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Peripheral Nerve Regeneration Through Blind-Ended Semipermeable Guidance Channels: Effect of the Molecular Weight Cutoff

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Synthetic nerve guidance channels are used to better understand the cellular and molecular events controlling peripheral nerve regeneration. In the present study, the contribution of wound-healing molecules to peripheral nerve regeneration was assessed by varying the molecular weight cutoff of the tubular membrane. Nerve regeneration through polysulfone tubular membranes with molecular weight (*Mw*) cutoffs of 10^5 and 10^6 Da was analyzed in a transected hamster sciatic nerve model. Cohorts of 6 animals received tubes of either type for 4 or 8 weeks with the distal end of the polymer tube capped. Other cohorts of 6 animals received tubes of either type for 4 weeks with the distal nerve stump secured within the guidance channel so as to create a 4 or 8 mm gap between both nerve stumps. Both types of channels contained regenerated tissue cables extending to the distal end of the guidance channel at both 4 and 8 weeks in the absence of a distal nerve stump. The cables regenerated in the 10^5 Da channels were composed of nerve fascicles surrounded by a loose epineurial sheath, whereas those regenerated in the 10^6 Da channels were composed mainly of granulation tissue. The numbers of myelinated and unmyelinated axons were significantly greater in the 10^5 Da than in the 10^6 Da channels at both 4 and 8 weeks. Both types of channel contained regenerated tissue cables with numerous nerve fascicles when the distal nerve stump was present with either gap length. However, when the gap distance was 8 mm, the 10^6 Da channels contained significantly fewer myelinated axons than the 10^5 Da channels. The present study reveals that the *Mw* cutoff of a semipermeable guidance channel strongly influences the outcome of peripheral nerve regeneration, possibly by controlling the exchange of molecules between the channel's lumen and the external wound-healing environment. These results suggest that the wound-healing environment secretes humoral factors that can either promote or inhibit the nerve-regeneration process.

Synthetic nerve guidance channels can help elucidate the cellular and molecular events underlying nerve regeneration. Wrapping of transected nerves with a silicone elastomer tube has revealed the contribution of the distal nerve stump in supporting peripheral nerve regeneration (Williams et al., 1983). Transected sciatic nerves do not regenerate if the distal insert of a silicone chamber is replaced by a tendon, skin, or muscle (Williams et al., 1984). In the rat, no regeneration occurs if the distance between both nerve stumps is >10 mm or if the distal end of the silicone tube is capped (Lundborg et al., 1982; Shine et al., 1985). The same observation has been made with other synthetic polymer tubes such as polyethylene (Madison et al., 1985) and polyvinyl chloride (Scaravilli, 1984), which are impermeable to watery solutes and effectively isolate the regenerative environment from the surrounding tissues, so that only cells or fluids within the tube can influence regeneration. These tubes, therefore, eliminate the effect of potential external factors that could affect the regeneration process. Cultured PNS cells suspended in a collagen matrix and placed in capped polyethylene tubes supported limited axonal regeneration in the absence of a distal nerve stump (Shine et al., 1985). Only isolated fascicles of axons were observed. The number of myelinated axons also decreased considerably along the length of the tube. Blind-ended tubes filled only with media conditioned by peripheral neurons did not support regeneration. In contrast to impermeable guidance channels, blind-ended semipermeable tubes with a molecular weight (*Mw*) cutoff of 50,000 Da allowed peripheral nerve regeneration in the absence of a distal nerve stump, suggesting that factors other than those produced by the distal nerve stump can support regeneration (Aebischer et al., 1988). Growth or trophic factors secreted by the wound-healing process around the guidance channel were thought to diffuse within the guidance channel's lumen and support nerve regeneration. By varying the *Mw* cutoff of the tube wall, it may be possible to assess the relative contribution to wound-healing molecules to peripheral nerve regeneration. The ability of different *Mw* cutoff membranes to support peripheral nerve regeneration in the absence of a distal nerve stump was tested in the sciatic nerve model of hamsters.

Materials and Methods

Guidance channel characterization and preparation. Segments of polysulfone tubes (i.d., 1.1 mm; o.d., 2.0 mm), 10 cm long, with various *Mw* cutoffs were kindly provided by the Amicon Corporation (Lexington, MA). The tubes featured a partially open outer skin separated from a semipermeable inner skin by an open trabecular network. Membranes with *Mw* cutoffs of 10^5 and 10^6 Da were tested. Membrane *Mw* cutoff was determined by measuring the rejection coefficient of various size molecules. All tubing materials were cleaned and sterilized prior to implantation as previously described (Aebischer et al., 1986, 1988). The channels were then cut into 8-, 10-, and 12-mm-long segments.

