Evaluation of acellular and cellular nerve grafts in repair of rat peripheral nerve

ADARSH K. GULATI, PH.D.

Department of Anatomy, Medical College of Georgia, Augusta, Georgia

Nerve grafts composed of basal lamina scaffolds and lacking viable Schwann cells have recently been shown to be effective in supporting axonal regeneration. As only short grafts were used in those studies, the present investigation was conducted to evaluate the ability of long acellular basal lamina nerve grafts and equivalent cellular grafts to support axonal regeneration for nerve gap repair. Cellular grafts consisted of nerve segments that had degenerated in situ for 4 weeks. Acellular grafting material consisted of similar segments that were repeatedly frozen and thawed to kill all cells prior to grafting. The results show that host axons can regenerate through the entire 4-cm length of cellular grafts but not through acellular basal lamina grafts. However, in the acellular grafts numerous axons were seen in the proximal 2-cm region. It is concluded that basal lamina grafts possess limited ability to support axonal regeneration. As in cellular grafts, viable Schwann cells appear to be important for regeneration to occur over longer distances.

KEY WORDS • nerve graft • nerve gap repair • axonal regeneration • basal lamina • peripheral nerve • rat

SUCCESSFUL regeneration of axons is known to occur over considerable distances through grafts consisting of peripheral nerve placed between injured peripheral nervous tissue as well as in central nervous tissue. The "endoneurial tube(s)" in the peripheral nerve graft is believed to be important in guiding and promoting the growth of the regenerating axon.

Several recent investigations have attributed particular importance to the Schwann cell basal lamina in supporting axonal growth. Basal lamina components such as laminin and fibronectin have been shown to stimulate axonal growth both in vivo and in vitro. Furthermore, nerve grafts composed of basal lamina and devoid of viable Schwann cells allow axonal regeneration through them. Acellular basal lamina grafts derived from skeletal muscle are also effective in supporting axonal growth and maturation. The regenerating axons invariably grow along the inside of the basal lamina scaffolds, implying that the basal laminae provide a favorable substrate and/or microenvironment for axonal growth. Because of the relatively short length of the grafts (less than 1 cm) used in the above studies, it remains to be determined whether longer grafts composed of basal lamina alone would be similarly effective in permitting regeneration through them. Accordingly, the present study analyzes the ability of long (4-cm) acellular basal lamina nerve grafts to support axonal regeneration and compares it to cellular grafts of equal length. A preliminary report of this work has been published elsewhere in abstract form.

Materials and Methods

Animals and Nerve Graft Preparation

Isogeneic male Fischer rats were used in this study in order to exclude the possibility of immune rejection of the graft nerves by their hosts. The nerve grafts were obtained from rats weighing 350 to 400 gm. Donor animals were anesthetized with intraperitoneal chloral hydrate (40 mg/100 gm body weight), and their sciatic nerves were exposed. The nerve in each leg was then cut a few millimeters distal to the sciatic notch and ligated. The nerves were allowed to undergo in situ degeneration for 4 weeks. After this time the donor rats were re-anesthetized, and the degenerated sciatic nerves were exposed and traced to the ankle region. The nerve was cut distally to remove a 4-cm segment and then placed in alpha-minimum essential culture medium. These nerve segments were used for cellular basal lamina nerve grafting without further preparation.

Acellular basal lamina grafts were prepared as fol-
lows. Degenerated nerve segments were prepared as above and were placed on a steel spatula, frozen by
immersing the spatula in liquid nitrogen, and then thawed to room temperature. The freezing time was
about 20 seconds and the thawing time 60 seconds. Nerves were frozen and thawed in this manner five
times. This freeze-thaw procedure was similar to that used by others to prepare acellular basal lamina
grafts.6,8,11 Treatment of nerves in this manner is known to kill all cell types, including Schwann cells, with no
apparent effect on the basal laminae (see the Results section). The frozen-thawed nerves served as acellular
basal lamina grafts.

Nerve Grafting Procedure

The nerve grafting procedure was similar to that used in our earlier studies.6,8,22,28 In brief, the recipient rat
was anesthetized with chloral hydrate, after which an acellular basal lamina graft was placed in the left leg and a
cellular graft in the right. This involved exposing and cutting the peroneal nerve of the recipient rat in mid-
ith. The proximal cut end of the peroneal nerve was then joined to the distal end of the nerve graft by
inserting both ends into a 2- to 3-mm sleeve of carotid artery also removed from the donor rat at the time of
nerve removal. A small amount of fibrinogen (type IX) was topically applied to strengthen the union site. Only
a proximal nerve anastomosis was performed, since this procedure was adequate to determine whether host
axons could traverse each graft without being influenced by the distal host stump. The nerve graft was
looped in the thigh region in order to accommodate its 4-cm length.

