Contribution of the proximal and distal nerve stumps to peripheral nerve regeneration in silicone chambers


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Summary. The specific contribution of the proximal and distal nerve stumps across an 8 mm gap within silicone chamber regeneration models was studied. For this, proximal and distal (Group A), distal and distal (Group B) and proximal and proximal (Group C) nerve stumps were placed in opposite ends of silicone chambers. In all the groups, a tissue cable forms between the nerve stumps, demonstrating that, without distinction, proximal or distal stumps can stimulate the growth of other proximal or distal stumps. Furthermore, in Group B, the newly formed pseudo-nerve, in the absence of regenerating axons, contains a number of Schwann cells significantly similar to Group A, which confirms that proliferation and migration of Schwann cells do not require axonal presence or contact. Likewise, the findings demonstrate that, with the exception of the axons, the distal stump contributes to the peripheral nerve regeneration in the same way as the proximal stump. Finally, when proximal stumps are placed in both the opposite ends of the silicone chamber, Schwann cells and regenerating axons grow into the chamber gap from both inserts, and myelination also proceeds from both ends to the centre of the chambers.

Key words: Peripheral nerve regeneration, Chamber model, Schwann cells, Axons, Myelination

Introduction

Recently, numerous studies on peripheral nerve regeneration, using artificial chambers as nerve guidance tubes to bridge transected nerves, have been undertaken (Lundborg and Hansson, 1980; Lundborg et al., 1982a,b; Seckel et al., 1984; Da Silva et al., 1985; Henry et al., 1985; Jenq and Coggeshall, 1985; Madison et al., 1985; Hurtado et al., 1987; Fields et al., 1989; Aebsicher et al., 1989; Knoops et al., 1990). The technique consists of the exposure of a peripheral nerve, usually the sciatic nerve, resection of a 2 mm segment of the nerve, and insertion of the proximal and distal nerve stumps into the ends of a cylindrical synthetic (Aebsicher et al., 1989) or biological (Lundborg and Hansson, 1980; Smahel and Jenitsch, 1983) chamber. In these conditions, a neonerve, consisting of regenerated axons, Schwann cells and perineurium-like sheath can be created between nerve segments if the interstump gap length is less than 10 mm (Lundborg et al., 1982a,b). This length is enhanced to 15 mm when some substances, such as neurotrophic factor, are added to the chamber lumen (Derby et al., 1993).

All the authors agree that the nerve regeneration across the chamber gap requires humoral and/or cellular contributions available from the distal nerve stump. Likewise, it has been demonstrated that Schwann cells can grow into an entubulation repair site in the absence of regenerating axons (Zhao et al., 1992; Williams et al., 1993; Madison and Archibald, 1994). Given these findings, the present study was undertaken to assess the precise contribution of the proximal and distal nerve stumps during nerve regeneration using artificial chambers. For this, three «in vivo» experimental models were performed. In the first, both proximal and distal nerve stumps were introduced into the ends of a tunnelled silicone chamber (proximal-distal system). In the other two models, developed in our laboratory, two distal or two proximal nerve stumps, with preserved microcirculation, were inserted into the opposing ends of the chamber (distal-distal system and proximal-proximal system, respectively).

Materials and methods

Adult Sprague-Dawley rats (average weight 300 gr) were used in accordance with the guidelines of the Animal Care Advisory Committee of the University of La Laguna. The rats were fed standard rat chow and water ad libitum and were maintained under pathogen-free conditions.

To achieve the proximal - distal (Group A), distal-distal (Group B), and proximal-proximal (Group C) systems the following procedures were undertaken. The rats (n=84) were anesthetized with Ketamine (150 mgr/Kg i.p.). Using a surgical microscope, the right
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sciatic nerve and its peroneal and tibial branches were exposed. In Group A (n=28), both the tibial and peroneal branches of the sciatic nerve were transected 2 mm from their bifurcation, with a razor blade. In 14 cases, the proximal stump of the tibial nerve and the distal stump of the peroneal nerve were respectively inserted into the opposite ends of a sterilized 10 mm length tunnelled silicone chamber (SILASTIC - Dow Corning 602-235, inner diameter 1.47 mm) with a single 10-0 suture to yield an 8 mm nerve gap distance. In the other 14 cases, a similar procedure was undertaken, but the proximal stump of the peroneal nerve and the distal stump of the tibial nerve were used. In Group B (n=28), both tibial and peroneal branches of the sciatic nerve were transected close to their bifurcation. Without damaging their microcirculation, the distal nerve branches were dissected from their neighbouring tissues and their stumps were respectively introduced into the opposite ends of a silicone chamber by a similar procedure to that in group A. In Group C (n=28), the tibial and peroneal branches of the sciatic nerve were transected 12 mm from their bifurcation, without damaging the proximal microcirculation. The proximal nerve stumps of both branches were respectively introduced into the opposite ends of a silicone chamber. In all the groups, the remaining nerve stumps were reflected and distanced from the silicone chamber. Furthermore, at each time point of the experiment, the number of cases in which the peroneal nerves were inserted, or considered, as proximal or distal stumps, was the same as for the tibial nerves. With this procedure, the possible variations of the results in the different areas of the regenerate depending on the nerve calibre were avoided.

