EDITOR'S CHOICE

Evaluation of the Induced Membrane for Neurotrophic Factors

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Purpose Despite gaining popularity as a bridge for small and moderate nerve gaps, an acellular nerve allograft (ANA) lacks many of the neurotrophic characteristics of a nerve autograft. Pseudomembranes induced to form around temporary skeletal spacers are rich in growth factors. Induced membranes may have beneficial neurotrophic factors which could support ANA.

Methods Twenty-two male Sprague-Dawley rats underwent resection of 2 cm of the sciatic nerve. A silicone rod was inset in the defect of 11 experimental rats, and marking sutures only were placed in the nerve stumps of the remaining 11 control rats. After allowing 4 weeks for tissue maturation, tissue samples harvested from the induced membrane (experimental group) and the tissue bed (control group) were analyzed using Luminex multiplex assay to quantify differences in detectable levels of the following neurotrophic factors: nerve growth factor, glial-derived nerve factor, vascular endothelial growth factor, and transforming growth factor ß (TGF-ß) 1, 2, and 3, interleukin-1ß, and monocyte chemoattractant protein 1.

Results No difference was detected between the control and experimental groups in levels of vascular endothelial growth factor. Higher levels of TGF-ß1, TGF-ß2, TGF-ß3, glial-derived nerve factor, nerve growth factor, monocyte chemoattractant protein 1, and interleukin-1ß were detected in the experimental group.

Conclusions In the setting of peripheral nerve injury, an induced membrane has higher levels of several neurotrophic factors that may support nerve regeneration compared to wound bed cicatrix.

Clinical relevance This investigation provides impetus for further study examining the utility of using a staged induced membrane technique in conjunction with delayed nerve grafting in reconstruction of some peripheral nerve defects. *(J Hand Surg Am. 2022;47(2):130–136.*

Key words Induced membrane, Luminex, nerve injury, nerve reconstruction, neurotrophic factors.

SEGMENTAL PERIPHERAL NERVE injuries cause significant morbidity and offer unique reconstructive challenges. Current repair methods involve the use of an allograft or autograft. Acellular nerve allograft (ANA) is an "off the shelf" bridging tool for overcoming small and medium gaps in peripheral nerve surgery. With the same 3-dimensional architecture as a nerve autograft, ANA provides an internal scaffold to support and guide axonal regeneration without donor site morbidity. However, in longer length grafts, unsatisfactory axonal ingrowth is generally attributed to degradation of the neurotrophic environment within the ANA.[1]

Many strategies have been pursued to improve the clinical effectiveness of ANA. Based on normal physiology, the number of possible therapeutic

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Received for publication October 29, 2020; accepted in revised form August 24, 2021.

No benefits in any form have been received or will be received related directly or indirectly to the subject of this article.

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0363-5023/22/4702-0003$36.00/0
https://doi.org/10.1016/j.jhsa.2021.08.023

targets is extensive. Following peripheral nerve transection, through Wallerian degeneration, Schwann cells dedifferentiate, remove myelin debris, and secrete trophic factors that recruit macrophages and promote axon regeneration.[2] Macrophages in turn also remove debris in the distal stump and release factors promoting axon regeneration.[2] Dedifferentiated Schwann cells recruit macrophages with secretion of interleukin-1ß (IL-1ß), chemokine (C-C motif) ligand 2 (CCL2) (also known as monocyte chemoattractant protein 1 [MCP-1]), and transforming growth factor ß (TGF-ß), among other factors. Recruited macrophages also express IL-1ß, TGF-ß, MCP-1, and vascular endothelial growth factor (VEGF).[3–15] Axonal regeneration is dependent on, and contiguous with, the process of Wallerian degeneration and is associated with several additional neurotrophic factors, such as nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF), also secreted by Schwann cells and macrophages.[16–23]

The induced membrane technique described by Masquelet et al[24] is an accepted, reproducible, staged method of spanning osseous defects.[25] The success of this technique[25] has been attributed, at least in part, to the high concentration of growth factors such as VEGF and TGF-ß that have been identified within the membrane at 4 weeks.[26,27] Based on the well-established application in tendon surgery, Zadegan et al[28,29] introduced the concept of 2-stage nerve reconstruction using a silastic rod to induce a vascularized and scar-free pseudomembrane tunnel for delayed nerve grafting. Histologically, increased vascularity was noted in rats treated with this 2-stage technique compared with delayed grafting in an induced scar bed model, though their evaluation did not assess either growth factor levels or axon histomorphology. Brief delays in nerve grafting may be clinically relevant and allow demarcation of the injured nerve tissue requiring resection prior to repair, and similar to the application of this technique in tendon surgery, 2-staged nerve reconstruction may offer additional benefits in limiting perineural scarring.

