

The Role of the Basal Lamina in Nerve Regeneration

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Abstract

Introduction: In the context of axon regeneration, certain molecules containing the basal lamina, particularly fibronectin, laminin and heparin sulfate promote axonal elongation *in vitro* and *in vivo*. This structure could lead the axons to distal stump in nerve defect repair.

Material and Methods: In twenty male Wistar rats, a critical defect of 15 mm was created in the sciatic nerve, which was bridged by an acellular muscle graft, which were obtained from the gluteus medius muscle of two donor rats, and chemically treated to cellular extraction by protocol consisted of 7 h in distilled water changed three times every 2-3 h, one night in Triton detergent, and 24 h in deoxycholate. After 90 days, the explanted pieces of sciatic nerve with grafts were carved and cut into seven pieces equally in all animals; pieces were processed in resin (toluidine blue staining) and also for paraffin embedding; hematoxylin-eosin and Masson trichrome.

Results: Microscopically, proximal ends appeared as a normal nerve, with proper, regular and orderly fascicular distribution, perfectly defined by its three wraps, with little connective tissue. At the graft, regenerated axons used the muscle as a bridge, with many small nerve fascicles separated by host tissue and plenty of blood vessels. Axons followed their basal lamina, defining small fascicles with large vessels and abundant connective tissue. At the distal end, there was no a clear division in fascicles in some animals. There was little connective tissue between the fibers and the epineurium was quite developed.

Conclusions: The selected biological method for chemical treatment of skeletal muscle achieves acellular grafts. We successfully could repair a critical lesion of the sciatic nerve in rats using this acellular muscle graft. Acellular muscle that we employed as a graft provides a bridge tool for the advancement of the regenerating axons, perhaps due to the structural proximity of laminin to nerve sheaths.

Keywords: Nerve regeneration; Basal lamina; Acellular muscle graft

Introduction

The purpose of a nerve graft is to replace a defect so it can lead the maximum number of regenerated nerve fibers to their target organs. But the limited availability of nerve tissue that serves as autograft and the incomplete functional recovery obtained in most cases and problems from the donor area, have other alternatives. Striated muscle contains basal lamina tubes from sarcolemmal, which persist even when myocytes found within them, have been destroyed chemically or thermally.

In the context of axon regeneration, certain molecules containing the basal lamina, particularly fibronectin, laminin and heparan sulfate promote axonal elongation *in vitro* and *in vivo* [1-8]

In a broader biological context it has been shown that cell-laminin binding *in vitro* affects phenomena as diverse as cell migration, cell division, and maintenance of the differentiated phenotype [9,10].

The basal lamina Schwann cell-derived seems to be a very durable structure. It survives the penetration by the invasion of macrophages during acute phases of Wallerian degeneration or to primary demyelination, and persists in the distal stumps chronically denervated, where it surrounds the cytoplasm of Schwann cells which constitute the survivors Büngner bands.

Presumably the basal lamina can undergo repair time, because their redundant loops associated with Büngner bands newly denervated are absent in the endoneurium of chronically denervated nerves [10,11].

Axons and Schwann cells grow preferentially along the inner side of the basal lamina rather than in association with its exterior, the extracellular face.

Objective

Study the ability of peripheral nerve regeneration in non-repairable injuries, by using allogeneic acellular muscle grafts, to study the role of the basal lamina.

Material and Methods

From two donor rats, muscle grafts for all animals to study subsequently obtained from the gluteus medius. Special detail was given to the section of the grafts along the longitudinal axis of the muscle, not to disrupt the histological structure of muscle fibers. Obtaining acellular grafts was completed following the decellularization protocol published by Baptista in 2009, the British Society of Medicine and Biology [12]. It consists of 7 h in distilled water needs to be changed three times every 2-3 h; one night in a detergent Triton® X-100 (# BDH-Prolabo, Briare, France) and 24 h in Sodium deoxycholate (5 g/l, Merck, Darmstadt, Germany); washed in 0.4 M phosphate buffer,

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Received September 28, 2016; **Accepted** November 04, 2016; **Published** November 14, 2016

Citation: García-Medrano B, Pérez CS, Sanz PB, García MG, Martín-Ferrero M, et al. (2016) The Role of the Basal Lamina in Nerve Regeneration. J Cytol Histol 7: 438. doi: [10.4172/2157-7099.1000438](https://doi.org/10.4172/2157-7099.1000438)

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pH 7.4 at 25°C; the whole process is repeated; finally passed to another buffer and stored at 4°C.

