Nerve growth factor: Increased angiogenesis without improved nerve regeneration

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Nerve growth factor (NGF) and laminin are important factors for neural development and regeneration. We examined the effects of increasing the local concentration and duration of action of NGF and laminin on peripheral nerve regeneration in the adult mouse sciatic nerve. A Silastic (silicone rubber) channel with intraluminal NGF solution was secured between transected nerve ends. The second channel tested, formed from a polysaccharide called chitosan, was prepared with NGF and laminin in the channel walls and provided a sustained release of NGF. At six weeks post-implantation, no improvement in nerve regeneration was identified in those channels prepared with NGF when comparing electromyographic thresholds (μA), maximum potentials (mV), nerve diameter, myelin sheath thickness, myelinated axon counts, or diameter. However, increased angiogenesis was demonstrated within the chitosan and Silastic channels prepared with NGF compared to those channels without NGF. Silastic exhibited minimal inflammation. Chitosan was associated with inflammation in many nerve channels. (OTOLARYNGOL HEAD NECK SURG 1991;105:12.)

The pioneering work of Levi-Montalcini[1] and subsequent developmental neurobiology studies support the tenet that mature neurons as well as developing neurons are dependent on intrinsic neuron maintenance factors and neuron-promoting factors for normal cellular and metabolic activity. In addition, mature peripheral neurons are dependent on neurite-promoting factors for regeneration after trauma.[2,3] The present study evaluates whether peripheral nerve regeneration can be improved by artificially increasing the local concentration of factors in the environment of the regenerating nerve ends.

Protein trophic factors have been implicated in several ways for promoting nerve regeneration. Nerve growth factor (NGF) and laminin exert a synergistic effect on rate of neurite outgrowth and neuron survival.[4,5] Neuritic growth is dependent on the presence of NGF at the nerve growth cone.[6] Concentrations of NGF receptors and NGF-mRNA increase significantly within Schwann cells after injury to the peripheral nerve. Once regeneration has occurred, the NGF-mRNA and NGF receptor concentrations return to low levels.[7-10]

In vivo, peripheral nerve regeneration has been extensively studied using nerve guidance channels.[2,11-15] Previous investigators have tested a variety of different nerve guidance channels in attempts to improve regeneration. Modifications have included channel wall materials such as selectively permeable polyvinylchloride and bioresorbable materials composed of synthetic polylactates.[12,14] Silicone channels reportedly have been associated with more connective tissue compared to vein or no channel at all.[13]

Nerve channel intraluminal materials also have been tested for improving nerve regeneration, including extracellular protein gels containing laminin and collagen, as well as plasma.[12,14,15] More recently, Chen et al.[16] studied facial nerve regeneration in the rabbit using silicone nerve guidance channel filled with Ringer's solution or a NGF solution (3.6 μg/mL). They reported an increase in the number of regenerated axons per fascicle and an increase in the diameter of the regenerated nerve trunks in those channels prepared with NGF; however, only the latter was reported as statistically significant.

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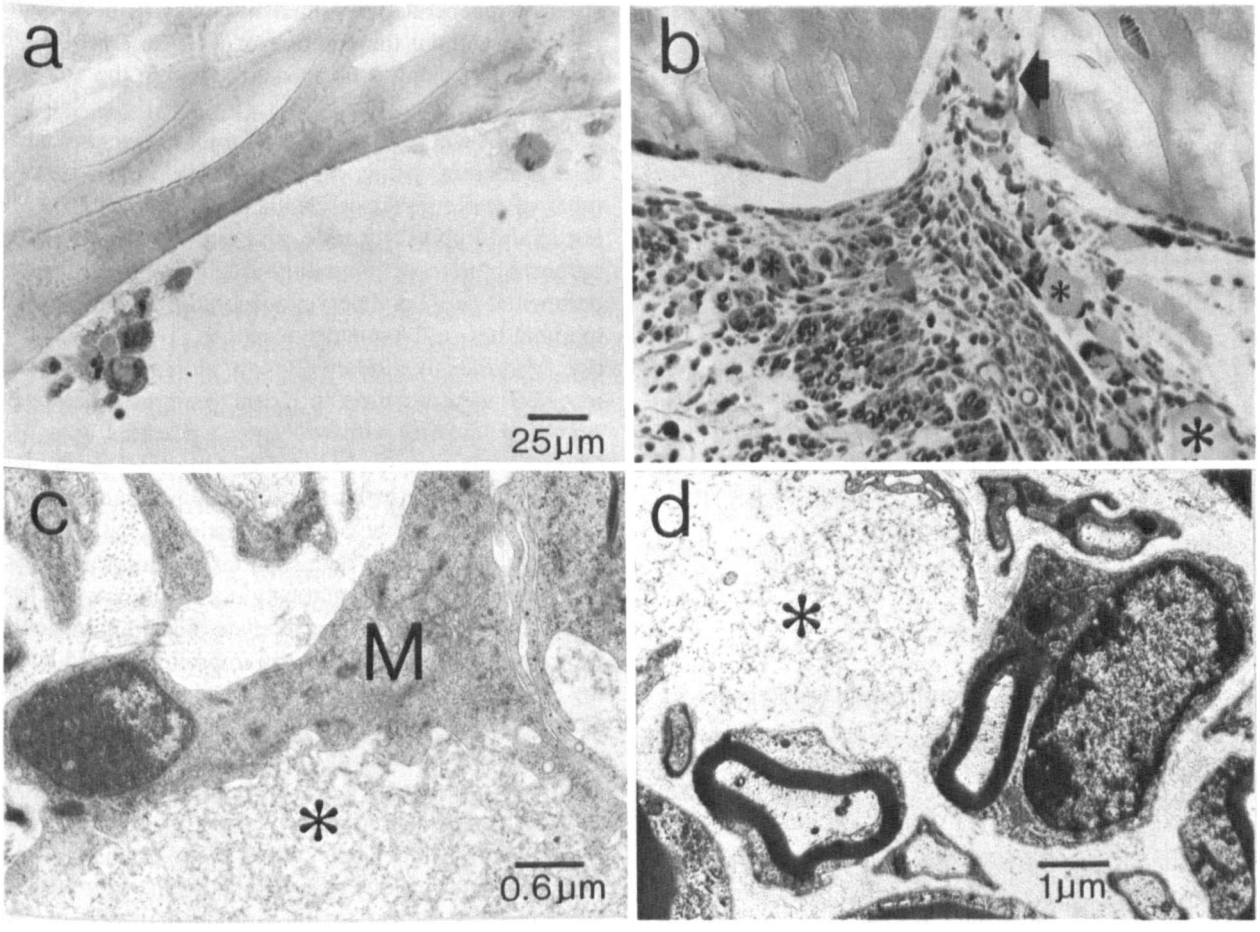

Fig. 1. a, Photomicrograph two weeks after implantation of a channel wall prepared from chitosan. Few inflammatory cells are present along the intact chitosan wall (Toluidine blue stain; original magnification × 400.) **b,** Photomicrograph six weeks after implantation of a nerve guidance channel prepared from chitosan. Regenerated nerve is associated with inflammation, sparse myelinated axons, multiple small chitosan fragments (*), and wall dehiscence with ingrowth of fibroblasts (*arrowhead* indicates ingrowth of fibroblasts) (Original magnification × 400.) **c,** Electron micrograph of a chitosan fragment (*) phagocytized by a macrophage *(M)* (Original magnification × 15,800.) **d,** Electron micrograph of normal myelinated axons adjacent to a chitosan fragment (*) with no associated inflammatory cells surrounding the chitosan (Original magnification × 9500.)

