

in the endoneurial areas of the nerve. By comparison, the regenerated nerves in the GGCs displayed a structure with a thin epineurium surrounding by thick fibrous tissues. The endoneurium was cellular and vascularized in which Schwann cells organized in clusters surrounding groups of unmyelinated axons were present. These axon-Schwann cluster formations, termed as regeneration units, are common organization structures seen under nerve cuff bridging conditions. In addition, Schwann cell columns were also seen, which may participate in the early scaffold formation for the migration of advancing axonal tips. As seen in the GGCs, it is still difficult to discriminate between the epineurium from the surrounding thick fibrous tissues of the regenerates in the ENGs. However, the regenerated nerves in the ENGs were relatively more mature compared to those in the GGCs, displaying a cellular and vascularized endoneurium in which numerous myelinated axons had been seen.

Morphometric studies revealed the nerve regenerates in the SRCs and the ENGs had a significantly larger endoneurial area containing more myelinated axons compared to those in the GGCs at $p<0.05$ (Figure 4). In the Western blot analysis, the nerve regenerates in the SRCs had a significantly higher expression of GAP-43 and synapsin I compared to those in the GGCs and the ENGs. However, the TGF-β expression was significantly higher in the ENG group as compared to the groups of SRC and GGC at $p<0.05$ (Figure 5).

4. Discussion

A wide range of soft materials has been developed for use as nerve grafts.[10] Although the ideal material for a nerve guide has not been identified, successful materials must fulfill the following requirements: be inert (biocompatible), be thin and flexible, inhibit the proliferation of fibroblasts and connective tissue surrounding the

injured nerve, and promote healing and regeneration.[11] Based on these criteria, silicone rubber could be the most acceptable material used to make the nerve bridging conduit because of its stable properties which can aid guidance of growing fibers along appropriate paths by mechanical orientation and confinement, and enhance the precision of stump approximation.[12,13] Recently, degradable materials have widely been used for tubulization to bridge damaged nerves, such as polyglycolic acid and polylactic acid.[14] In addition, collagen has also been used to fabricate the nerve conduit with favorable results.[15] However, collagen is rather expensive and may express antigenicity in physiological conditions.[16] With respect to collagen, gelatin, which is essentially denatured collagen, is much cheaper and biocompatible.[17] Therefore, it has a myriad of uses in food, pharmaceutical, cosmetic, industries, wound dressings, bone substitutes, extracellular matrices, and neuro-plastics. However, the gelatin must be cross-linked if it is expected to be used as a stable nerve conduit material without losing its integrity. Different cross-linkers have been used to fix the gelatin, such as the genipin and the EDC/NHS used in the present study to make nerve substitutes for transplantation resistant to natural biodegradation.[18,19] In the present study, 100% of the animals receiving the GGC and the ENG implants exhibited regeneration across the nerve gaps, whereas only 50% had regenerated in the SRC. However, the quality of these successfully reconnected nerves in the SRCs was much better than those in both the degradable bridging conduits. We noted a thick collagen capsule resembling fibrous scar tissue with scattering contractile cells surrounded the regenerated nerve in the GGCs and the ENGs. In contrast to both the degradable chambers, the nerve regenerate in the SRC had thin layers of myofibroblasts around the perimeter of the nerve trunk. While examining the axonal development, we found the regenerates inside SRCs and ENGs were clearly superior

to those regenerated inside GGCs. These results imply that the maturity of regenerated nerves depended very strongly on the type of bridging conduits. It is most likely that the degraded materials of the GGCs triggered the cellular activity vigorously, causing an inflammatory reaction accompanied with scar tissue formation. The fibrous scar tissue formed a contractile cell capsule around the regenerating nerve that appeared to restrict axonal growth by application of circumferential mechanical forces. However, penetration by the regenerated axons in the ENGs seemed not delayed. The gap inside the ENG had been successfully bridged by a relatively larger number of myelinated axons though they were also surrounded by a thick fibrous capsule. Therefore, we believe that the pressure capsule may not be the only factor causing the poor regenerated nerve tissue in the GGCs. We have reported earlier that the GGCs remained with only mild degradation even after 8 weeks of implantation. The reduced elasticity of the chamber and an increased hydrophobic surface after genipin-treatment may obstruct cell attachment on the GGCs, resulting in mobilization and activation of Schwann cells was not perceptible. This could be another reason that the nerve regenerates in the GGCs were less mature than those in the ENGs, which degraded at a rate in accordance with the speed of axonal elongation during regeneration. By comparison, the cellular bridge in the SRC was well established with a circular form surrounded by circumferential cell layers, in which a large number of myelinated axons had been seen. Accordingly, we can conclude that the SRC could present a continuous framework for the nerve fibers to regenerate across the gap because of its non-resorbability. Ironically, the highly stable property of the silicone rubber may also be the reason that only 50% of the animals had successfully reconnected nerve inside the SRC. Without the rapid progress of a continuous inflammatory reaction triggered by degraded materials as seen in the GCC

and the ENG, formation of such a tissue cable inside the SRC could be more challenging. Nevertheless, silicone rubber is still a good choice used to make the bridging chambers. It has been demonstrated that SRCs are well tolerated in humans even after 3 years of implantation.[20]