There were 3 goals for our experiment: (1) to induce a membrane around a silicone rod in a segmental nerve defect in an animal model; (2) to use high throughput technology (ie, Luminex) to evaluate levels of neurotrophic factors in the induced membrane; and (3) to compare the levels of neurotrophic factors between the induced membrane tissue and a control scar tissue bed. We hypothesized that an induced membrane within a nerve defect would be reliably generated and would exhibit higher levels of neurotrophic factors compared to control tissue.

MATERIALS AND METHODS

After study protocol approval by our institutional animal care and use committee, 22 male Sprague-Dawley rats were used in accordance with the guidelines of the authors' institution, the National Institutes of Health, and any national law on the care and use of laboratory animals.

Sample size determination was based on previous protein quantifying studies of "Masquelet-type" membranes in rabbits.[30] Though effect size and SD varied for different proteins, a statistically significant difference for levels of VEGF and TGF-ß1[29] allowed an *a priori* sample size estimate with the following input parameters: single tail, normal parent distribution, effect size 1.5, alpha error probability 0.05, and power 0.95.

Under inhaled isoflurane anesthesia and using aseptic technique, the right sciatic nerve was exposed via a standard bicep femoris-semitendinosus muscle splitting approach. Two centimeters of sciatic nerve were excised in all 22 animals. In the experimental group (n = 11), a sterile 6-F silicone pediatric Foley catheter (Medline) was placed in the nerve defect and secured to the adjacent epineurium with 8-0 nylon suture. In the control group (n = 11), after nerve resection, the cut nerve ends were tagged with the same sutures to mark the scar bed. The skin and subcutaneous tissue were sutured close. All animals received analgesia after surgery and were monitored per institution protocol.

Four weeks later, the hind limb was reopened, and the induced membrane from the experimental group and connective tissue from the center of the scar bed of the excised sciatic nerve in the control rats were harvested using the previously placed sutures as a guide. Tissue was immediately stored in sterile sealed DNase-, RNase-free tubing that was flash frozen and stored in -80 °C freezer. In preparation for analysis, the tissue was both physically and chemically lysed using liquid nitrogen chilled mortar and pestle (Scienceware Liquid Nitrogen Cooled Mini Mortar & Pestle Set) and Luminex-compatible buffer (Invitrogen tissue extraction reagent with cOmplete, Mini, EDTA-free protease inhibitor cocktail), then centrifuged. Supernatant was used for analysis.

In order to verify that our 2-step lysis procedure yielded sufficient protein content and to subsequently determine the appropriate dilution of supernatant, the prepared tissue underwent rat-specific enzyme-linked immunosorbent assays for NGF, GDNF, brain-derived neurotrophic factor, and VEGF (Sigma