In a similar study, Rich et al.[17] studied rat sciatic nerve regeneration (four weeks post-transection) comparing histologic parameters using a silicone nerve guidance channel, either with or without NGF (1 mg/ml). No difference was measured in regenerated nerve cross-sectional areas; however, in the distal nerve stump they reported a greater number of myelinated axons, greater percentage of myelinated axons to unmyelinated axons, and thicker myelin sheaths in those channels with NGF compared to those without NGF. This work by Rich et al.[17] supports a trophic activity of NGF on Schwann cells in the distal nerve segment; i.e., the portion of nerve distal to the nerve gap. Unfortunately, no parallel data of the number of myelinated axons and myelin thickness were presented for regenerated nerve in the middle section of the nerve channel. Thus, no comment can be made regarding a trophic effect of NGF on the regenerated axons or Schwann cells in the middle section of the nerve channel. Also, the time frame at which regeneration was evaluated (4 weeks) is just 1 week beyond the time at which axons are regenerating across the proximal to distal nerve stump.[18] The study by Rich et al.[17] was not designed to evaluate regeneration at a later time. Therefore, it is not clear if NGF only enhanced the temporal progress of Schwann cell maturation of the distal segment or if NGF would also increase the ultimate outcome of Schwann cell maturation. Temporal acceler-

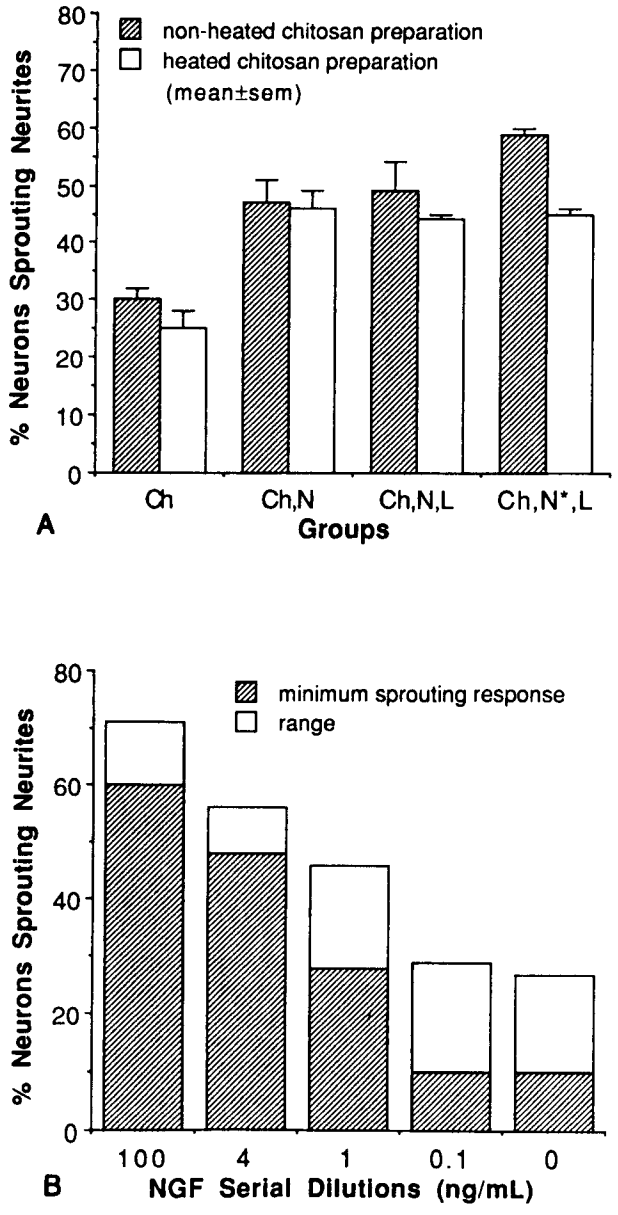

Fig. 2. A, Chart depicts the percent of neurons sprouting neurites in the rat pheochromocytoma cell line (PC12) bioassay for heated (100° C) and nonheated chitosan preparations with NGF. Heated chitosan preparations with NGF at 400 ng/mL (Ch,N*,L) demonstrated a 12% decrease in biologic activity compared to nonheated chitosan preparations with NGF. However, preparations with 40 ng/mL (Ch,N,L) did not exhibit a decrease in biologic activity after heating. *Ch*, Chitosan; *N*, NGF 40 ng/mL; *N**, NGF 400 ng/mL; *L*, laminin. **B,** Chart demonstrates serial dilutions of NGF (100 ng/mL to 0 ng/mL) vs. the percentage of neurons sprouting neurites in the rat pheochromocytoma cell line (PC12) bioassay. Note that at a NGF concentration of 0 ng/mL, there is a 10% to 25% spontaneous neurite sprouting response, and at 100 ng/mL, 60% to 70% of the neurons sprout neurites. The sprouting response values from this control panel of known serial dilutions of NGF concentrations are compared to Fig. 2, *A*, where final concentrations of biologically active NGF are unknown (see text).

ation of regenerated myelinated axons has been demonstrated without the addition of NGF in a rat sciatic nerve model using a fibrin matrix within the silicone channel.[15]

In the present study, NGF and laminin were presented to regenerating nerve ends within the lumen of two types of nerve guidance channels, silicone rubber (Silastic) and a polysaccharide, chitosan. Silastic (silicone rubber) is the most commonly used biomaterial for experimental peripheral nerve regeneration because of the minimal host inflammatory response. The polysaccharide, chitosan, is a relatively new biomaterial and has received wide attention in recent years as a naturally occurring material with widespread potential uses.[19-21] Chitosan has unique chemical and physical properties compared to other naturally occurring polysaccharides. This is largely attributed to its distinctly basic nature (other polysaccharides being neutral or acidic). Because chitosan resembles ubiquitously occurring polysaccharides, it is theoretically less likely to have immunogenic properties. Chitosan reportedly improves wound healing when compared to cartilaginous preparations (Catrix).[20] Suture materials and contact lenses have been constructed with chitosan.[19,21] Chitosan may have bacteriostatic properties.[19] In this study, chitosan was shaped into nerve guidance channels impregnated with NGF and laminin. Channels were designed to serve dual functions: a conduit for directing nerve regeneration and a reservoir for the sustained release of supraphysiologic concentrations of NGF and laminin within the channel lumen. Thus, the silicone channel tested the efficacy of immediate delivery of NGF to the transected nerve ends and the chitosan channel tested the efficacy of sustained release of NGF and laminin to the transected nerve ends. The following hypotheses were tested in the present study:

1. The presence of short-term supraphysiologic concentrations of NGF solution within the lumen of a silicone nerve guidance channel should improve nerve regeneration compared to a silicone nerve guidance channel without NGF.
2. Chitosan nerve guidance channels should be more physiologic and better tolerated than silicone nerve guidance channels and result in improved peripheral nerve regeneration.
3. Locally sustained and supraphysiologic concentrations of NGF released into the lumen of the chitosan nerve guidance channel should improve nerve regeneration more than chitosan or silicone alone.
4. NGF and laminin should have synergistic activity with regard to nerve regeneration compared to NGF alone in the chitosan model.

METHODS AND MATERIAL

Materials

Using an established protocol, 2.5S NGF (β subunit) was isolated and purified from mouse submandibular gland saliva.[22] Nerve growth channels prepared with chitosan and NGF had approximately 560 ng of NGF/channel. Nerve growth channels of silicone were filled with a phosphate-buffered NGF solution at a concentration of 30 μg/mL; approximately 60 ng of NGF was present in the channel lumen.

Laminin, a glycoprotein, was used at a concentration of 100 μg/mL in the chitosan solution (Collaborative Research, Lexington, Mass.). The chitosan channels prepared with laminin had approximately 5.6 μg of laminin/channel.

Silicone, an elastomeric resin of polydimethylsiloxane, is manufactured in a medical biomaterial called Silastic (Dow Corning, Midland, Mich.). We used a 6-mm long channel, with an inner diameter of 0.8 mm and outer diameter of 1.7 mm. Preparation of the channel includes: wash in 2% Alconox-distilled water (6 times), ultrasonicator to remove all dust, rinse (6 times) in distilled water, and sterilize with ethylene oxide.

Chitosan is a copolymer consisting of 81% glucosamine and 19% N-acetyl glucosamine (Protan Laboratories, Commach, N.Y.). Various techniques to construct chitosan channels were examined. The optimum chitosan concentration for channel construction was 36-mg chitosan/mL of 5% aqueous acetic acid. Construction of the channels used 22G stainless steel needles. Sterile needles were dipped into the chitosan solution. After air-drying, the same needle was again dipped and dried until eight coats were applied. Heating of the chitosan on the needle was found to prevent significant swelling and subsequent dissolution of the chitosan channels. Optimum heating parameters were 100° C for 2 hours. After cooling, the channel was carefully cut to the appropriate length and pushed off the needle. The heating procedure served as the sterilization step for the chitosan channels. The dimensions of the chitosan channel approximated the Silastic channel dimensions.