Animal model and guidance channel implantation. Young male LVG hamsters (Charles River Laboratories, Wilmington, MA) weighing 80–100 gm were anesthetized by inhalation of methoxyflurane (Metofane®). The left sciatic nerve was exposed through a lateral skin incision and carefully dissected from the surrounding connective tissue. Channel

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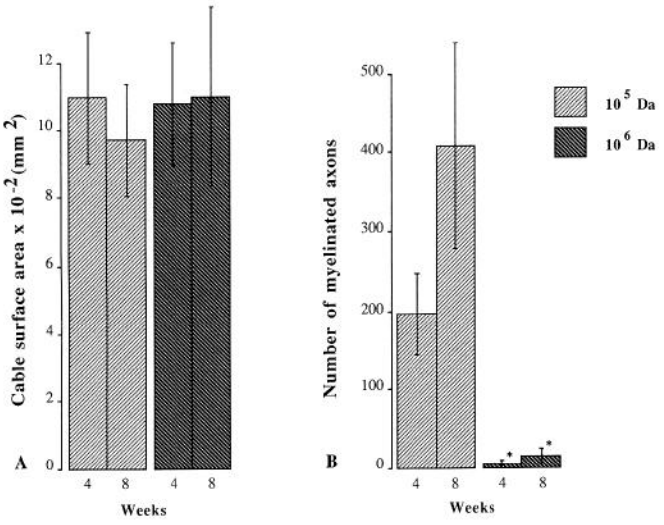

Figure 1. Histogram showing (*A*) the cable surface area and (*B*) the number of myelinated axons of cables regenerated in 10^5 and 10^6 Da guidance channels 5 mm from the proximal stump in a blind-ended guidance channel at 4 and 8 weeks postimplantation.

implants were made either with or without the distal nerve stump present. For the former group, a 4 mm segment of nerve immediately proximal to the tibioperoneal bifurcation was excised and discarded. The proximal and distal nerve stumps were secured 4 or 8 mm apart within 8- or 12-mm-long tubes, respectively, using single 10-0 Nylon sutures placed through the epineurium. For the latter group of implants, the sciatic nerve was transected about 5 mm proximal to the tibioperoneal bifurcation. The remaining distal nerve branches were resected up to their muscle penetration and discarded. The proximal nerve stump was secured 2 mm within a 10-mm-long channel using a single 10-0 Nylon suture placed through the epineurium. The distal end of the channel was then capped with a compatible acrylic glue. The wound was closed as previously described (Aebischer et al., 1988). Surgery was performed aseptically under a Zeiss operating microscope.

Cohorts of 6 animals with their distal nerve stump secured within the distal end of 10^5 and 10^6 Da channels were implanted for 4 weeks. Cohorts of 6 animals received 10^5 and 10^6 Da blind-ended channels for 4 and 8 weeks. The animals were caged in groups of 6 and housed in a controlled environment with 12 hr on–off light cycles. They received food and water *ad libitum*.

Implant retrieval and evaluation. Upon retrieval, the animals were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/ml) and transcardially perfused with 40 ml of heparinized physiologic saline followed by 40 ml of a modified Karnovsky's solution consisting of 3.0% paraformaldehyde and 2.0% glutaraldehyde in PBS at pH 7.4. The wound was reopened and the guidance channel