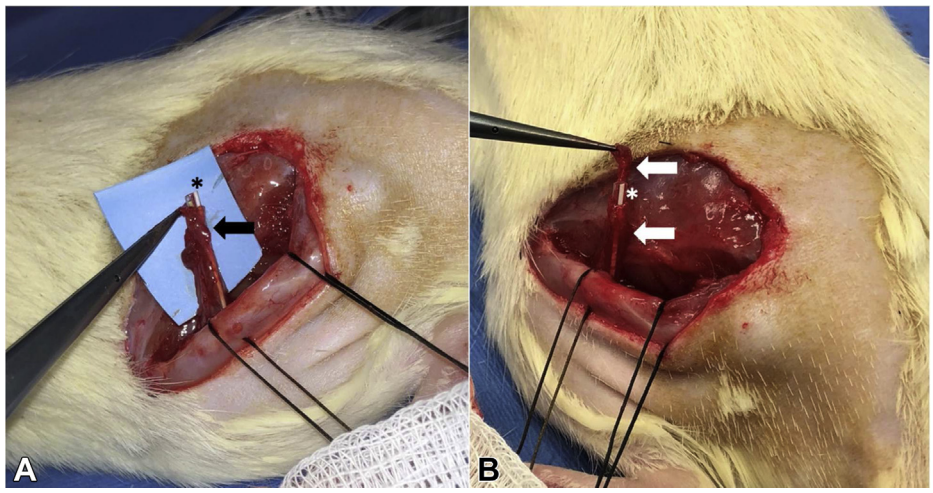

FIGURE 1: A Demonstrates the membrane induced around a silicone rod. The silicone rod is labeled with an black asterisk and the membrane tissue is marked with a black arrow. **B** Another example of the induced membrane (white arrows) around a silicone rod (white asterisk).

Aldrich RAB0883, RAB1144, RAB1138, and RAB0512, respectively).

Processed tissue from the control and experimental samples was then analyzed for the presence and quantities of the following neurotrophic factors using Luminex assays: TGF ß-1, 2, 3, and GDNF (11 matched samples tested); and IL-1B, VEGF, MCP-1, and NGF (10 matched samples tested based on available quantities of reagents). The following Emmanuel Merck, Darmstadt Millipore MILLIPLEX MAP plates were used: HADCYMAG-61K containing antihuman NGF; HNDG4MAG-36K containing antihuman GDNF; RECYTMAG-65K containing antirat VEGF, MCP-1, IL-1ß; TGFBMAG-64K-03 for antihuman TGF-ß 1, 2, and 3. Quantifying and comparing levels of these factors were completed using commercially available software (xPONENT 3.1 and Milliplex Analyst 5.1). These programs allowed for raw fluorescent intensity per bead to be recorded and quantified.[31] The concentration values and detection limits were determined from the standard curves generated from each analyte kit standard using a 5PL weighted curve fitting procedure.

Levels of factors were not normally distributed; therefore, the statistical comparisons of levels were performed using nonparametric tests (Wilcoxon signed-rank test for 2 groups). Values below the limits of detection were assigned a value equivalent to the lower limit of detection. A P value of .05 or lower was considered significant.

RESULTS

At 4 weeks postimplantation, the formation of a uniform, identifiable membrane around the silicone rod was observed in all 11 experimental specimens (Fig. 1). The induced membrane (experimental tissue) and tissue from the deep wound bed (control tissue) were excised in similar volumes as recorded by weight.

There were significantly higher levels of TGF-ß1, TGF-ß2, TGF-ß3, GDNF, IL-1ß, NGF, and MCP-1 in the induced membrane tissue compared with control tissue (Fig. 2). No statistically significant difference ($P = .153$) was detected between the control and experimental groups for levels of VEGF.

DISCUSSION

We achieved our goal of inducing a membrane around a silicone rod placed in a peripheral nerve and successfully demonstrated higher levels of NGF, GDNF, TGF-ß1, TGF-ß2, TGF-ß3, IL-1ß, and MCP-1 in this tissue. Elevations in VEGF levels were not found to be statistically significant compared with the normal tissue bed. We focused on these specific growth factors based on their potential neurotrophic properties previously documented in the literature. Silicone was chosen as a flexible, practical, and clinically relevant implant material, and the 4-week end point was chosen by extrapolating data from studies demonstrating optimal growth factor levels in polymethylmethacrylate (PMMA)-induced membranes at this time point.[30,32]

Nerve growth factor is involved in neural survival, development, and function in peripheral cells and is implicated in regulating Schwann cell differentiation and axon remyelination.[19] In a rat sciatic nerve crush injury model, administration of NGF improved motor function after 30 days, reduced atrophy of the target muscle, and increased the number of myelinated