Rat Pheochromocytoma Cell Line Bioassay (PC12)

One of the obvious difficulties arising from chitosan channel preparation was that in order to maintain chitosan channel wall integrity and lumen patency we subjected both the NGF and the chitosan to 100° C temperatures. Such heating placed the NGF polypeptide at risk of denaturation and loss of biologic activity. The rat pheochromocytoma cell line bioassay (PC12) was used to test for residual NGF activity after heating.[23]

Table 1. Nerve regeneration channel groups and normal nerve, by abbreviations, number of subjects in each group, and by number (%) of unsuccessful nerve regenerations

Animal groups	Abbreviation for group	No. of mice per group	No. of unsuccessful regenerations (% failure)
Chitosan channels			
Without NGF or laminin	Ch	7	0 (0%)
With NGF only	Ch,N	8	3 (38%)
With NGF and laminin	Ch,N,L	8	1 (14%)
Silastic channels			
Without NGF	Si	9	0 (0%)
With NGF	Si,N	7	0 (0%)
Normal sciatic nerve without previous manipulation	Norm	6	–

Cells were grown in Delbecco's medium containing calf and horse serum on polystyrene tissue culture dishes. Subconfluent cells at a density of 1×10^4 cells/cm^2 were used after reaching logarithmic growth phase.

Scoring of NGF activity in the PC12 bioassay is a function of the percentage of cells sprouting neurites in a 24-hour period. Chitosan channels prepared for the bioassay were sectioned in 1-mm lengths and placed in 5% aqueous acetic acid. An aliquot of this solution was then placed into the well containing PC12 cells. The final NGF concentration tested from the chitosan groups were 40 and 400 ng/mL. Known serial dilutions of NGF served as a control and ranged from 0 to 100 ng/mL.

Nerve regeneration groups. Six groups of Swiss-Webster adult mice, weighing 35 to 40 grams each, were used. Three types of chitosan channels were prepared, with NGF and laminin as variables, and two types of Silastic channels were prepared with NGF as the variable. A control group of mice with normal sciatic nerves and with no previous transection or pharmacologic manipulation was also included (Table 1).

Surgical technique. Animal care was maintained in compliance with the principles set forth by the University of Washington Animal Care Committee and Principles of Laboratory Animal Care. Mice were anesthetized intraperitoneally with Nembutal (80 mg/kg), then locally with 1% lidocaine. After cleansing of the shaved area overlying the gluteal muscle groups with Betadine, sharp incision through the skin and subsequent retraction of the gluteus muscle exposed the sciatic nerve. In preliminary studies, chitosan channels

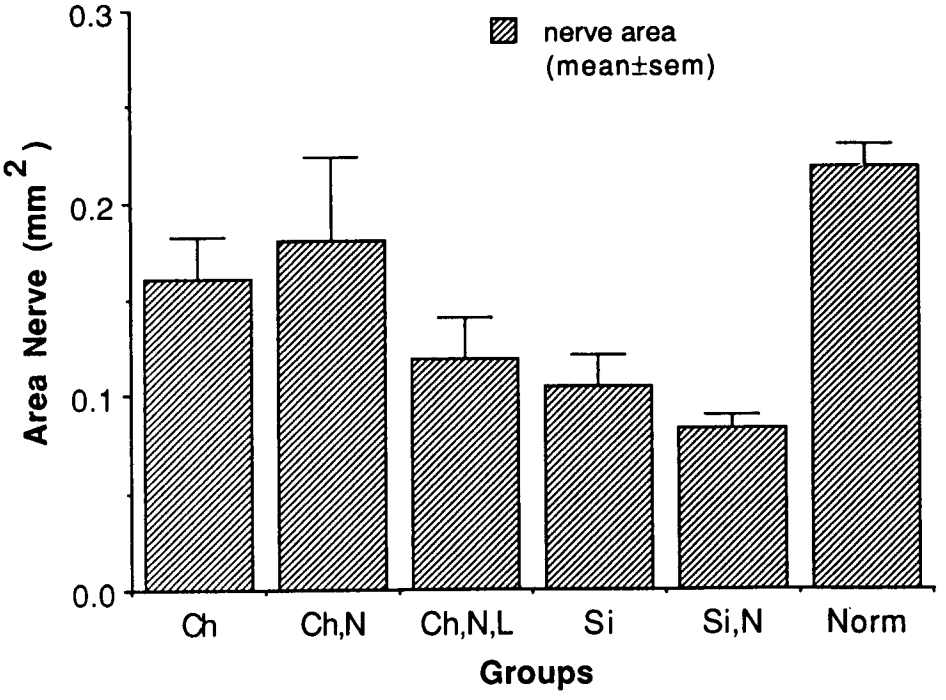

Fig. 3. Mean cross-sectional area (mm^2) of the regenerated nerves for each type of nerve guidance channel is plotted. The cross-sectional area in the regenerated nerve was not affected by the addition of NGF and/or laminin in any of the chitosan or Silastic channels. *Ch,* Chitosan without NGF or laminin; *Ch,N,* chitosan with NGF only; *Ch,N,L,* chitosan with NGF and laminin; *Si,* Silastic without NGF; *Si,N,* Silastic with NGF; *Norm,* normal sciatic nerve without previous manipulation.

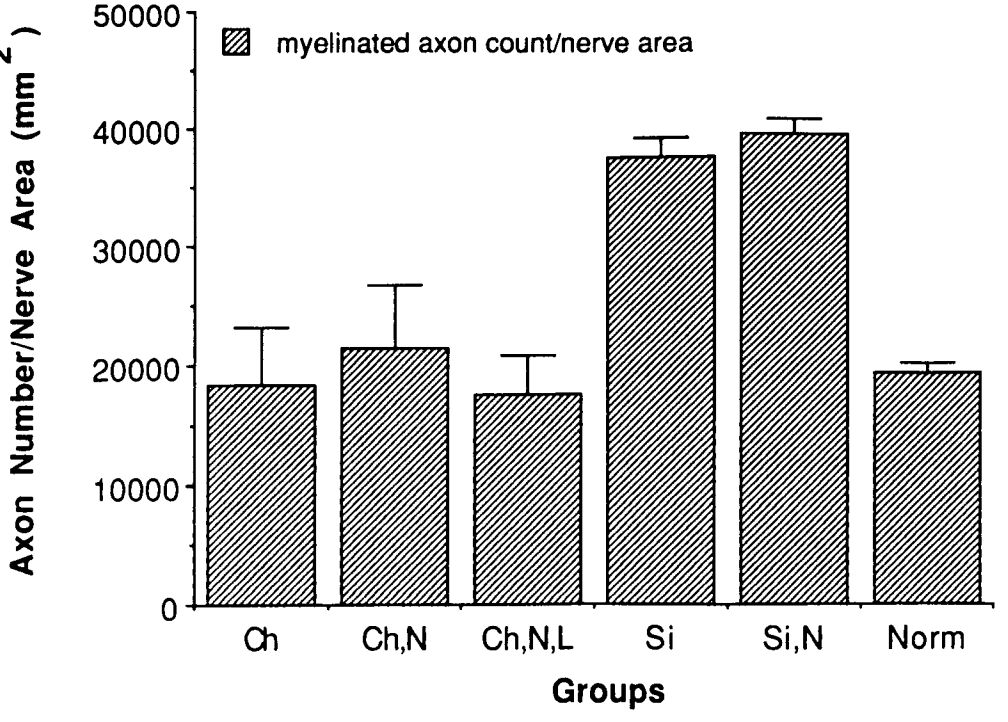

Fig. 4. The mean density of myelinated axons [myelinated axon number/sample area (mm^2)] for the regenerated nerves in each type of channel is plotted. Axonal density in the regenerated nerve was not affected by the addition of NGF and/or laminin in any of the chitosan or Silastic channels.