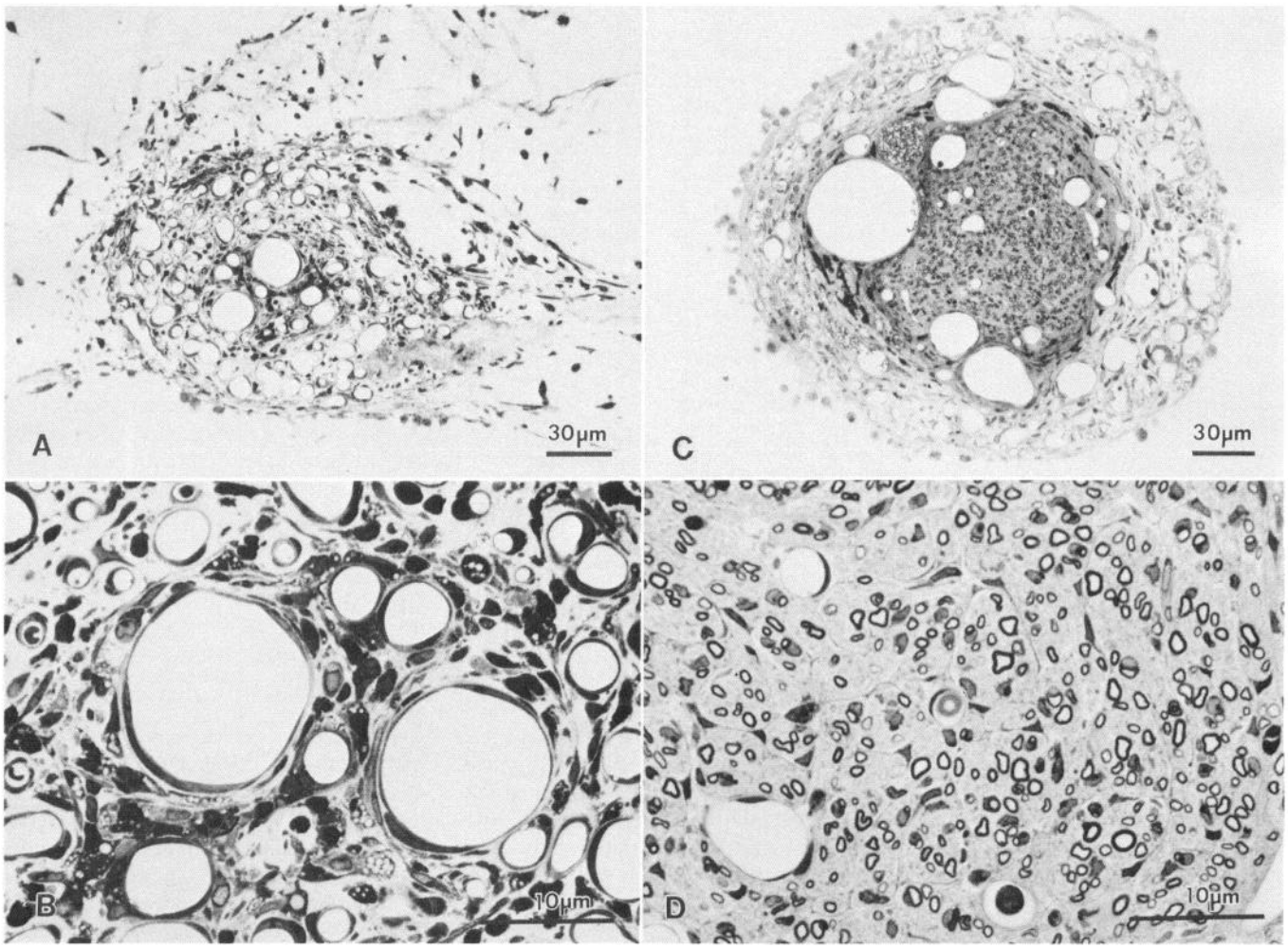

Figure 2. Light micrographs of cables regenerated in blind-ended guidance channels 8 weeks postimplantation. *A,* Loose granulation tissue lacking a well-defined border is observed in the 10^6 Da channels. *B,* At higher power, collagenous tissue and macrophage-like cells can be observed. No nerve fascicle is present. *C,* Numerous nerve fascicles surrounded by an epineurial-like tissue containing large amounts of blood vessels are seen with the 10^5 Da channels. *D,* At higher power, note the presence of numerous myelinated axons.