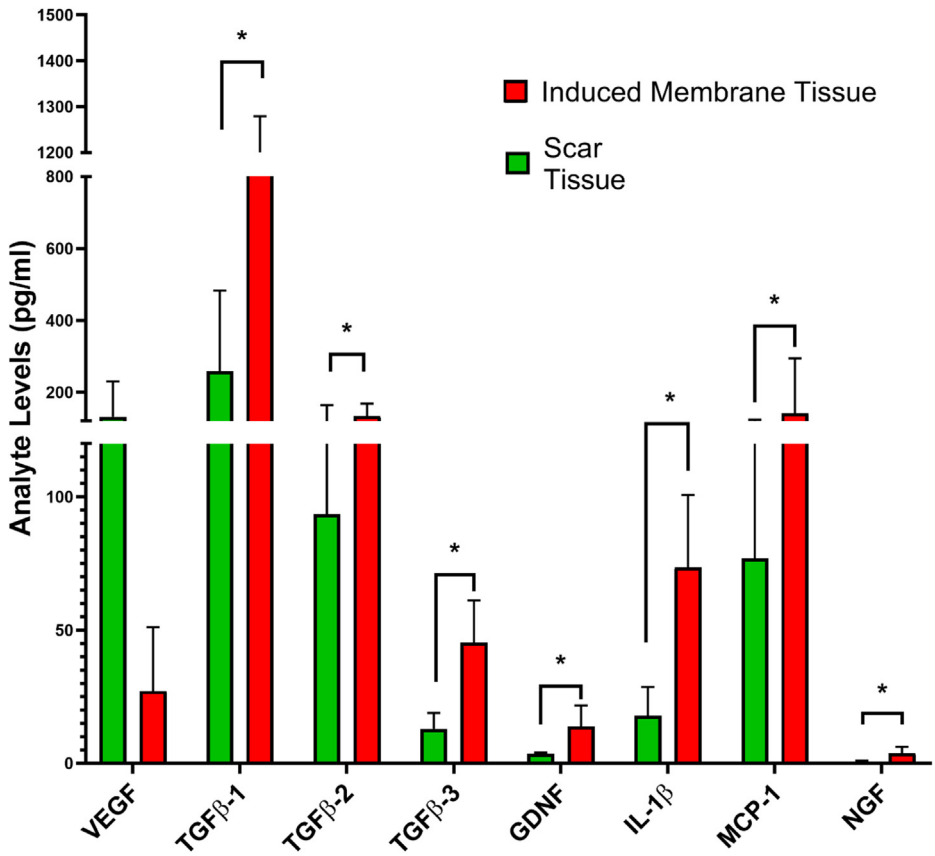

FIGURE 2: Comparison of target growth factors measured in induced membrane versus scar tissue (*$P < .05$).

axons compared to control.[20] Acellular nerve allograft soaked in NGF resulted in higher axon counts when implanted in a rodent model; one study noting improved motor regeneration[33] and a separate study noting improved sensory regeneration.[34]

Glial-derived neurotrophic factor is associated with promotion of survival and function of neuronal populations in the peripheral nervous system.[22] In a rat sciatic nerve injury model, defects treated with GDNF demonstrated faster recovery of regenerated axons compared to controls as seen by improved functional assessment; increased nerve conduction velocity; increased target muscle weight; and greater nerve fiber, axon diameter, and myelin sheath thickness.[23] Specifically, a sustained GDNF delivery system applied around an implanted ANA resulted in a more robust axonal regeneration.[35]

The cytokine IL-1ß and chemokine MCP-1 are associated with increased expression after rat sciatic nerve injury.[7] In one study, IL-1ß mRNA was not detected in intact sciatic rat nerves but was detected in increasing amounts shortly following injury.[8] When IL-1ß and MCP-1 antibodies were infused near the site of cut rat sciatic nerve, reduced recruitment and activation of macrophages as well as delayed myelin phagocytosis was observed.[7] Macrophage chemotaxis was also decreased in the presence of MCP-1 antibodies in vitro.[9]

Vascular endothelial growth factor has a well-defined role in angiogenesis, the development of new blood vessels, as well as vessel maintenance.[36–38] Delayed axonal regeneration has been associated with slow revascularization of ANA.[39] Vascular endothelial growth factor improved vascularization in ANA[40] and increased axonal regeneration in a proximal nerve graft.[41] Axon growth enhancement was marginal in a VEGF-treated 20 mm ANA.[42] Vascular endothelial growth factor is consistently increased within pseudomembranes induced by PMMA implanted for osseus defects,[32,43] so the lack of increased levels within the silicone rod induced membrane was unexpected. Pseudomembrane formation (and presumably growth factor content) is influenced by implant material and the subsequent tissue bed reaction.[44,45] Silicone may not induce the same tissue response as PMMA, though in the only comparative study identified in the literature, nonirradiated silicone-induced membranes and PMMA-induced membranes had similarly weak VEGF levels on Western-blot, and microscopic

vessel quantification between the 2 groups were not statistically significant (*P* value not published).[46] Additionally, VEGF has been shown to be elevated in tissue healing in general, which would affect the levels detected in the tissue bed harvested for analysis, even in our control group.[47]