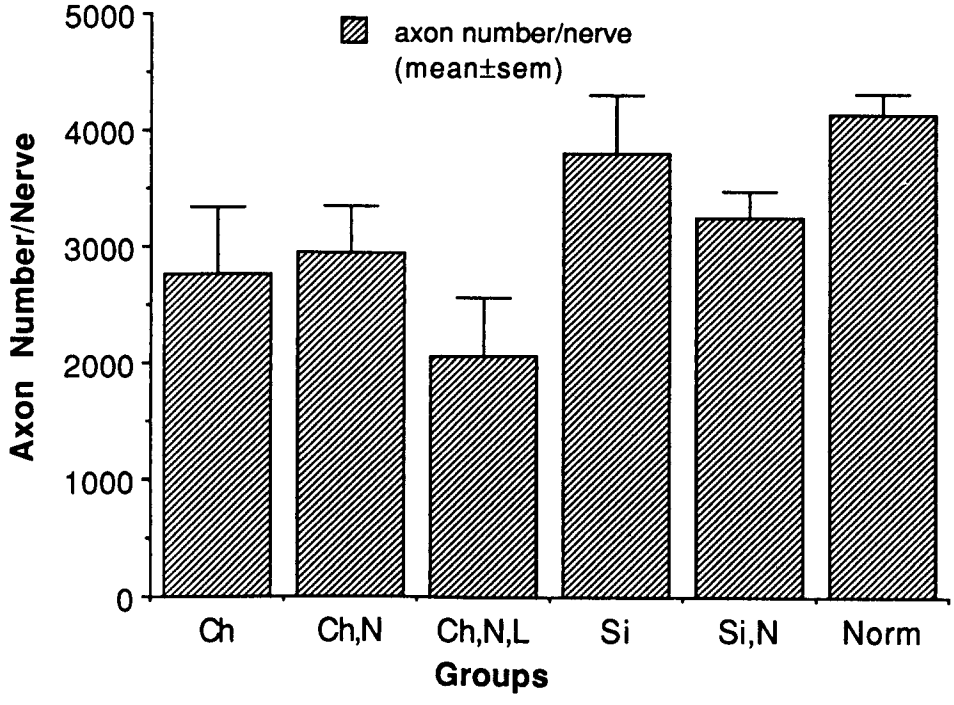

Fig. 5. Mean total number of myelinated axons in the regenerated nerve for each type of channel is plotted. The mean total number of myelinated axons in the regenerated nerve was not affected by the addition of NGF and/or laminin in any of the chitosan or Silastic channels. Significantly more myelinated axons were seen in the Silastic channels without NGF than in the chitosan channels prepared with NGF and laminin ($p < 0.05$).

were placed adjacent to the gluteus muscle without nerve section for 2 weeks to determine biocompatability. In the final long-term implantation study, 6-mm long channels were positioned between the proximal and distal nerve ends of the sharply transected sciatic nerve. A 3-mm gap between the proximal and distal nerve end was maintained with 10-0 nylon suture passed through the epineurium and channel wall. Only one sciatic nerve was used in each animal. Normal saline was placed within the lumen of channels made with chitosan. NGF solution or normal saline was placed in Silastic channels. All air bubbles were removed from the channel lumen because bubbles block regeneration.[10] The surgical field was closed in a deep and superficial layer.

Electromyography

At the time the animals were killed, 6 weeks after nerve transection, the sciatic nerve and nerve guidance channel were exposed after the animal was anesthetized with Nembutal. The nerve was stimulated proximal to the site of regeneration with an insulated bipolar platinum electrode and driven by a Grass SD9 stimulator (Grass Instrument Co., Quincy, Mass.) fed into a Bak Biphasic stimulus isolator (Bak Electronics Inc., Germantown, Md.). Muscle potentials were recorded from paired stainless steel electrodes placed into the gastrocnemius muscle. Potentials were preamplified through a Grass P15 preamplifier and recorded on a Tektronix 561 oscilloscope (Tektronix Inc., Beaverton, Ore.). A reliably visible EMG potential was used to determine current threshold (mA), and input/output (I/O) functions were determined for each nerve. Current threshold levels ranged from 80 to 320 mA, with a stimulus duration of 0.1 msec at 1 Hz. The maximum EMG potentials (mV) were determined from the oscilloscope as the amplitude at which no further increase was seen with increases in nerve stimulation. The contralateral sciatic nerve not operated on was also tested in each animal and served as a control. Data were compared using a one factor ANOVA.

Histology

After EMGs were obtained the animals were euthanized with Pentobarbital. For light microscopic analysis, the sciatic nerve was fixed in 2% glutaraldehyde and 2% paraformaldehyde by transcardial perfusion and subsequent immersion. After the sciatic nerve and channel was dissected away from the tissues, it was postfixed in 1% osmium tetroxide and 1.5% potassium ferricyanide.[24] Tissue was dehydrated in alcohol and imbedded in plastic (Spurr; Polysciences, Warrington, Pa.).

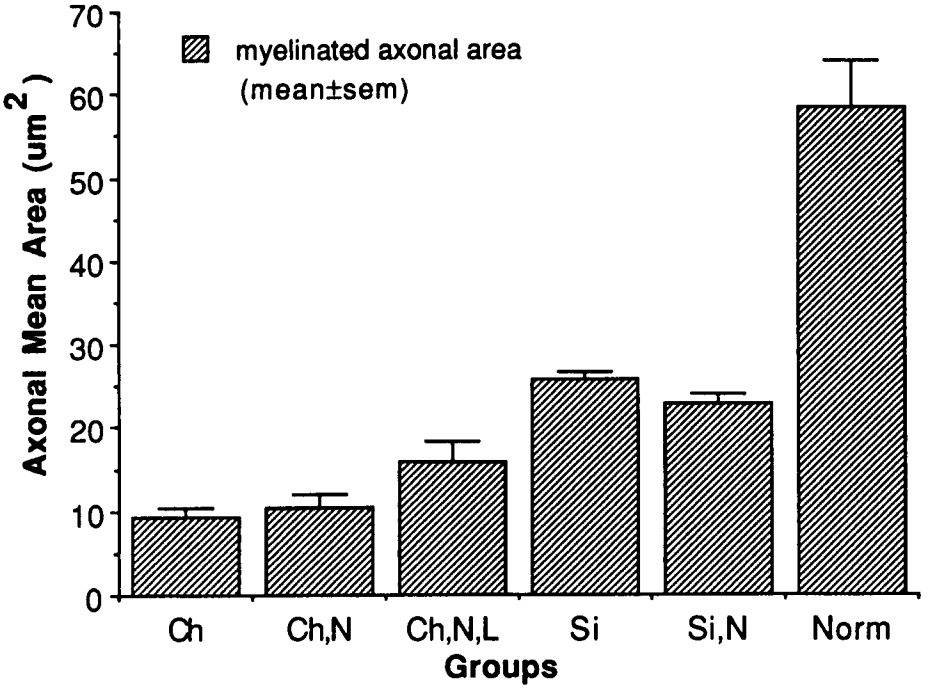

Fig. 6. Mean myelinated axonal areas (μm^2) of the regenerated nerve for each type of channel is plotted. The myelinated axonal area in the regenerated nerve was not affected by the addition of NGF in any of the chitosan or Silastic channels; however, the addition of laminin in the channel prepared with chitosan and NGF (Ch,N,L) was associated with an increase in myelinated axonal areas compared to channels prepared with chitosan alone or with NGF (Ch), (Ch,N). The increase in area associated with (Ch,N,L) is still less than the area seen in the Silastic channel without NGF (Si).

Transverse semi-thin sections, 1 to 2 μm each, were collected at the nerve channel midpoint and stained with toluidine blue. For electron microscopic analysis, a sample of thin sections (70-nm thickness), were collected at the nerve channel midpoint from selected nerves representative of each group and stained with uranyl acetate and lead citrate. Sections were examined and photographed on a Philips 420 EM (Philips Medical Systems North America Inc., Shelton, Conn.) at an accelerating voltage of 60 kV.

Data Analysis

A computer-assisted microscopy system (Zeiss Videoplan, Carl Zeiss, Inc., Thornwood, N.Y.) was used for morphometric analysis of the transverse nerve sections. Total nerve cross-sectional area, myelinated axon density, total myelinated axon count, myelinated axon area, myelin thickness, total blood vessel count/nerve, mean blood vessel surface area, and percentage vessel area to nerve area ratios were calculated. Myelinated axon densities were approximated from 1000 to 1500 counts per nerve. The sampled areas were in the same four regions of each nerve. The sampled areas totaled approximately 15% to 25% of the total nerve area. Total myelinated axon counts were calculated from the product of the nerve area and axon density. Myelinated axon maturity was evaluated by measuring the area of each of the largest 50 myelinated axons within each nerve.