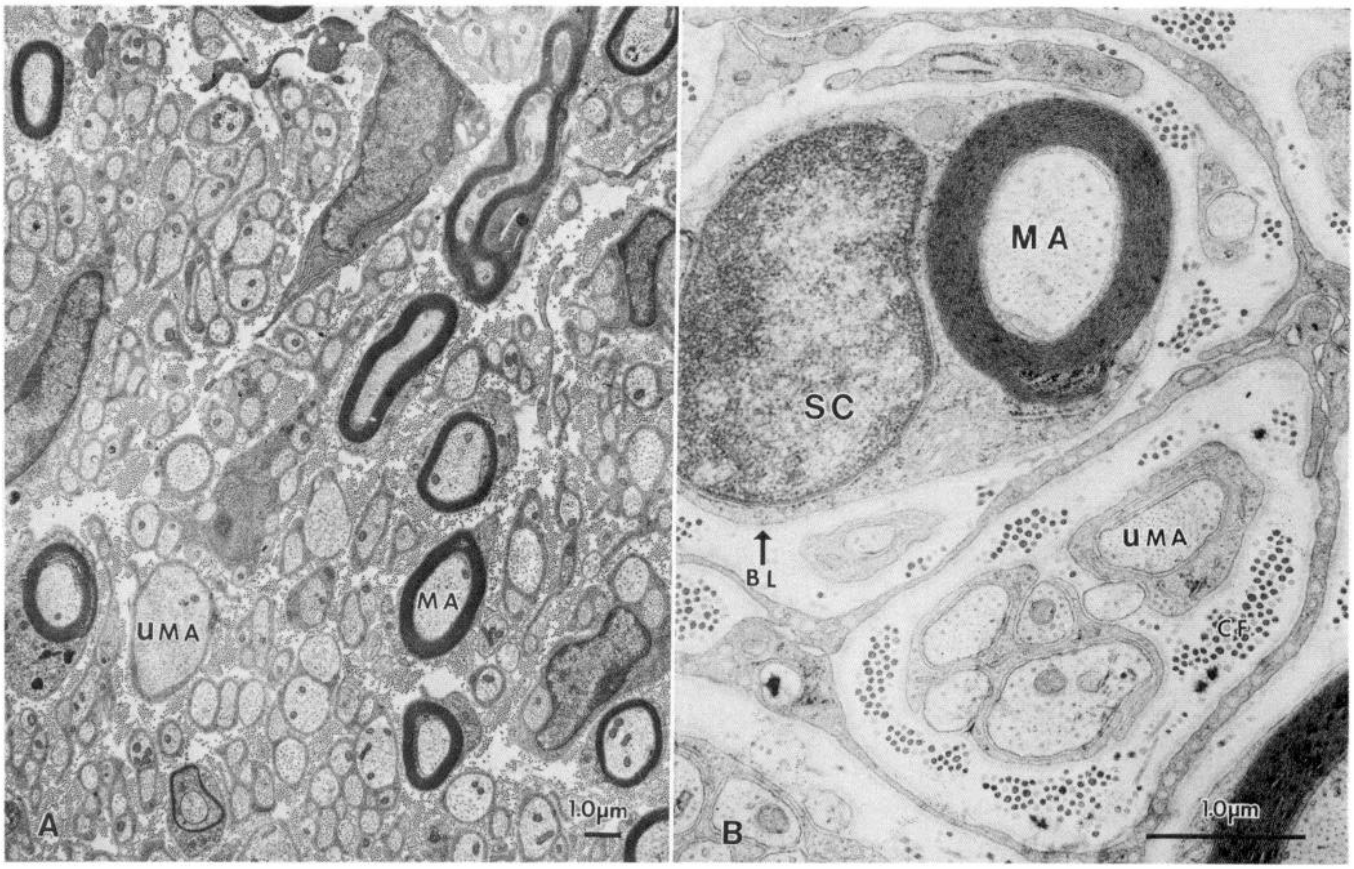

Figure 3. *A*, Electron micrograph taken at the midpoint of a 10^5 Da channel 8 weeks postimplantation showing myelinated (*MA*) and unmyelinated (*UMA*) axons with their Schwann cells. Note the relatively small diameter of the myelinated axons and the very high number of unmyelinated axons. *B*, Higher-power micrograph revealing Schwann cells (*SC*) with their myelinated (*MA*) and unmyelinated (*UMA*) axons with their typical basal laminae (*BL*) and collagen fibrils (*CF*).

removed with a 5-mm-long piece of native nerve. The specimens were soaked overnight in the same fixative and cut transversely at their midpoint. Specimens were postfixed, dehydrated, and embedded in Spurr resin as previously described (Aebischer et al., 1988). The cable area, number and area of blood vessels, and number of myelinated axons were analyzed at the channel's midpoint on serial transverse semithin sections with the aid of a morphometric analysis system (CUE-2, Olympus Corp., Lake Success, NY) interfaced with an IM35 Zeiss microscope. Ultrathin sections (70 nm) were cut on a Reichert Ultracut E microtome, stained with Reynold's lead citrate and uranyl acetate, and examined on a Philips 410 transmission electron microscope. Unmyelinated axon populations were evaluated at the channel's midpoint by averaging the number of unmyelinated axons counted on 10 randomly taken transmission electron micrographs at an original magnification of 3000× and extrapolating the average to the total nerve cable area.