Evaluation of Nerve Grafts

Cellular and acellular basal lamina nerve grafts were examined at 1, 2, 4, 8, and 12 weeks after transplanta-
tion. At each time interval at least five grafts of each type were evaluated. Pieces 4- to 5-mm long were cut
along the entire length of the nerve graft, placed in slabs of skeletal muscle, and frozen in liquid nitrogen.
Longitudinal sections and cross sections 8 and 16 ~m thick, respectively, were cut in a cryostat set at −20°C and
mounted on glass slides. Cut nerve pieces were placed in muscle slabs to facilitate sectioning. They were po-
tioned in order, so that the extent of axonal regeneration through the graft could be monitored in a proxi-
mal to distal direction. Some slides with 8- ~m thick sections were stained with periodic acid-Schiff-hema-
toxylin for general histological analysis. Other slides with 8- ~m thick sections were stained with antibodies
against laminin, an integral component of basal lamina,5,24 to monitor Schwann cell basal lamina. The
indirect immunofluorescence technique used for basal lamina analysis was identical to that described earlier.5,6
The slides with 16-~m thick sections were stained by the cholinesterase-silver technique26 to determine ax-
onal growth into each graft.

Results

Morphological Features of Cellular and Acellular Grafts

The cellular basal lamina grafts (obtained from nerves degenerated in situ for 4 weeks) exhibited many
Schwann cells arranged in columns along with some myelin debris (Fig. 1A). The perineurium surrounded
the Schwann cell columns. Laminin, a basal lamina marker, was localized in the region of the Schwann cell
basal lamina. These basal laminae were of various sizes and shapes, and a majority had a circular configura-
tion (Fig. 1B). Laminin staining was also observed in the perineurium and blood vessels (Fig. 1B). These
morphological features of degenerated nerves are similar to those reported earlier.3,19

Upon placement of acellular basal lamina grafts (obtained from nerve segments degenerated for 4 weeks
then frozen and thawed repeatedly), it has been shown that dead cells disintegrate and are slowly absorbed by
the invading host macrophages; however, the basal lamina scaffolds remain.8,19 Thus, in this study, ex-
amination of nerves immediately after the freeze-thaw process revealed the presence of Schwann cells as well
as their basal lamina (that is, features identical to those of cellular grafts, as shown in Fig. 1A and B). A few
days after grafting, the dead Schwann cells and other cells disappeared, and these nerves appeared acellular
(Fig. 1C). In spite of the absence of Schwann cells, the basal laminae persisted (Fig. 1D) and exhibited a variety
of forms similar to those seen in cellular grafts.

Regeneration Through Cellular Grafts

Regeneration of host axons through cellular basal lamina grafts occurred rapidly, and many axons traversed
their entire length. In the proximal region of the cellular grafts, some regenerated and myelinated axons
were seen as early as 1 week after grafting (Fig. 2A). Regeneration progressed distally with increased time,
and by 8 weeks the axons had crossed the entire 4-cm length of these grafts. Figure 2B to D illustrates the
presence of regenerated and myelinated axons in the proximal, middle, and distal regions of cellular grafts,
respectively. In all of the long-term cellular grafts examined, numerous myelinated axons, perineurium, and
vasculature were observed along the entire graft length. The cholinesterase-silver stain also revealed the pre-
sence of regenerated axons throughout the cellular grafts (Fig. 2E). Each of the regenerated and myelinated axons
was enclosed by a basal lamina as determined by laminin antibody staining (Fig. 2F). In many instances,
clusters of three to six regenerated axons were seen adjacent to each other forming a fascicle. The peri-
nneurium also showed clear staining with laminin antibodies.