After intraperitoneal preoperative anesthesia, and by posterolateral approach, the sciatic nerve of twenty Wistar male rats was exposed and transected generating a 15 mm defect. An acellular muscle block was added, where the epimysium-epineurium were sutured with polypropylene 7/0 at each end (Figure 1).

Rats were sacrificed at 90-100 days post-surgery (body-weight of 450-500 g). Same surgical approach was repeated dissecting segment sciatic nerve including muscle graft, performing in a second time, a post-fixation of samples for 4 h at 40°C, to prevent cell shrinkage in paraformaldehyde solution (4 % w/v, 4 g of solute are dissolved in 100 ml of solution) in 0,1 Molarity phosphate buffer (pH=7,4). Samples were obtained for macro and microscopic preparations. They were cut into seven fragments, all different from each other in cross section and size, but equal in all parts, dedicating pairs to inclusion in resin (blue toluidine staining) and impairs for paraffin embedding and hematoxylin-eosine and Masson trichrome (Figures 2 and 3).

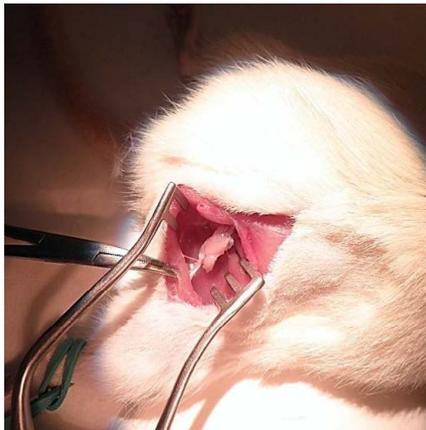


Figure 1: Suture of the graft. Detail of suture muscle graft acellular sciatic nerve defect, with Prolene sutures 7/0.

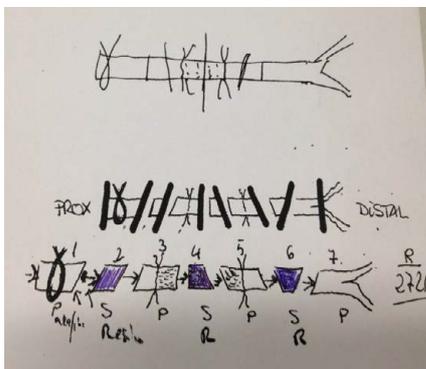


Figure 2: Diagram of the sections of each piece. Scheme protocol sizing of each histological piece. The tie on left side means the Vicryl suture to mark the proximal end after dissecting the piece. The wires in the middle of the piece are the proximal and distal sutures of the graft, leaving the sides of the ends of the sciatic nerve. Division into 7 segments, all different from each other in order to recognize them. Impairs slices are intended for inclusion in paraffin and smaller, for thin slices and inclusion in resin.

Results

Microscopical findings

Section 1 (proximal end): At the proximal end, with usual techniques of hematoxylin-eosin and Masson trichrome staining, a characteristic structure of healthy peripheral nerve, rounded, organized in homogeneous bundles, separated from each other, with symmetry in the density of axons with myelin coverage regularly seen depending on Schwann cells. With Masson trichrome method, is clearly distinguished an abundant epineurium and relatively low perineurium and endoneurium. The space between the fascicles is relatively broad and nerve fibers are clearly differentiated from connective tissue by Masson method. Blood vessels are in normal proportions, one for every four fields 100x on average (Figure 3).