All data are presented as means ± SEM. The Student *t* test was used to determine statistical differences between the various populations.

Results

Upon retrieval at both time periods, the polysulfone tubes exhibited a limited external tissue reaction, consisting of a layer of macrophage-like cells (identified as such by their interdigitating membranes) spread over the channel's outer wall. This macrophage layer was covered with 3–6 layers of fibroblasts with elongated nuclei and extensive rough endoplasmic reticulum, alternating with collagen fibrils. Macrophage-like cells with elaborate pseudopodia and multinucleate giant cells invaded the channel's trabecular network.

Distal nerve stump absent

All 10^5 Da and 5 of 6 10^6 Da channels contained tissue cables extending to the distal end of the guidance channel at both 4 and 8 weeks. There was no significant difference in cross-sectional area between the cables regenerated in the 10^5 and 10^6 Da channels at both 4 and 8 weeks (Fig. 1*A*). No increase in cable size was noticed for either channel type between 4 and 8 weeks.

The cables regenerated in the 10^6 Da channels were composed mainly of granulation tissue (Fig. 2*A*). The granulation tissue was composed of macrophage- and fibroblast-like cells surrounded by large numbers of collagen fibrils and blood vessels (Fig. 2*B*). Small, isolated nerve fascicles were spread in the granulation tissue. Cable delineation was poorly defined. In contrast, well-defined circular cables surrounded by an acellular gel were present in the 10^5 Da channels. The cables were composed of nerve fascicles surrounded by a loose epineurial sheath containing numerous blood vessels (Fig. 2*C*). The nerve fascicles were composed of myelinated and unmyelinated axons with presumptive Schwann cells (Fig. 2*D*). The Schwann cells were identified by their typical basal laminae and the presence of axons in their cytoplasm (Fig. 3*A*).

In the 10^6 Da channels, 2 of 5 and 3 of 6 regenerated cables contained myelinated axons at 4 and 8 weeks, respectively, whereas in the 10^5 Da channels, 5 of 6 cables and 6 of 6 regen-

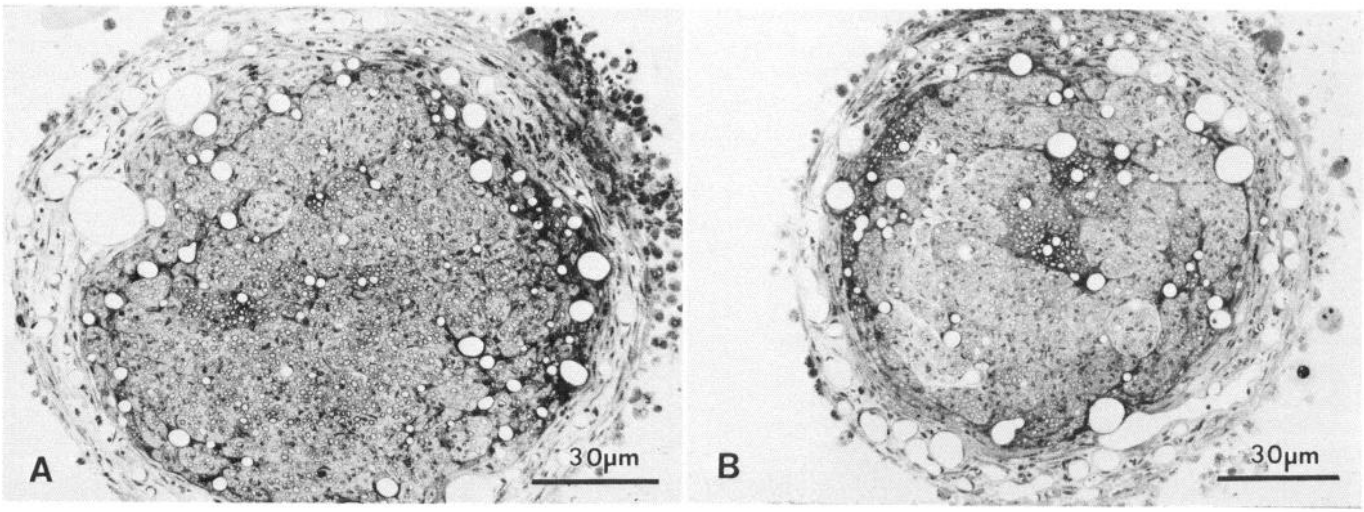

Figure 4. Light micrographs of cables regenerated in (*A*) 10^5 Da and (*B*) 10^6 Da guidance channels with a 4 mm gap 8 weeks postimplantation. Numerous nerve fascicles surrounded by an epineurial-like tissue containing blood vessels are seen with either type of channels.