Blood vessel cross-sectional surface areas were determined by measuring the lumen area of all blood vessels within each nerve. In the case of blood vessels running tangentially to the long axis of the nerve fibers, the portion of the vessel with the smallest diameter was identified and a circle was traced out to represent the vessel cross-sectional area. Total number of blood vessels and total area of blood vessels/nerve area ratio were determined. Myelin thickness was determined from direct measurements of myelinated axons from EM photographs. An average of 55 myelinated axons were measured for myelin thickness in each group.

Statistical analyses were performed using a one-factor ANOVA for each independent variable comparing: (1) all groups together; (2) subgroups (channels prepared with chitosan as one subgroup and Silastic channels as another subgroup).

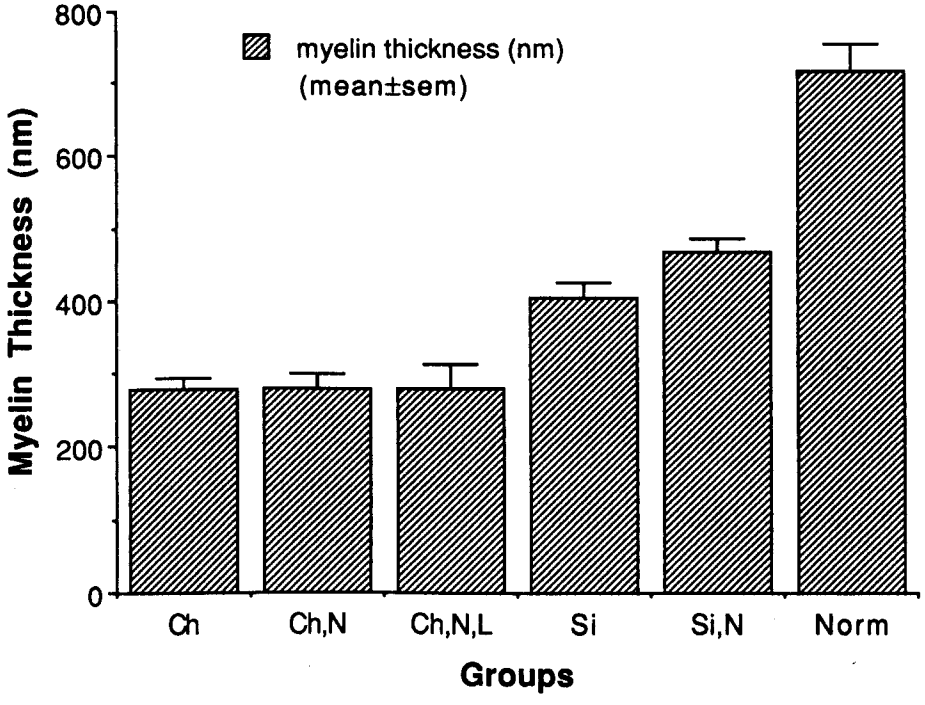

Fig. 7. Mean myelin sheath thickness (nm) of myelinated axons for each type of channel is plotted. Normal nerve had the thickest myelin sheaths, followed by regenerated axons in Silastic channels. Myelin sheaths from regenerated nerves of chitosan channels were the thinnest. The addition of NGF to the Silastic and chitosan channels had no effect on myelin thickness.

RESULTS

Nerve Guidance Channel Preparation from Chitosan

Preliminary work in creating the chitosan nerve guidance channels with NGF and laminin focused on three areas: the first was to establish if chitosan could be shaped into a nerve guidance channel and maintain structural integrity in vivo, the second was to determine biocompatability, and the third was to determine if NGF activity was maintained and biologically available from the chitosan after preparation of the channel.

Heat-treated chitosan channels were harvested from the four implanted mice without nerve transection 2 weeks after implantation. Channel wall integrity was maintained by gross and histologic evaluation. A thin vascularized membrane on the chitosan surface was present. No fibrous encapsulation or evidence of infection was seen. Light microscopic analysis in Fig. 1, *A* shows that the channel wall of chitosan material has minimal inflammation present.

Results of the rat pheochromocytoma cell line (PC12) bioassay (Fig. 2, *A*) revealed that 100° C heating of chitosan prepared with NGF at 400 ng/mL resulted in a small loss of neurite sprouting activity (12%) compared to non-heated chitosan prepared with NGF at 400 ng/mL (group represented by Ch,N*,L). However, no loss of activity was apparent with heating of the chitosan prepared with NGF at 40 ng/mL (Ch,N,L). To determine an estimate of the NGF concentration available from heated chitosan channels, Fig. 2, *A* is compared to Fig. 2, *B*. Figure 2, *B* shows the sprouting response values from known serial dilutions of NGF, ranging from a concentration of 100 ng/ml to 0 ng/ml. Comparison of the percentage of neurons sprouting neurites from the heated chitosan channels prepared with NGF in Fig. 2, *A* with the known serial dilutions of NGF in Fig. 2, *B* reveals that the heated chitosan and NGF had the same amount of neurite sprouting activity as did the serial dilution group with an NGF concentration of 4 ng/ml. Laminin had no obvious synergistic effect when added to the chitosan NGF preparation. In summary, NGF activity is maintained and available for release from heat-treated channels prepared with chitosan and NGF.

*Bothwell MA, Schatteman G: unpublished observation.

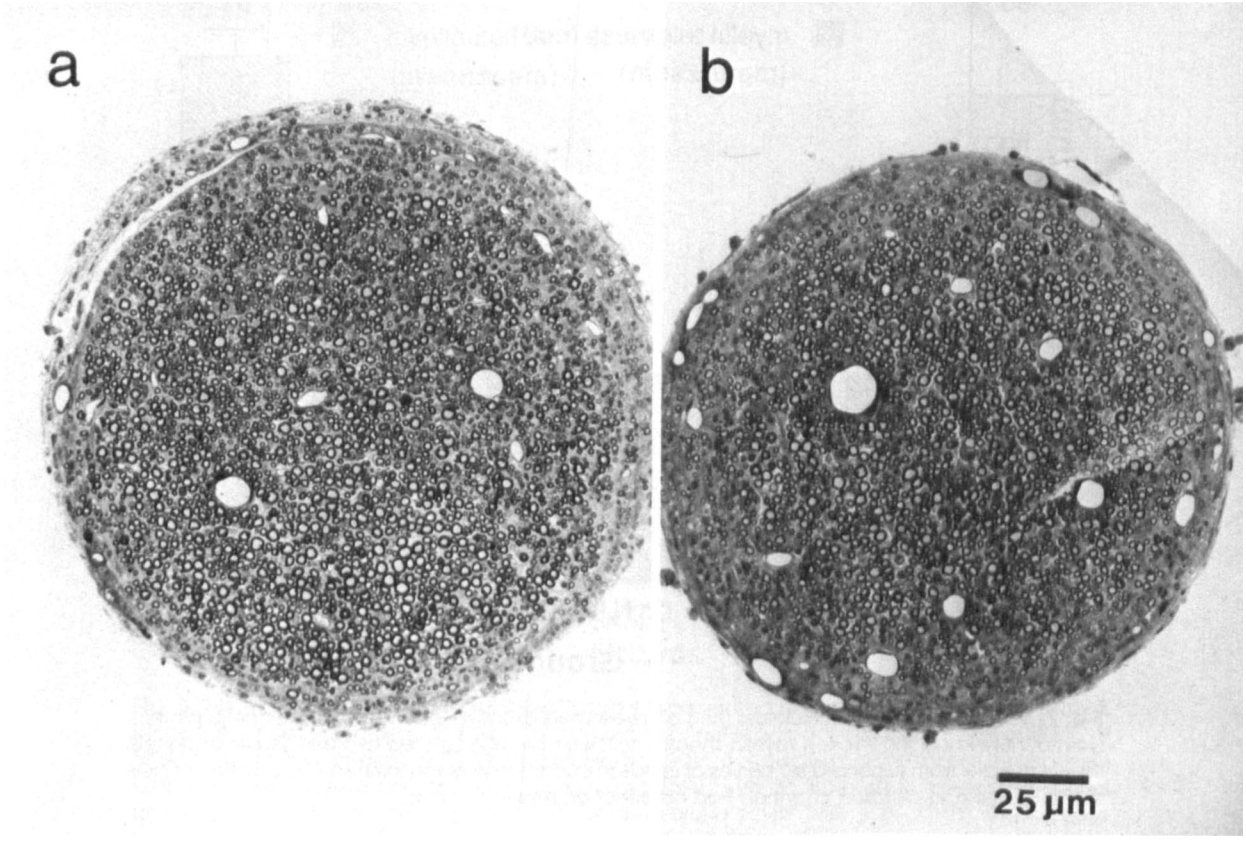

Fig. 8. Photomicrograph of **a**, a regenerated nerve from a Silastic channel prepared without NGF solution, with fewer and smaller blood vessels than **b**, the regenerated nerve from a Silastic channel with NGF. Neither regenerated nerve has evidence of any inflammation. (Toluidine blue stain; original magnification ×400.)