Transforming TGF-ß is a cytokine associated with a variety of functions including bone metabolism, extracellular matrix synthesis, angiogenesis, macrophage activation, and scar tissue.[3–5,48] Transforming TGF-ß has multiple isoforms (1, 2, and 3), and TGF-ß1 is secreted by macrophages and Schwann cells.[3] Transforming TGF-ß is not only secreted by macrophages, but also attracts macrophages.[3] Following nerve injury, TGF-ß1 levels increase and are associated with inducing expression of neurotrophic factors in Schwann cells.[4,5] One study incubated chronically denervated Schwann cells in TGF-ß and found that those treated with TGF-ß promoted more than twice the number of motoneurons to regenerate axons across a bridge between proximal and distal nerve stumps compared to the control group.[6]

The use of the Luminex system allowed the measurement of multiple proteins of interest within the same tissue. Luminex is a commercially available bead-based flow cytometric multiplex array system that offers the ability for high throughput, simultaneous analysis of multiple analytes with a single, low-volume sample in less time and with increased reproducibility compared to traditional immunoassays.[49–51] A traditional enzyme-linked immunosorbent assay test requires that each sample can only be run against a single analyte at a time. In contrast, a multiplex assay allows for each sample to be run against multiple analytes concurrently. Each Luminex bead has 2 identifiers: a unique color profile and a unique biomarker/reporter molecule (eg, antibody) that allows proteins to bind with the bead and is coupled with a fluorescent marker. This technology allows for multiplex assays (multiple specimens run against multiple analytes concurrently) on a standard 96-well plate to be completed in a few hours. For some proteins in this study, antihuman markers were used when antirat markers were not available. This type of multiplex assay cross-reactivity has been previously reported across species.[52] Conservation of structure and immunological properties between human and rodent NGF, GDNF, TGF-ß, and IL-1ß has been previously demonstrated,[53–56] so the cross-reactivity between antihuman markers for rat proteins should be high. This was, however, not validated in our study, and these results should be considered as relative differences and not absolute values.

While our study is able to evaluate and describe the level of neurotrophic factors in an induced membrane model for peripheral nerve defects, there are several limitations. First, this is a pilot study with a small sample size, and although adequately powered, the variability in protein concentrations (ie, levels of neurotrophic factors) within tissue samples would be improved with larger sample sizes. Second, this is an animal study and may not accurately reflect similar biologic mechanisms in humans. Third, we did use markers for antihuman NGF, GDNF, TGF-ß 1, 2, and 3, and IL-1ß. Follow-up validation studies evaluating cross-reactivity for these markers with the assay system are necessary.

There are important implications for further study based on the findings in our pilot study. While a membrane was induced reliably around a silicone rod placed in a peripheral nerve defect in rats at 4 weeks, the ideal time of membrane "maturity" is unknown, and the ideal size and material of the implant might vary depending on the clinical scenario. For this study, we used a 6-F (2 mm outer diameter) Foley catheter, which was appropriately larger than the average diameter of an adult rat sciatic nerve (approximately 1 mm to 1.5 mm). Although there is a significant elevation in several neurotrophic factors in the induced membrane tissue, the threshold level required to promote nerve regeneration as well as dynamic interaction of these factors in axonal regeneration is unknown. For example, supraphysiological levels of GDNF attract axons, though these axons become trapped in the area of "high GDNF" and do not continue to regenerate.[57] Most importantly, there is impetus for further study using the induced membrane technique in a peripheral nerve injury model with autograft or allograft reconstruction.

ACKNOWLEDGMENTS

The authors acknowledge Geetanjali Bendale, PhD, and Satya Mallu, MD. This study was supported by a 2018 American Foundation for Surgery of the Hand Resident/Fellow Fast Track Grant.

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