After examining the morphological differences, the expression of GAP-43, synapsin I, and TGF-β was determined over the same time period of nerve regeneration among the 3 types of bridging conduits. GAP-43 is a marker for growth cones and elongating axon of developing neurons.[21] High levels of GAP-43 expression are correlated with the beginning of neurite outgrowth. As for the synapsins, they are abundant phosphoproteins essential for regulating neurotransmitter release and increase in level with the formation of mature synapses in developing cell cultures.[22] In the present study, the expression of GAP-43 and synapsin I of nerve regenerates was significantly higher in the SRCs as compared to the GGCs and ENGs. This result is similar to the morphological measurements of nerve regeneration in that the regenerates in the SRCs had a more mature microstructure with a relatively larger area containing more myelinated axons. These results imply that though the SRCs were nondegradable, they still provided a good growth environment for the nerve fibers to regenerate across the gap and toward the distal stump.

As for the TGF-β, it is a potent mitogen for purified rat Schwann cells which can stimulate DNA synthesis in quiescent cells and increase their proliferation rate.[23] In adult animals, Schwann cells can provide their basal lamina as a substratum for the regenerating axons to adhere and grow.[24] In the present study, the expression of TGF-β of nerve regenerates was higher in the ENGs and the GGCs as compared to the SRCs. In particular, the difference between the TGF-β of the ENGs and the SRCs

reached the significant level at $p<0.05$. This result is not consistent with our morphometric measurements since if the Schwann cells are beneficial to regenerating axons, the expression of TGF-β of nerve regenerates in the SRCs should be the highest than the other two types of bridging conduits. This result inspired us to look deeper into the TGF-β for its role playing in the nerve regeneration. In the literature, we found that the TGF-β had versatile functions. In addition to the Schwann cell proliferation-promoting capability, the TGF-β can also activate monocytes, generating fibroblast growth-promoting factors to induce fibrosis.[25] This means that the implanted nerve scaffold could produce immune reaction, evaluating levels of cytokine genes in the implanted region, such as the expressions of IL-1b, IL-18, IL-33, IL-6, and IL-24.[26] Based on these theories, we believe the high levels of TGF-β in the ENGs and GGCs were coming from the thick fibrous tissues at the outer layers of the nerve regenerates, whereas the nerve regenerates in the SRCs had no such mass with only a thin epineurium surrounding the inner endoneurium.

5. Conclusion

The present study shows that the *in vivo* morphological and neurochemical measurements can be used to speculate how bridging conduit parameters affect the quality of nerve regeneration. The use of the two assessing methods with nerve bridging conduits should increase greatly the ability of investigators to select proper soft materials for nerve regeneration. In addition, investigators should examine all the criteria, including morphological alterations, electrophysiological recovery, and the neurochemical indices before defining a so-called ‘successful nerve regeneration’ within a guidance tube.

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Captions

Fig. 1. Macrographs of (a) SRC, (b) GGC, and (c) ENG.

Fig. 2. (a) SRC, (c) GGC, and (e) ENG 8 weeks of implantation. Regenerated nerve exposed after trimming the remained bridging materials of (b) SRC, (d) GGC, and (f) ENG.

Fig. 3. Photomicrographs of regenerated sciatic nerve sections 8 weeks after surgery stained with Toluidine Blue. (a) Regenerated nerve repaired with SRC had a well-defined structure in which the endoneurium was highly cellular and vascularized surrounded by a thin layer of epineurium. By comparison, regenerated nerve repaired with (c) GGC and (e) ENG formed a thick fibrous capsule surrounding the inner endoneurium. Numerous myelinated axons had been seen in the regenerated nerve repaired with (b) SRC and (f) ENG, whereas mostly unmyelinated axons in (d) GGC. Scale bars in a, c and e = 100 μm; b, d and f = 30 μm. Arrow: myelinated axon;

Circle inside: endoneurial area.

Fig. 4. Morphometric analysis from regenerated nerves in bridging conduits, including (a) endoneurial area and (b) myelinated axon count. Data are means ± S.E. (n=12). *Significantly different at $p<0.05$.

Fig. 5. Representative immunoblots for (a) GAP-43, (b) synapsin I, and (c) TGF-β, and quantification of the three protein levels in regenerated nerves. *Significantly different at $p<0.05$.