Section 2 (just proximal to the graft): Section 2, which also belongs to the proximal end, discovers, with toluidine blue staining, relatively normal nerve fibers although it shows some irregular thickened, pleated shapes and alterations of the myelin sheath, evidence of degeneration and degradation mechanisms suffered after neurotmesis, which also move retrograde. It is occupied by cords of regenerated fibers, which seem to want to organize themselves to form mature structures. Typically, axons remain parallel to the major axis of the graft paths, although sets of fibers are seen in other orientations. In longitudinal sections, the proximal end of the nerves of both groups of animals, show a normal arrangement of epineurium as well as both fibrillar and cellular elements that constitute them. Likewise, it differs myelin sheath of nerve fibers and connective tissue of endoneurium and perineurium.

Section 3 (proximal graft suture): An abundant inflammatory infiltrate around the muscle fascicles decellularized is observed. We observe a regenerative stroma colonized by cords of axons, loosely packed and quite disorganized, but with some direction in parallel (in the longitudinal sections). In some animals, we have found some irregular nerve fascicles which could be considered small neuromas, because of their morphology and cellular organization. In some areas of the implant, bundles of nerve fibers that penetrate, not very deep, are observed between acellular muscle fibers.

Section 4 (into the graft): At this level is often observed small nerve fascicles closed to acellular muscle fibers, although with an abundance connective tissue separating nerve fibers from muscle. Nerve fibers are of varied size, coexisting very thick myelinated fibers with other thin and non-myelinated, hardly identifiable in this size of sections (Figure 4). In the perineurium, abundant blood vessels are distinguished,

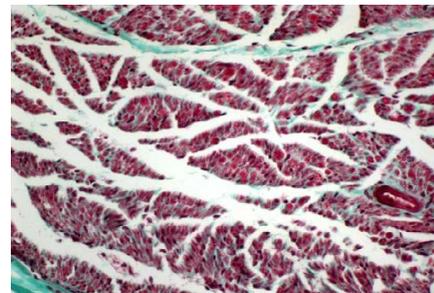


Figure 3: Section 1 (proximal end). With Masson trichrome staining, the collagen fibers can be seen dyed green in the epineurium and perineurium. 20x detail to observe the organized structure of a nerve with signs of regeneration, which maintains homogeneity in their fascicles (Masson trichrome staining; 20x magnification).

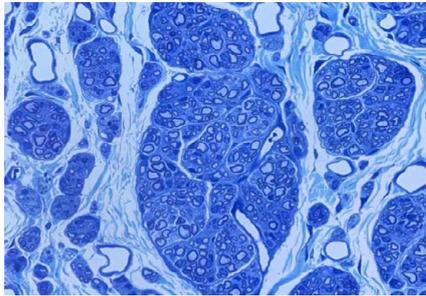


Figure 4: Section 4 (thin slices of the graft). Separated, irregular in size fascicles. Abundant connective tissue between the fascicles and lax, many glasses (Blue toluidine 40x).

ensuring good irrigation regenerative tissue. In addition to the nerve bundles near the muscle fibers, they have inflammatory cells, especially in relation to acellular muscle fibers.

Section 5 (distal graft suture): In this region, it shows nerve fascicles that run on the surface of acellular muscle, although both are always separated by abundant connective tissue structures, along a random path. Inside the nerve trunks, connective tissue is more lax and less abundant. Some slices show nerve fascicles regenerated into the graft, with the arrangement of a nerve completely regenerated, eg, forming fascicles, surrounded by well-organized epineurium and relatively small amount of perineurium and endoneurium.

Section 6 (distal to the graft): It shows the presence of a number of minifascicles with myelinated axons, which colonize the distal stump. These groups are usually distributed over the periphery of the cut, leaving a central more unpopulated area. Myelin thickness at this level is less than the above, and the diameter of the nerve fibers themselves. Epineurium is well developed and it has moderate amount of perineurium and endoneuro. In some sections, the grouping of several axons associated with the process of a Schwann cell is observed. No inflammatory cells were observed.

Section 7 (distal end): In the distal stump, it was observed from 1 to 4 nerve fascicles relatively normal. Axons form cords of various elements, which turn out to be continuity of those small bundles of the central segments (in the longitudinal sections) (Figure 5).