Influence of NGF on Nerve Regeneration

Electromyography. Six weeks after implantation, all mice that had undergone sciatic nerve transection and channel implantation had gross muscular wasting and paresis of the lower ipsilateral extremity. There was no clinical difference observed in the severity of these findings between animals groups. Analysis of the data by ANOVA revealed no significant differences in minimum thresholds (mA) or maximum stimulation levels (mV) when animal groups receiving channels with NGF were compared to those without NGF. Likewise there were no differences between animal groups with channels of chitosan and Silastic.

Histologic observations. All regenerated nerves from animals that were implanted with any of the channels constructed with chitosan (i.e., Ch; Ch,N; Ch,N,L) were characterized by mild-to-moderate inflammation within the lumen of the channel and on the outer surface on the channel. Approximately half of the chitosan channels had one or more wall dehiscences, often with the ingrowth of fibroblasts creating an irregular epineurium, as demonstrated in Fig. 1, *B*. Within these regenerated nerves, small fragments of chitosan were found. Generally the chitosan fragments were surrounded by fibroblasts and macrophages (Fig. 1, *B*). Electron microscopic analysis demonstrated macrophages phagocytizing chitosan fragments within the regenerated nerve (Fig. 1, *C*); however, there were several normal-appearing myelinated axons adjacent to small fragments of chitosan that were not surrounded with macrophages (Fig. 1, *D*). In contrast to the inflammation associated with the chitosan channels, Silastic channels with or without NGF demonstrated no or few inflammatory cells.

Quantitative Analysis of Histologic Preparations

Nerve regeneration failed in several of the channels constructed with chitosan and trophic factors. However, nerve regeneration was seen in all of the Silastic channels (Table 1). In three of the channels contructed with chitosan and NGF (Ch,N), nerve regeneration failed and fibroblasts only bridged the proximal and distal

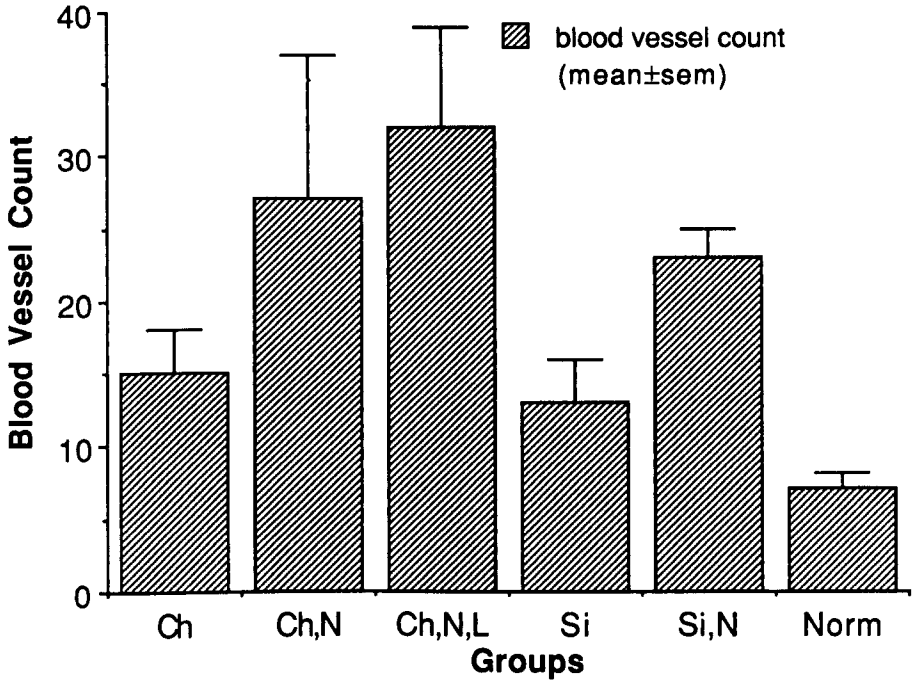

Fig. 9. Total blood vessel count of the regenerated nerve for each type of channel is plotted. Channels prepared with chitosan, NGF and laminin (Ch,N,L) were associated with twice the number of vessels compared to chitosan alone (Ch). Silastic channels with NGF (Si,N) were also associated with nearly twice the number of vessels compared to Silastic alone (Si). Both findings were significant ($p < 0.05$).

nerve ends or no bridging occurred. No axons were present.

Figure 3 shows the mean cross-sectional area of the regenerated nerve in each of the groups. Channels prepared with chitosan and NGF (Ch,N) or chitosan, NGF, and laminin (Ch,N,L) did not promote larger nerves than channels prepared with chitosan alone (Ch). Similarly, no effect of NGF was demonstrated in the Silastic channels prepared with or without NGF (compare Si to Si,N). However, greater cross-sectional areas were found in the regenerated nerves from the channels constructed with chitosan than in the channels of Silastic. Inspection of the tissue sections revealed that this effect resulted from a greater abundance of inflammatory cells in the chitosan channels and not more axons. Thus, no meaningful effect on cross-sectional area of the regenerated nerve was demonstrated by the addition of NGF and/or laminin.

Figure 4 shows the mean myelinated axonal density of the regenerated nerves for each group. Between the three groups of channels constructed from chitosan, there was no increase in the axonal density when comparing the channels with NGF or NGF and laminin (Ch,N; Ch,N,L) with the chitosan channels without NGF and laminin (Ch). Likewise, axonal density did not differ between the Silastic groups with and without NGF (Si; Si,N). Figure 4 also demonstrates that the axonal density within the Silastic channels (Si; Si,N) was significantly greater than those channels constructed with chitosan (Ch; Ch,N; Ch,N,L), ($p < 0.05$).

Figure 5 shows the mean total number of axons in the regenerated nerves for each group. In those channels prepared with chitosan and NGF and laminin (Ch,N; Ch,N,L), no improvement was identified in the total number of axons compared to chitosan alone. Similarly, the total number of axons in the regenerated nerve appeared to be the same in the Silastic channels prepared with and without NGF (Si; Si,N). Figure 5 reveals that significantly more myelinated axons were seen in the regenerated nerves from the Silastic channels without NGF (Si) than in the channels prepared with chitosan, NGF, and laminin (Ch,N,L), ($p < 0.05$).