Regeneration Through Acellular Grafts

In contrast to the regenerative success through the cellular grafts, regenerating axons did not traverse the
Nerve regeneration through grafts

FIG. 1. Photomicrographs of a nerve after in situ degeneration for 4 weeks. PAS-hematoxylin, × 85. A: Longitudinal section of a fresh specimen used as a cellular graft. Many Schwann cells arranged in columns are seen. Arrows point to the perineurium. B: Cross section of a fresh specimen stained with antibodies against laminin. The Schwann cell basal lamina region and the perineurium (arrow) are clearly stained. Staining is also seen in association with blood vessels (V). C: Longitudinal section of a specimen after freezing and thawing. 7 days after grafting into a host rat. Schwann cells are absent (compare with A) and the nerve appears acellular. Arrows point to the perineurium. D: Cross section of a specimen after freezing and thawing. 7 days after grafting. This section is stained with antibodies against laminin. The Schwann cell basal lamina, the perineurium (arrow), and the blood vessel basal laminae (V) are identifiable (compare with B).

entire length of the acellular basal lamina grafts. Regenerated axons were seen only in the proximal 2 cm of the acellular grafts. Axonal regeneration and Schwann cell migration were observed in the proximal region of the acellular grafts. However, the axonal growth did not progress distally, and in no case did it reach the most distal portion of the graft. Figure 3 shows the distribution of regenerated and myelinated axons in the different regions of the acellular basal lamina grafts. Numerous axons were present in the proximal 2 cm of these grafts (Fig. 3A and B). Further distally, the axons became very sparse (Fig. 3C) and were completely absent in the most distal region (Fig. 3D). Absence of regenerated axons in the most distal region of the acellular grafts was also observed in additional grafts analyzed 24 weeks after grafting. The distal graft contained unidentifiable connective tissue cells interspersed in a collagenous-type matrix (Fig. 3D). The perineurium in these acellular grafts was not as easily identifiable as in the cellular grafts. The marked superiority of cellular grafts over acellular grafts for supporting axonal regeneration over long distances can be appreciated by comparing Fig. 2C and D with Fig. 3C and D. Laminin staining of the proximal region of the acellular graft revealed basal lamina around the regenerated and myelinated axons, similar to that shown in Fig. 2F. However, the basal lamina scaffolds in the distal graft region were collapsed and appeared as dots that are not shown in the figure.

Discussion

The present study shows that host axons can grow through the entire 4-cm length of cellular grafts but not through the acellular grafts. Numerous axons were present in the proximal region of the acellular grafts. Earlier studies with acellular basal lamina grafts used only short grafts.8-11 Thus, the extent to which regenerating axons can grow through such grafts could not be determined.
FIG. 2. Photomicrographs of cellular basal lamina grafts. PAS-hematoxylin. A: Cross section of the proximal region of a cellular graft 1 week after grafting. Some regenerated and myelinated axons (R) can be identified within the graft. Arrow points to the perineurium. × 88. B: Cross section of the proximal region of a cellular graft 12 weeks after grafting. Numerous regenerated and myelinated nerve fibers are shown surrounded by the perineurium (arrow). × 88. C: Longitudinal section of the middle region of the cellular graft shown in B. Numerous regenerated nerve fibers (long arrow) and the perineurium (short arrow) are also seen in this section. × 88. D: Cross section of the distal region of the cellular graft shown in B and C. Numerous regenerated nerve fibers surrounded by a perineurium (arrow) are present at this region also. × 88. E: Cross section of the distal region of a cellular graft, 12 weeks after grafting, stained with cholinesterase-silver. Many axons can be seen (arrows). The perineurium (P) is also visible. × 123. F: Cross section of the distal region of a cellular graft, 12 weeks after grafting, stained with laminin antibodies. The perineurium (P) and the basal lamina around the regenerated nerve fibers (arrow) are stained. × 228.
Fig. 3. Photomicrographs of an acellular basal lamina graft 12 weeks after grafting. PAS-hematoxylin. A: Cross section of the proximal region showing numerous regenerated and myelinated nerve fibers similar to those observed in cellular grafts (compare with Fig. 2B). Arrow points to the region of perineurium. × 84. B: Longitudinal section of the middle region of the graft showing many regenerated nerve fibers (arrow) surrounded by the perineurium (P) in this section also. × 218. C: Cross section of the distal region (over 2 cm from the regenerating nerve) of the graft showing a few regenerated and myelinated nerve fibers (long arrows) arranged in fascicles (compare with Fig. 2C and D). Short arrow points to the perineurium. × 84. D: Longitudinal section of the most distal region of the graft. No regenerated nerve fibers are visible, and the graft consists of unidentifiable cells dispersed in a connective tissue matrix. Arrows point to the region of perineurium. × 84.