Axon density: It is kept in the center slices at the graft level and distal end, an average of 63 axons per field (36-89), which get through the graft and reaching the distal stump of the nerve.

Axon diameter: The average of the diameters is not a significant data, the variety between fibers within the same section (0.071 microns average). is achieved not decipher any distribution thereof; we have found the number of nerve fibers is lower in the more peripheral areas, but it has not yet seen that at this level the difference in the average diameter of axons is significant, compared to the average diameter central fields.

Myelin thickness: Prior to grafting, the average value of 0.00062 is (0.00061 to 0.000625). At graft level, the average myelin is 0.00039 mm (from 0.000375 to 0.000405). Distal to the graft, the average myelin is 0.0005389 (0.00053 to 0.000545).

Electron microscopical results in distal stump: The regenerated nerve graft shows the typical characteristics of myelinated and unmyelinated fibers. Myelinated fibers have a varying size (Figure 6), all within the same nervous fasciculus. They are distinguished either

by the myelin sheath that surrounds them, although the core of the Schwann cell is found only in some sections, in others only see a section of the membrane. Schwann cells show typical characteristics: abundant rough endoplasmic reticulum, free polyribosomes, mitochondria, nucleus and chromatin condensation some near the cell membrane. Myelin presents, depending on the section plane of the myelin sheath, a clear organization in concentric layers more particularly in sections perpendicular to the major axis. In some areas, we can see dilations of the myelin sheath containing cytoplasm of Lantermann schmidt sulci (Figure 7).

The unmyelinated fibers, which are not seen with light microscopy, are observed in the electron microscope, with his usual characteristic: wraps cytoplasm of Schwann cells surrounding the bundles of nerve fibers, but without forming myelin (Figure 6). They measure a tenth of myelin fibers. Although smaller, unmyelinated fibers have the same microstructure as myelinated: neurotubules, and some mitochondria neurofilament.

Both types of fibers are separated from connective tissue by a basal lamina membrane surrounding the Schwann cell (Figure 7). Outside the basal lamina, connective space in which they can be distinguished collagen fiber bundles, endoneurial fibroblasts (Figure 7) and blood vessels is. The endoneurial fibroblasts are characterized by a core of chromatin, grouped into submembranal clusters, and not very abundant cytoplasm with numerous free polyribosomes and cisterns of rough endoplasmic reticulum. The extensions of these cells are very

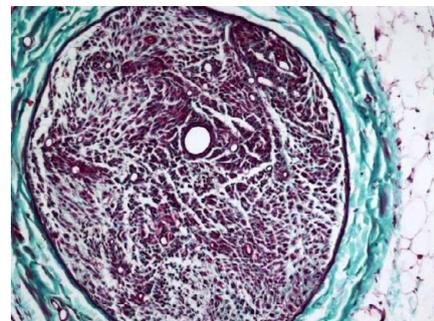


Figure 5: Section 7 (distal stump). After distal bundle of nerve. Among the abundant vascularization, it highlights the great central blood vessel (Masson trichrome 10x).



Figure 6: Electron microscopical image. Ultrastructural image of a regenerating nerve fascicle. The variety of sizes characteristic of myelinated fibers is distinguished. Myelinated nerve fibers show a normal relationship with Schwann cells, some of whose nucleus (CS) in this section. Unmyelinated nerve fiber bundles, surrounded by cytoplasm of Schwann cells (arrows) are distinguished. 10000x increases.