Figure 6 shows shows the mean myelinated axonal data within the regenerated nerves for each of the groups. Channels constructed with chitosan and NGF (Ch,N) showed no reliable increase in axon area compared to channels made of chitosan alone (Ch); however, in channels with the addition of NGF and laminin (Ch,N,L) there was a 70% increase in myelinated ax-

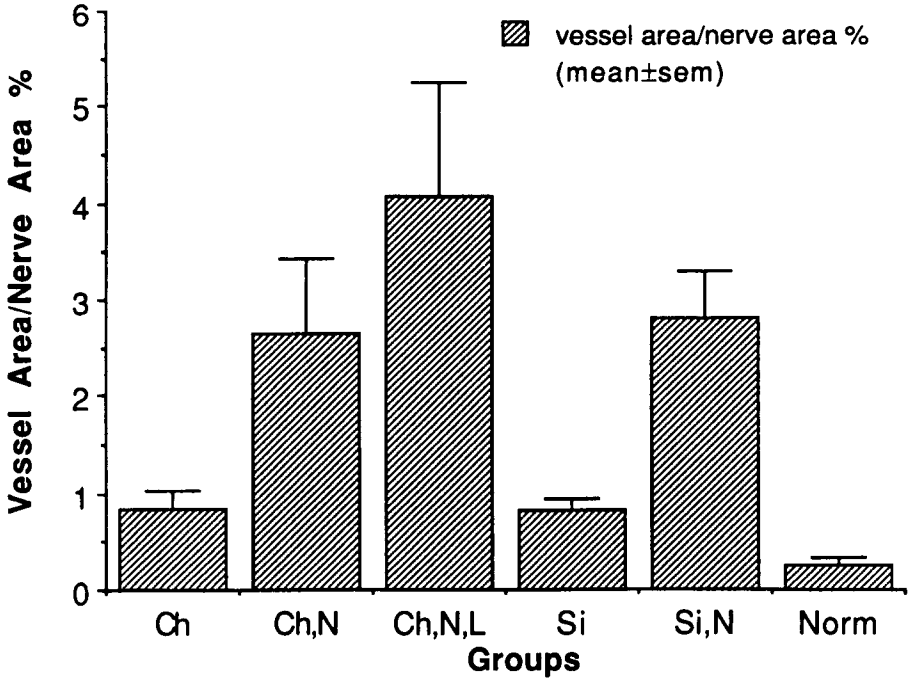

Fig. 10. The mean sums of the vessel areas to nerve area ratio (expressed as a percentage, %) are plotted for each channel type. Regenerated nerves from channels prepared with chitosan, NGF and laminin (Ch,N,L) demonstrated a greater vessel area to total nerve area than chitosan alone (Ch), ($p < 0.01$). Similarly, regenerated nerves from Silastic channels with NGF (Si,N) demonstrated a greater vessel area to total nerve area than Silastic channels alone (Si), ($p < 0.01$). Regenerated nerves from Silastic channels with NGF (Si,N) revealed a greater vessel area to total nerve area than did channels prepared with chitosan alone (Ch), ($p < 0.05$).

onal area compared to channels made of chitosan alone (Ch), ($p < 0.05$). On the other hand, the Silastic channels with NGF did not reveal an increase in the axonal area compared to those Silastic channels without NGF. The regenerated nerves from the Silastic channel group prepared without NGF (Si) still had a greater axonal area than the chitosan channels prepared with NGF and laminin (Ch,N,L).

Figure 7 shows mean myelin sheath thickness of myelinated axons within the regenerated nerves for each of the groups. Myelin thickness was greater in the normal nerve than any of the regenerated nerves. Regenerated nerves in the Silastic channel groups had greater myelin thickness than any of the chitosan groups. Within each of these Silastic and chitosan channel groups no measurable effect on myelin thickness was seen in those channels with NGF or laminin compared to those channels without.

Influence of NGF on Angiogenesis

Evaluation of the regenerated nerves for trophic effects associated with NGF and laminin revealed a greater number of blood vessels, as well as larger blood vessels within the regenerated nerve tissues exposed to NGF, than nerves without exogenous NGF. This effect is demonstrated in the photomicrograph in Fig. 8 and graphically in Figs. 9 and 10.

Counts of the total number of blood vessels in each nerve demonstrated a 100% increase when channels were made with chitosan, NGF, and laminin (Ch,N,L) than in chitosan channels alone (Ch) ($p < 0.05$) (Fig. 9). A 70% increase in vessel number was observed in the Silastic channel with NGF (Si,N) when compared to Silastic alone (Si) ($p < 0.05$). Normal nonregenerated nerve was found to have fewer and smaller blood vessels than either the Silastic or chitosan channels prepared with NGF.

The sum of the vessel areas from each regenerated nerve was divided by the corresponding regenerated nerve area to give the proportion of vascular area in each sample. These figures were converted to percentages and averaged for each group (Fig. 10). Regenerated nerve from channels prepared with chitosan, NGF, and laminin (Ch,N,L) demonstrated 350% greater blood vessel area/nerve area than chitosan alone (Ch) ($p < 0.01$). A 200% increase in the blood vessel area/nerve area was associated with the Silastic and NGF channel (Si,N) compared to the Silastic channel alone (Si) ($p < 0.01$). In spite of the inflammatory response associated with chitosan alone, the Silastic and

NGF channel (Si,N) demonstrated a 200% increase in the percentage blood vessel area /nerve area compared to the channels prepared with chitosan alone (Ch) ($p < 0.05$).

DISCUSSION

Neurotrophism

The present study was undertaken to determine if peripheral nerve regeneration after transection in the adult mouse could be improved by increasing the local concentration of NGF and laminin. Two parameters were manipulated during the nerve regeneration period. The first parameter was to increase the local concentration of the neurotrophic factor(s). The second parameter was to alter the duration of availability of these factors: short-term availability of NGF from the Silastic channel lumen vs. prolonged availability of NGF with and without laminin from the chitosan channel wall. Evaluation of prolonged availability of NGF from chitosan was complicated by inflammation associated with chitosan.

Peripheral nerve regeneration was not improved by physiologic and anatomic criteria evaluated in the groups exposed to increased concentrations of NGF or laminin. The only significant finding was an increase in myelinated axonal diameter in the channels prepared with NGF and laminin when compared to chitosan alone. This effect was probably a result of laminin alone and not NGF and laminin, because there was no measurable difference brought about by the addition of NGF alone in either the chitosan or Silastic channels. The axonal density of the regenerated nerves in the Silastic channels was greater than the normal nonsectioned nerve; however, when the greater nerve area of the normal nerve is taken into account, there was little or no difference between the total number of axons of the Silastic channels vs. normal nerve.

This study also indicates that short-term availability of supraphysiologic concentrations of NGF within a Silastic channel lumen does not improve sciatic nerve regeneration in the adult mouse. The results suggest that the regenerating nerve and associated Schwann cells have sufficient concentrations of endogenous NGF to maximally promote nerve regeneration. This is supported by the finding that the number of regenerated axons in the Silastic channel without NGF added to the lumen is the same as the number of axons in the normal sciatic nerve.

Searching for a more sensitive indicator of supraphysiologic concentrations of NGF on axon regeneration, we studied myelinated axonal maturation. Our study model, after 6 weeks of regeneration, did not demonstrate increased maturation either by increased myelinated axon diameter or myelin sheath thickness. Chen et al.[16] showed an increase in nerve trunk diameter and a trend toward an increased number of axons per fascicle in rabbit facial nerves exposed to NGF compared to those without. Mouse sciatic nerve fascicles are much less defined than in the rabbit facial nerve and are most accurately quantified using a collage of EM photographs of the entire regenerated nerve. This was not performed in our study.

Rich et al.[17] reported a quantitative increase in and maturation of fascicles in the distal nerve stump in those channels with nerve growth factor. Our findings are consistent with those of Chen et al.[16] and Rich et al.,[17] who were unable to demonstrate a significant increase in the number or diameter of regenerated myelinated axons, respectively, in the presence of increased concentrations of NGF within the middle portion of the Silastic channel. Our study used NGF at a concentration ten times greater than that of Chen et al.[16] and we were still unable to document a neurotrophic effect.

In the study by Rich et al.[17] it is important to emphasize that the trophic effect seen was only reported for Schwann cell activity and only in the distal nerve stump, not in the middle portion of the channel of regenerated nerve. Their results support an important NGF trophic effect of improving Schwann cell activity in the distal nerve stump, thereby myelinating more axons that regenerate across the nerve gap. However, the total number of regenerated axons (myelinated and unmyelinated) were the same from both NGF treated and non-NGF treated groups. Our data did not reveal an increase in Schwann cell activity in the middle portion of the channel of regenerated nerve.

Considering the findings from the three nerve regeneration studies of Chen et al.,[16] Rich et al.,[17] and Santos et al. (current study), it appears that supraphysiologic concentrations of NGF in the nerve guidance channel during nerve regeneration do not augment the number or diameter of the regenerated and myelinated axons of the middle section of the channel, but does have a trophic effect of improving Schwann cell activity in the distal nerve stump, thereby increasing the number of myelinated axons, the diameter of the myelin sheath, and the number of fascicles distally. Further investigations need to address: (1) if the trophic effects noted are permanent or merely accelerating maturational events with a common endpoint, and (2) what trophic effects might be occurring in the proximal nerve stump.