After surgical procedure, the animals were sacrificed, under general anesthesia, at days 2, 4, 7, 10, 14, 21 and 30: 4 rats per time point for each group. Specimens were fixed in a glutaraldehyde solution, diluted to 2% with sodium cacodylate buffer, pH 7.4, for 6 h at 4 °C, washed in the same buffer, postfixed for 2 h in 1% osmium tetroxide, dehydrated through a graded acetone series, and embedded in epoxy resin. For light microscopic histology, 1.5 µm-thick sections were cut, mounted on acid cleaned slides, and stained with 1% toluidine blue. Thin sections were obtained from selected areas, double stained with uranyl acetate and lead citrate, and examined under an electron microscope.

To calculate the degree of nerve regeneration across chamber gaps, we have chosen a simple and reproducible procedure, similar to that used previously in other conditions (Radek et al., 1986). After obtaining selected areas by transversal thick sections (Fig. 1), at an approximate distance of 2.5 mm from both stumps, ultrathin sections were performed in the regenerated tissue for quantitative studies. A series of photographs at a magnification of x2,400 was obtained along 0.3 mm of a diameter of the regenerated tissue, and a montage was assembled. The number of Schwann cell nuclei and of myelinated and unmyelinated axons was counted. Following that, the mean value and standard deviation of the mean were calculated for day 30 of each group. Statistical analysis was made with analysis of variance (ANOVA), followed by t-test comparisons. Analysis was carried out using Statistic Software programme (NH Analytical Software). Statistical significance was defined as a p<0.05.

Results

Group A (proximal-distal system)

Between 2 and 7 days after chamber implantation, a newly-formed matrix linked the transected nerve stumps, along the central axis of the chamber, appearing surrounded by a pinkish fluid. The matrix consisted of fibrin strands arranged in a predominantly longitudinal orientation, erythrocytes at different stages of hemolysis, cellular debris and extracellular fluids. The fibrin matrix was progressively infiltrated by cells emigrating from both ends of the transected nerve stumps towards the centre of the chamber. Macrophages, with intracytoplasmic vacuolization and phagocytosis, and fibroblasts, whose orientation was parallel to the long axis of the chamber, were the first to migrate. Budding vascular sprouts were also observed aligned along the axis of the

Fig. 1. Group A (proximal-distal system). Transverse section of the neonerve regenerated across the chamber gap (2.5 mm from the proximal stump) 30 days postsurgery. The section corresponds to a selected area for ultrathin sections. A minifascicular arrangement is observed. Semithin section; toluidine blue. x 125
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Between 7 and 21 days, the matrix appeared invaded from both stumps by Schwann cells (Fig. 3), which were identified by the presence of continuous basal laminae and, in those originated from the proximal stump, by their association with unmyelinated (Fig. 4) and myelinated (Fig. 3) axons. Therefore, myelination seemed to proceed from the proximal towards the distal stump.

Between 21 and 30 days, a nerve structure was partially reformed, with the presence of numerous microfascicles which contained Schwann cells and myelinated and unmyelinated axons (Fig. 5). The neovascularization was made up of longitudinal and radial microvessels. Next to the latter, macrophages filled with phagocytosed particles were present. The entire regenerated structure was delineated by several layers of interlaced fibroblasts and collagen fibres, acquiring epineurial characteristics.

The quantitative studies of the Schwann cell nuclei, as well as of myelinated and unmyelinated axons in the reformed nerve structure, at a distance of 2.5 mm from both stumps, are shown in Table 1. All the cases of this group significantly showed the capacity of inducing the growth of Schwann cells, with the presence of regenerated axons. The numbers of myelinated axons were significantly greater in the sections closer to the proximal than to the distal nerve inserts, the opposite occurring with the unmyelinated axons.