erated cables contained myelinated axons at 4 and 8 weeks, respectively. The number of myelinated axons was significantly greater in the 10^5 Da than in the 10^6 Da channel types ($p < 0.005$ at 4 and $p < 0.05$ at 8 weeks; Fig. 1*B*). Nerve cables regenerated in the 10^5 Da channels contained more myelinated axons at 8 than at 4 weeks, although the difference was not statistically significant. Transmission electron micrographs of the regenerated cables displayed abundant unmyelinated axons in both channel types (Fig. 3*B*). At 8 weeks, the number of unmyelinated axons was significantly greater in the 10^5 Da channels than in the 10^6 Da channels (11,172 ± 2901 vs 4167 ± 1583; $p < 0.05$). The percentage of myelinated versus unmyelinated axons was 3.8 ± 1.1% in the 10^5 Da channels and 0.6 ± 0.3% in the 10^6 Da channels.

Distal nerve stump present

With the exception of one 8 mm gap 10^5 Da channel, all channels contained regenerated cables bridging the nerve stumps. The regenerated cables were centrally located within the tube and surrounded by a gel-like matrix. A well-defined epineurial sheath surrounded numerous nerve fascicles (Fig. 4, *A, B*). The nerve fascicles were composed of myelinated and unmyelinated axons with their presumptive Schwann cells. No morphological difference was observed between the 10^5 and the 10^6 Da channels for either gap distance.

Quantitative analysis, however, revealed differences between the various groups. The cable area and the number of myelinated axons were significantly smaller in the channels with an 8 mm versus a 4 mm gap in both the 10^5 and 10^6 Da channels (p = 0.01–0.005; Fig. 5, *A, B*). There was no significant difference in cable size between the 10^5 and 10^6 Da channels for both the 4 and 8 mm gaps (Fig. 5*A*). There was no significant difference in the number of myelinated axons between the 10^5 and 10^6 Da channels with a 4 mm gap. However, the 10^5 Da channels contained significantly more myelinated axons than the 10^6 Da channels when the gap distance was 8 mm ($p < 0.05$; Fig. 5*B*).

Discussion

The present study shows that the *Mw* cutoff of the tubular membrane exerts a significant influence on the ability of the transected nerve to regenerate in blind-ended semipermeable guidance channels. Both the 10^5 and 10^6 Da channels support the regeneration of a tissue cable in blind-ended tubes. This observation suggests that growth or trophic factors diffuse into the guidance channel lumen and promote the formation of a tissue cable. The presence of myelinated and unmyelinated axons in the regenerated tissue cable is, however, strongly influenced by the *Mw* cutoff of the channel. Only the 10^5 Da channels supported the regeneration of well-differentiated peripheral nervous tissue containing a significant number of myelinated axons. When the distal stump was secured at the distal end of the tube, no difference between channels was observed if the gap distance separating both nerve stumps was 4 mm. However, when the gap distance was increased to 8 mm, the 10^6 Da tubes contained significantly less myelinated axons than the 10^5 Da tubes.

Various peptides have been reported to promote the survival

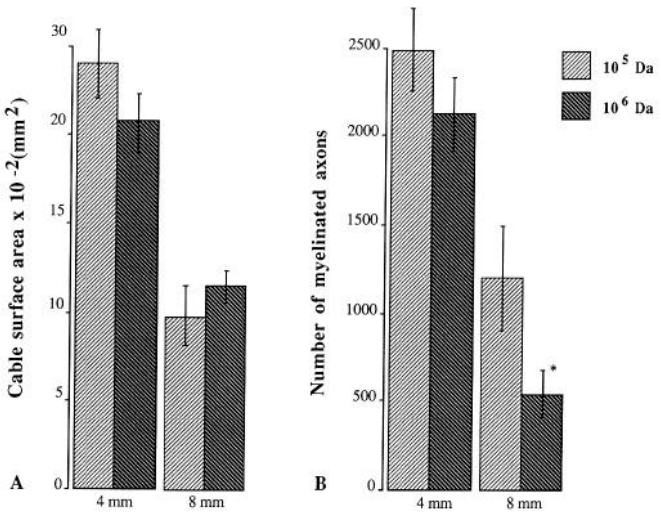

Figure 5. Histogram showing (*A*) the cross-sectional area and (*B*) the number of myelinated axons in cables regenerated in 10^5 and 10^6 Da guidance channels with a 4 and 8 mm interstump gap at 4 weeks postimplantation.