The present results provide evidence that basal lamina grafts are not effective in supporting axonal regeneration over long distances. As in cellular grafts, viable Schwann cells seem to be necessary for regeneration to occur over longer distances. Fawcett and Keynes have recently reported successful regeneration through acellular grafts 4 cm long prepared from skeletal muscle after transplantation to repair nerve gaps in rabbits. Such a success over a 4-cm distance was not observed through the acellular grafts in the present study. This variance in regenerative success may be due to the species differences in the two studies, especially in consideration of the diameter of the nerve being repaired (that is, the larger nerve diameter in rabbits as compared to rats).

It can only be speculated why, after good initial regeneration into the proximal portion of the acellular graft, the axons failed to grow distally. It has been shown that, during axonal regeneration through hollow tubes and blood vessels, Schwann cells along with other cells migrate (after proliferation) into the hollow interior and somehow modify the environment to initiate and sustain axonal growth. If the interior chamber length is too great, the migrating Schwann cells cannot completely populate it, preventing axonal growth. A similar situation may exist during regeneration through acellular grafts. After grafting of frozen-thawed nerve segments, the dead cell debris is removed by host macrophages. Simultaneously, migration of Schwann cells and other cells into the grafts occurs from the proximal host nerve. In the case of long grafts, as used in the present study, the Schwann cells cannot extend and populate the entire length of the graft, thus affecting the success of axonal regeneration.

The fact that host axons did not traverse the entire length of the acellular grafts should not obscure the ability of such grafts to support axonal regeneration. It is important to note that good axonal regeneration was
observed up to a distance of 2 cm through basal lamina grafts. The extent of this regeneration is particularly good when compared to that observed through regeneration chambers (maximum distance of 1 cm) which originally lack both the Schwann cells and the basal lamina.\textsuperscript{13,16,23,27} Similarly, only a few axons grow into the proximal 1-cm region of immunologically rejected nerve allografts\textsuperscript{6,29,30} and frozen grafts of normal nerve.\textsuperscript{17,28} Schwab and Thoenen\textsuperscript{22} have recently reported differences in the extent of neurite growth into grafts of sciatic nerves with viable or dead Schwann cells. In addition, the rate of regeneration into cellular grafts is considerably faster than into acellular grafts.\textsuperscript{8} Taken together, these results imply that the growing axons do not display an absolute requirement for their growth but rather exhibit a graded preference.

It is notable that the Schwann cell basal lamina persists relatively unchanged, even after the disintegration and removal of Schwann cells killed by the freeze-thaw treatment. These observations indicate that the basal laminae are not degraded and remain in the form of circular conduits to allow subsequent axonal regeneration. Regenerating axons invariably grow along the inner surface of the basal lamina scaffold.\textsuperscript{8,9,11,12} Some form of mechanical obstruction external to the basal lamina scaffold has been proposed as the factor responsible for selective growth of axons inside the basal lamina.\textsuperscript{9} Another possibility may be that the inner surface of the basal lamina possesses a favorable molecular composition for sustaining axonal growth. In support of this, differences in the molecular nature of the inner and outer surfaces of the basal lamina have been shown to exist.\textsuperscript{25}

The Schwann cell basal lamina initially appeared similar in the cellular and acellular grafts, but its fate was very different at later time intervals. In cellular grafts, the original basal lamina disappeared with time and was replaced by a new basal lamina that formed around the regenerated nerve fibers. Similar turnover in the basal lamina was observed in the case of acellular grafts, but only in the proximal half of the graft. In the more distal region of the acellular grafts, no such turnover was apparent and the original basal lamina became occluded. The same observations have been reported earlier in the case of regeneration through various types of nerve grafts.\textsuperscript{6,18}

In conclusion, the present results show that the basal lamina grafts do possess a limited ability to support axonal regeneration; however, axons cannot extend for long distances along such grafts. The presence of viable Schwann cells appears to be essential for regeneration to occur through long grafts. We are currently exploring alternative methods of preparing basal lamina grafts in an attempt to further enhance the ability of such grafts to support axonal regeneration and to improve their usefulness in the surgical repair of nerve gaps.

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Address reprint requests to: Adarsh K. Gulati, Ph.D., Department of Anatomy, Medical College of Georgia, Augusta, Georgia 30912.