Group B (distal-distal system)

Similar results to those of group A were observed

Fig. 2. Group A (proximal-distal inserts). Vascular sprouts (V), macrophages and fibroblast-like cells (F) are seen migrating into the fibrin matrix (FM) that links the transected nerve stumps. Semithin sections; toluidine blue. x 900
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during the first days of the experiment, with the presence of a fibrin matrix between the nerve stumps, infiltrated by macrophages, fibroblasts and vascular sprouts. From day 7 onwards, Schwann cells were present within the newly-formed tissue connecting the opposing stumps. The migrating Schwann cells were grouped in columns orientated parallel to the long axis of the chamber (Fig. 6), and they showed numerous processes, which appeared to be confined within a continuous basal lamina (Fig. 7). Axons were not present in the Schwann cell columns. At day 30, some of the Schwann cells in the tissue cable showed degeneration, with intracytoplasmic membrane-limited vacuoles containing densely packed vesicles, disintegrating cellular organelles and myelin figures (Fig. 8), resembling the cells of the granular cell tumor.

Fig. 3. Group A (proximal-distal system). Sections at 2.5 mm of the proximal (A) and distal (B) inserts. Migrating Schwann cells are present in the tissue regenerated between both inserts. The Schwann cells are orientated parallel to the long axis of the chamber and some of them are in mitosis (arrowheads). Some myelinated fibres (arrows) are observed in the section next to the proximal insert. Semithin sections; toluidine blue. x 900

Fig. 4. Group A (proximal-distal system). Schwann cells (SC), some of them in the process of mitosis (SCM), associated with an unmyelinated axon (A), are observed. Ultrathin section; uranyl acetate and lead citrate. x 12,500

Fig. 5. Group A (proximal-distal system). A regenerated nerve, with myelinated and unmyelinated axons, is present between both inserts. Semithin section; toluidine blue. x 350
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The quantitation of Schwann cell nuclei showed similar results to Group A, while the number of myelinated and unmyelinated axons was always zero.

Group C (proximal-proximal System)

The tissue regenerated between both nerve inserts showed similar characteristics and sequence to those in group A (Fig. 9), the only difference being that regenerating axons and myelination were observed from both nerve stumps in the present Group. These findings were confirmed in the quantitative studies (Table 1), including the demonstration of similar numbers of myelinated and unmyelinated axons in the sections next.

Table 1. Number of Schwann cell nuclei, and myelinated and unmyelinated axons in the regenerate across the chamber gaps, at 2.5 mm from the stumps 30 days postsurgery. Schwann cell nuclei in group A vs group B and vs group C: not significant. Number of myelinated and unmyelinated axons in groups A and C versus group B: significant (myelinated and unmyelinated axons in group B: 0). Myelinated axons in proximal group A vs distal group A: significant (p< 0.05).

<table>
<thead>
<tr>
<th>STUMPS FROM 2.5 mm OF THE CONSIDERED AREA</th>
<th>SCHWANN CELL NUCLEI</th>
<th>MYELINATED AXONS</th>
<th>UNMYELINATED AXONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>402.00±129.78</td>
<td>770.75±162.63</td>
<td>1078.50±174.91</td>
</tr>
<tr>
<td>Distal</td>
<td>401.00±141.90</td>
<td>415.25±150.26</td>
<td>1475.00±217.61</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>422.75±162.21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Distal</td>
<td>391.50±195.88</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group C</td>
<td></td>
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<tr>
<td>Proximal</td>
<td>443.25±98.61</td>
<td>680.75±101.39</td>
<td>1133.75±144.08</td>
</tr>
<tr>
<td>Proximal</td>
<td>336.25±163.24</td>
<td>704.25±80.03</td>
<td>1143.00±227.09</td>
</tr>
</tbody>
</table>

Fig. 6. Group B (distal-distal system). Columns of migrating Schwann cells (SC), fibroblasts and newly-formed vessels, orientated parallel to the long axis of the chamber, are observed between both inserts. Semithin sections; toluidine blue. x 900
to the proximal and distal nerve inserts.

Discussion

The results in Group A, with proximal and distal nerve stumps inside opposite ends of a silicone chamber, confirm previous studies of other authors on the nerve regeneration across chamber gaps (entubulation repair), (Lundborg and Hansson, 1980; Williams et al., 1983, 1984, 1988; Jenq and Coggeshall, 1985; Henry et al., 1985; Hurtado et al., 1987; Fields et al., 1989; Knoops et al., 1990; Derby et al., 1993), the salient events being: a) accumulation of fluid, which probably contains neurotrophic factors; b) presence of a co-axial fibrin matrix, formed from plasma precursors in the nerve stump exudate, connecting the opposing stumps; c) immigration of Schwann cells, fibroblasts and endothelial cells into the coaxial matrix from cell precursors in the proximal and distal nerve stumps, originating a continuous tissue cable across the gap (Williams et al., 1983, 1984); d) axonal elongation (Williams et al., 1983, 1984; Zalewski et al., 1990); and e) reformation of a neonerve, including myelinated and unmyelinated axons. Schwann cells, blood vessels, endoneurial components, and a perineurial-like outer cell layer. With the exception of the axons, which only elongate from the proximal stump, the new tissue regenerated from the distal insert contained similar components to those originated from the proximal insert. In other words, Schwann cells, fibroblasts, vascular sprouts and macrophages enter the chamber from both the proximal and distal stumps. All the events involved in capillary growth in vivo were also observed (Díaz-Flores et al., 1994). Likewise, our quantitative studies, demonstrating that myelinated axons are more numerous next to the proximal nerve insert than in the distal regions of the reformed nerve structure, support the fact that myelination proceeds from the proximal towards the distal nerve stumps.