and neuritic extension of different types of neurons. Nerve growth factor (NGF) and basic fibroblast growth factor (b-FGF) promote the survival of dorsal root ganglia cells both *in vitro* (Levi-Montalcini, 1986; Unsicker et al., 1987) and *in vivo* (Hamburger et al., 1981; Otto et al., 1987). b-FGF adsorbed on synthetic substrates enhances the spatial progression of peripheral nerve regeneration (Danielson et al., 1988). The controlled release of b-FGF from synthetic nerve guidance channels promotes peripheral nerve regeneration over long nerve gaps (Aebischer et al., 1989), suggesting that b-FGF is a mediator of peripheral nerve regeneration *in vivo*. Activated macrophages are known to secrete various neurotrophic molecules, including b-FGF (Baird et al., 1985), NGF (Otten et al., 1987), and apolipoprotein E (Basu et al., 1981, 1982). Macrophages seen adhering to the outer surface of the guidance channels could secrete various growth or trophic factors which can diffuse into the guidance channel lumen and promote nerve regeneration in the absence of a distal nerve stump.

Utilizing the bifurcation of a rat abdominal aorta as a Y-channel system with or without the insertion of tendons and nerve stumps, Weiss and Taylor (1944) first reported the contribution of the wound-healing environment to peripheral nerve regeneration. The contribution of external wound factors was also suggested by the use of "holey" tubes as nerve guidance channels. Silicone tubes that were made porous by punching 2 macroscopic holes through their walls supported enhanced peripheral nerve regeneration in rats (Jenq and Coggeshall, 1985). When the holes were covered with Millipore filters of various sizes, only the filters which were large enough to allow the inward invasion of cells enhanced regeneration (Jenq and Coggeshall, 1987). The invasion of cells from the external wound was believed to be the basis of the effect. The fact that the present study demonstrates regeneration with tubes not allowing the inward invasion of cells may be due to the quantity of growth factors which are able to diffuse within the channel lumen. The use of a semipermeable tube provides a much greater diffusional surface, thus allowing increased levels of trophic factors to reach the regenerative environment.

The lower number of myelinated axons observed in the 10^6 Da channels, either blind-ended or with an 8 mm gap, may be explained by the presence of a large ($Mw > 10^5$ Da) inhibitory molecule secreted by the wound-healing environment. When the interstump distance is small, e.g., 4 mm, the neurotrophic effect exerted by the distal stump may supercede the effect of the inhibitory molecule.

Migration of neurites has been associated with the presence of growth cone-associated proteases (Krystosek and Seeds, 1981; Monard, 1988). Enhanced neurite outgrowth was observed with various primary cultures of central and peripheral neurons when the neurons were switched to serum-free medium (Luduena, 1973; Ziller et al., 1983). Addition of thrombin to neuroblastoma cells cultured in serum-free medium induces a rapid retraction of neurites (Gurwitz and Cunningham, 1988). Certain inhibitors of serine proteases such as the glia-derived nexin have been reported to stimulate neurite outgrowth from primary neurons (Monard, 1988; Zurn et al., 1988), while others such as inhibitors of plasminogen activators have been shown to impair sciatic nerve regeneration (Kalderon et al., 1987). These studies suggest that neurite outgrowth *in vitro* is regulated by a delicate balance between proteases and their inhibitors. The wound-healing environment may secrete some high-*Mw* protease inhibitors which could affect neurite extension *in vivo*.

We conclude that semipermeable guidance channels are capable of supporting peripheral nerve regeneration in the absence of a distal nerve stump, but that the *Mw* cutoff of the tubular membrane strongly influences the outcome of regeneration, possibly by controlling the exchange between the external wound-healing environment and the internal regenerative environment. The present study suggests that the wound-healing environment secretes humoral factors that can either enhance or inhibit the nerve regeneration process. Further studies aimed at the characterization of the wound-healing molecules affecting peripheral nerve regeneration should be considered.

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