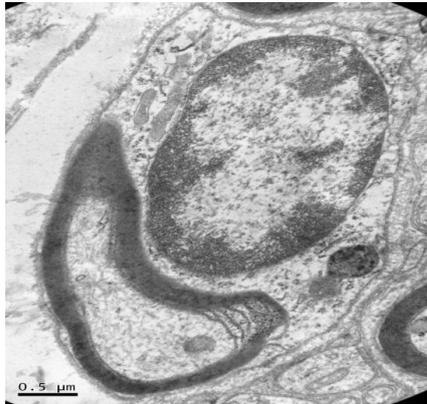


Figure 7: Myelinated nerve fiber surrounded by its corresponding Schwann cell. Outside the cell membrane, this is a basal lamina that separates the assembly cell-fiber and the endoneurial tissue. In an area of the myelin sheath, cytoplasm dilations are detected which are called Schmith Lantermann sulci. In the cytoplasm of the nerve fiber, plus some mitochondria, neurofilaments and neurotubules are distinguished. 40000x increases.

thin and sometimes surround multilayer fascicles of myelinated and unmyelinated nerve fibers.

Discussion

From the analysis of the results obtained, we can say that the acellular muscle we use as a graft is a useful tool for the advancement of regenerated axons bridge. The muscle has been described as the most effective histological structure for the local release of the factors in the injected [13,14].

This successful conclusion may be associated with structural proximity of the basal lamina of acellular muscle nerve sheaths. Endomysium acts as anatomical and biomechanical support to the growth of sprouting out axons towards the distal stump, motivated by the presence of Schwann cells in both ends initially. In addition, the cytoskeleton of degenerated Schwann cells, laminin +, persists to process cellular deterioration, acting as signal for regeneration [15].

Differences in percentage of success of the various decellularization techniques lie in the residual amount of antigenic cellular constituents and quality of the extracellular matrix preserved. Other chemical protocols processing of longer times, adversely affect the integrity of the microstructure of the grafts [16,17].

The most repeated histological findings show a growth of distal to proximal end, leaning on the support of muscle acellular graft, using it as a crossover vehicle; although we have detected two cases in which it is corroborated the passage of axons between muscle fibers of the graft. These findings are consistent with previously published by other authors, as Meek et al., who claimed that regenerating axons and Schwann cells of the injured sciatic nerve does not require the presence of the basal lamina of Schwann cells, but only the existence of any cellular basal lamina, including that coming from the skeletal muscle fibers [18].

Laminin, a major component of the basal lamina, promotes neurite outgrowth, induce mitosis of the Schwann cells, and plays a key role in peripheral nerve regeneration. The guiding role exerted the presence of extracellular matrix and the basal lamina in the acellular grafts is a subject of ongoing debate, with numerous support our results [19-21].

Acellular muscle graft coaxially aligned regenerate the nerve we

want behaves like a block of empty channels that appear to offer little or no resistance to cell growth. More importantly, the walls of the tubes containing molecules such as laminin and fibronectin, which possess domains known to support neurite outgrowth and cells of the neural crest *in vitro* [22-26].

Conclusions

The biological method for chemically treating selected skeletal muscle graft decellularization achieved adequate thereof by chemical detergents protocol selected. This method achieves a mioplasma Almost complete cleavage and other cellular components while preserving the basement membrane tubes (not affect the laminin or type IV collagen), and the original contour of the muscle fibers.

Shown criteria for this type of graft has to achieve optimal regeneration. First, the sample block acellular muscle similarly to the mechanical rigidity of a healthy nerve with great biocompatibility and minimal induction of an autoimmune response against foreign material, since it is considered allogeneic graft. Brings the extracellular matrix components of natural origin, such as collagen I/IV and laminin, which has a profound impact on the fate of nerve axons bridge and stimulate growth neuronal cells, in terms of migration and cellular repair.

Acellular muscle fibers basal lamina define tubes that could support axonal regeneration, acting as matrices for the growth of axons, when grafted into a nerve defect. The basal lamina of the muscle is likely to be able to “guide” the growing axons. It succeeds in restocking regenerating axons in distal stump, reaching a macroscopic and microscopic appearance of nerve after 90 days postoperative follow.

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Citation: García-Medrano B, Pérez CS, Sanz PB, García MG, Martín-Ferrero M, et al. (2016) The Role of the Basal Lamina in Nerve Regeneration. *J Cytol Histol* 7: 438. doi: [10.4172/2157-7099.1000438](https://doi.org/10.4172/2157-7099.1000438)

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