Chitosan

The use of chitosan for nerve guidance channels was complicated by approximately half of the chitosan channels becoming dehiscent and fragmented with a resultant inflammatory response and poorer nerve regeneration. Even in chitosan channels that remained intact

without fragmentation or inflammation, however, nerve regeneration was still stunted when compared to those nerves in the Silastic channel groups. In spite of the chitosan inflammation, NGF biologic activity within chitosan channels remained intact. This conclusion is based on the observation that increased angiogenesis was associated with all of the chitosan channels prepared with NGF.

In light of the inflammation and overall poorer nerve regeneration observed with chitosan, it is not possible from this study to comment on the effects of prolonged NGF exposure on peripheral nerve regeneration. It is interesting to note that some chitosan fragments were without associated cellular inflammatory response (as demonstrated in Fig. 1, *D*), which suggests that some fragments of chitosan may have been less inflammatory than others. One possible explanation for the differences in inflammation is protein contamination with an immunologic host vs. foreign body response. We were recently informed that chitosan derived from the squid induces a lesser immunologic response than chitosan derived from the crab (used in this study). This is thought to be a function of protein content (personal communication: Powell J, Minneapolis, Minn.). Other potential biomaterials that would serve the dual function of both nerve guidance channel and sustained release carrier of NGF have been evaluated. Thus far, none has proved ideal.

Angiotrophism

A surprising and apparently new finding from this study was the strong association between NGF and angiogenesis. The number of blood vessels per nerve and the ratio of vessel area to nerve area were significantly increased in both the regenerating nerve from chitosan and Silastic channels prepared with NGF compared to those channels prepared without NGF. In a separate study, NGF receptors have been identified lining blood vessel endothelium and perivascular cells within the adult rat brain.* It is as yet unclear what role the NGF receptors along the endothelium might play. Although NGF has not been described as an angiogenic factor, several angiogenic factors have been well-characterized, purified, and cloned.[25,26] No structural homology exists between the NGF peptide and known angiogenic factors.

Angiogenic growth factors were originally described in association with tumors. However, angiogenic factors are now known to also exist in normal proliferating tissue as well as nonproliferating tissue.[26,27] Angiogenic growth factors are a complex group of factors, but can be divided roughly into two categories: (1) factors directly responsible for endothelial cell proliferation and motility, and (2) factors stimulating other cells, such as macrophages, to release angiogenic factors. Fibroblast growth factors (FGF, acidic, and basic types) and transforming growth factor (TGF-alpha) have direct endothelial cell proliferative effects. TGF-beta and angiogenin have angiogenic activity but lack direct endothelial cell proliferative activity. Other factors have not been as well characterized; included are certain prostaglandins and small peptides from wound fluid and modulators of angiogenesis (heparin and copper).[26]

In light of the current information on angiogenic factors, it is unclear by what mechanism(s) NGF is associated with angiogenesis. It is possible that the angiogenic effect is merely caused by an inflammatory response to the NGF protein in high concentrations. Two pieces of evidence against an inflammatory mediated mechanism are: (1) the same level of angiogenesis in two disparate nerve channel models with NGF (i.e., chitosan channels with associated inflammation and Silastic channels without inflammation), and (2) known presence of NGF receptors lining endothelial cells, thus suggesting that NGF exerts a direct proliferative effect on endothelial cells. Clearly the nerve regeneration channel model is not the ideal model to study angiogenesis and studies to further the angiogenic activities of NGF are in progress.

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REFERENCES

1. Levi-Montalcini R. The NGF 35 years later. Science 1987;237:1154-62.
2. Longo FM, Manthorpe M, Skaper SD, Landborg G, Varon S. Neuronotrophic activities accumulate in vivo within Silastic nerve regeneration chambers. Brain Res 1983;261:107-17.
3. Varon S, Manthorpe M, Williams LR. Neuronotrophic and neurite-promoting factors and their clinical potentials. Dev Neurosci 1983/4;6:73-100.
4. Baron-Van Evercooren A, Kleinman HK, Ohno S, Marangos P, Schwarz JF, Dubois-Dalcq ME. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J Neurosci Res 1982;8:179-93.
5. Edgar D. Nerve growth factors and molecules of the extracellular matrix in neuronal development. J Cell Sci 1985;3:107-13.
6. Campenot RB. Local control neurite development by nerve growth factor. Proc Natl Acad Sci USA 1977;74:4516-9.
7. Enfors P, Henschen A, Olson L, Persson H. Expression of nerve growth factor receptor mRNA is developmentally regulated and

increased after axotomy in rat spinal cord motoneurons. Neuron 1989;2:1605-13.
8. Verge VMK, Riopelle RJ, Richardson PM. Nerve growth factor receptors on normal and injured sensory neurons. J Neurosci 1989;9:914-22.
9. Heumann R, Lindholm D, Bandtlow C, et al. Differential regulation on mRNA endocing nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: Role of macrophages. Proc Natl Acad Sci USA 1987;84:8735-9.
10. Taniuchi M, Clark HB, Johnson EM. Induction of nerve growth factor in Schwann cells after axotomy. Proc Natl Acad Sci USA 1986;83:4094-8.
11. Lundborg G, Dahlin LB, Danielsen N, et al. Nerve regeneration in silicone chambers: influence on gap length and of distal stump components. Exp Neurology 1982;76:361-75.
12. Madison R, da Silva CF, Dikkes P, Chiu TH, Sidman RL. Increased rate of peripheral nerve regeneration using bioresorbable nerve guides and a laminin-containing gel. Exp Neurology 1985;88:767-72.
13. Szal GJ, Miller T. Surgical repair of facial nerve branches. Arch Otolaryngol 1975;101:160-5.
14. Valentini RF, Aebischer P, Winn SR, Galletti PM. Collagen- and laminin-containing gels impede peripheral nerve regeneration through semipermeable nerve guidance channels. Exp Neurology 1987;98:350-6.
15. Williams LR. Exogenous fibrin matrix precursors stimulate the temporal progress of nerve regeneration within a silicone chamber. Neurochem Res 1987;12:851-60.
16. Chen Y, Wang-Bennett LT, Coker NJ. Facial nerve regeneration in the silicone chamber: the influence of nerve growth factor. Exp Neurology 1989;103:52-60.
17. Rich KM, Alexander TD, Pryor JC, Hollowell JP. Nerve growth factor enhances regeneration through silicone chambers. Exp Neurology 1989;105:162-70.
18. Williams LR, Longo FM, Powell HC, Lundborg G, Varon S. Spatial-temporal progress of peripheral nerve regeneration within a silicone chamber: parameters for a bioassay. J Comp Neurol 1983;218:460-70.
19. Allan GG, Altman LC, Bensinger RE, et al. Chitin, chitosan, and related enzymes, biomedical applications of chitin and chitosan. New York: Academic Press, Inc., 1984:119-33.
20. Balassa LL, Prudden JF. Applications of chitin and chitosan in wound healing acceleration. Proceedings of the First International Conference on Chitin/Chitosan. MIT Sea Grant Program, 1978:296-305.
21. Nakajima M, Atsumi K. Chitin, chitosan, and related enzymes, development of absorbable sutures from chitin. New York: Academic Press, Inc., 1984:406-10.
22. Burton LE, Wilson WH, Shooter E. Nerve growth factor in mouse saliva. J Biol Chem 1978;253:7807-12.
23. Heidemann SR, Joshi HC, Schechter A, Fletcher JR, Bothwell M. Synergistic effects of cyclic AMP and NGF on neurite outgrowth and microtubule stability of PC12 cells. J Cell Biol 1985;100:916-27.
24. Langford LA, Coggeshall RE. The use of potassium ferricyanide in neural fixation. Anat Rec 1980;197:297-303.
25. Presta M, Rifkin DB. New aspects of blood vessel growth: tumor and tissue-derived angiogenesis factors. Haemostasis 1988; 18:6-17.
26. Folkman J, Klagsbrun M. Angiogenic factors. Science 1987;235:442-7.
27. Folkman J. Tumor angiogenesis. Adv Cancer Res 1985;43:175-203.