In our experimental model of Group B, a regenerated pseudo-nerve between two peripheral nerve stumps connected to their target (distal-distal system) is demonstrated, indicating that a nerve distal insert has the ability of stimulating the growth of another nerve-distal insert. In our model, the microvascularization of the stumps was preserved, since the regeneration and final size of the neonerve seem to be dependent on the well preserved circulation (Zhao et al., 1992). This probably

Fig. 7. Group B (distal-distal system). Ultrastructural characteristics of the Schwann cells (SC) between both inserts. These cells are grouped in columns and confined within a continuous basai lamina (arrowheads). N: Nucleus. Ultrathin section; uranyl acetate and lead citrate. x 12,500
made a prefilled of the chambers with dialyzed plasma unnecessary, unlike that required in experimental models of other authors (Williams et al., 1993). Although the capacity of the axons to induce Schwann cells to proliferate has been pointed out (Pellegrino and Spencer, 1985; Ratner et al., 1988; Decoster and DeVries, 1989), in this group the pseudo-nerve contains Schwann cells, which are capable of bridging the chamber gap in the absence of regenerating axons. Indeed, the numbers of Schwann cells in Group B did not show significant differences to those of Group A, while the number of axons was always zero in all the cases of Group B. Therefore, the findings of the current experiments confirm that proliferation and migration of the Schwann cells into fibrin matrix do not require axonal presence or contact (Zhao et al., 1992; Williams et al., 1993; Madison and Archibald, 1994) and that Schwann cells may grow into a wound site in front of regenerating axons (Williams et al., 1993; Hall, 1986a,b; Kljavin and Madison, 1991). On the contrary, the reverse seems not to be true (Williams et al., 1983; Zhao et al., 1992), since activated Schwann cells are critical for axonal regeneration (Terenghi, 1995), which fails when Schwann cells are prevented from entering a nerve repair site (Williams et al., 1983, 1984; Hall, 1986a; Fields et al., 1989; Feneley et al., 1991). However, some authors have pointed out that regenerating axons can grow a limited distance without Schwann cells (Ide et al., 1983).

The presence in our observations of Schwann cell columns, resembling Bungner bands, surrounded by a continuous basal lamina agrees with previous studies of other authors indicating the possibility of basal lamina formation in the absence of axons (Bunge et al., 1980; Bunge and Wood, 1989; Obremski and Bunge, 1989). However, this fact does not exclude the participation of the axons in other conditions, since they induce...
Schwann cells to express specific proteins and basal lamina (Bunge et al., 1980, 1982; Cornbrooks et al., 1983; Bosch et al., 1989). Likewise, there are «in vitro» experiments demonstrating that the appearance of basal lamina could only be undertaken when fibroblasts were included in the culture environment (Bunge and Wood, 1989; Bunge et al., 1989; Obremski and Bunge, 1989).

Furthermore, the formation of a sheet of perierium-like cells surrounding the pseudonerve in group B also agrees with the fact that several components of a peripheral nerve can be expressed in the absence of axons (Zhao et al., 1992).

Taking the above mentioned facts into consideration, both experimental models, proximal-distal and distal-distal systems, demonstrate an important contribution of the distal nerve stump to the formation of a regenerated tissue bridging a transected nerve across a chamber gap. The only difference in both groups of experiments is that the tissue regenerated between the proximal and distal nerve stumps contains elongated axons, while it is non axonal in the distal-distal systems.

The purpose of group C was to assess whether a neonerve could form across chamber gaps between two nerve proximal inserts. The results indicate that a nerve proximal stump can also stimulate the growth of another nerve proximal stump, which leads to all the events of entubulation repair. Likewise, in this experimental model, Schwann cells and regenerating axons grow into the chamber gap from both inserts. Moreover, the morphological findings and the quantitative studies support the fact that myelination also proceeds from both ends to the centre of the chamber.

The ends of the axons, originating from both inserts, are probably found halfway along the chamber gap, although this fact has not been clearly demonstrated by the techniques used here. The exact behaviour of the regenerating axons when the growth fronts meet is a topic worthy of future exploration.

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