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NATURALLY SUPRAORGANIZED COLLAGEN INCREASES AXONAL REGENERATION AFTER TUBULIZATION REPAIR

Alexandre L.R. Oliveira[1], Benedicto de Campos Vidal[2] and Francesco Langone[3]

[1]Department of Anatomy, [2]Department of Cell Biology and [3]Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP) Campinas, SP, Brazil.

ABSTRACT

After axotomy, regeneration can be enhanced by bridging the transected nerve with a biocompatible tube, and the effect of trophic substances or molecules from the extracellular matrix can be investigated by filling the prosthesis. In this study, we assessed the importance of the molecular organization and aggregational state of collagen type I in axonal regeneration and guidance. Two types of collagen were used, namely, a collagen gel derived from bovine tendon that displays supraorganization after extrusion, and collagen from rat tail which does not self-organize under such conditions. Adult male Wistar rats were divided into four groups. In the first group (n=3), the polyethylene tube was filled with bovine collagen, while in the second (n=3), the prosthesis was filled with rat-derived collagen. In the third group (n=3), the tube was left empty, and the fourth group (n=3), consisted of unoperated rats. Six weeks after tubulization, the number of axons was significantly higher with bovine collagen than with rat collagen (7,661 ± 1,018 versus 4,110 ± 1,027, $p<0.05$), as was the degree of implant absorption. These results support the hypothesis that the use of extracellular matrix substances that self-assembly in an organized pattern can enhance nerve regeneration.

Key words: Collagen type I, nerve tubulization, sciatic nerve, self-assembly, supramolecular organization

INTRODUCTION

Nerve repair by the tubulization technique is an interesting paradigm in which the gap between the nerve stumps can be filled with different types of substances that may enhance the regenerative process. Over the years, several extracellular matrix (ECM) compounds have been used to increase the number of regenerated axons after peripheral nerve lesion and tubulization repair. The most studied ECM molecules include collagen, laminin, hyaluronic acid, fibronectin and more recently, glycosaminoglycans alone or associated with collagen [3,4,6,13,14,18]. The use of these substances has made it possible to increase the number of regenerating fibers and to fill gap between nerve stumps [9].

The molecular organization and aggregational state of the implant are important factors in providing a supportive environment for axonal regeneration and guidance [1,2,7,8,10]. Indeed, the artificial alignment of the ECM molecules by magnetic forces can be used to optimize nerve regeneration [1,2]. However, there has been no ultrastructural or polarized light microscopy analysis of the extent of implant integration and degradation, or of the maintenance of the supraorganization for longer periods of time. In addition, the use of ECM molecules that show autoassembly and macromolecular aggregational properties has not been fully investigated. Such naturally oriented proteins may facilitate the axonal growth and may be degraded more efficiently. Collagen from bovine tendon can self-assemble after dialysis and, when extruded through a needle, shows a patterned structure consisting of oriented fibrils [17]. In contrast, collagen from other sources or submitted to different treatments does not self-organize into helically oriented fibers and chiral objects after extrusion. A supraorganized implant may benefit cellular recognition and the interaction of neuronal and non-neuronal cells, enhancing axonal guidance. In addition, the rate of degradation of the implant may be accelerated.

In this study, we investigated the influence of collagen type I from two distinct sources, namely bovine calcanear tendon and rat tail tendon, on axonal regeneration six weeks after sciatic nerve transection and tubulization. The integration and degradation of the implant were assessed using polarized light and transmission electron microscopy, and the number of myelinated axons at the tube midpoint was obtained.

Correspondence to: Dr. Alexandre L.R. Oliveira
Departmento de Anatomia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, SP, Brazil. Tel: (55) (19) 3788-6295, Fax: (55) (19) 3289-3124. E-mail: alroliv@unicamp.br.

MATERIAL AND METHODS

Animals

Adult male Wistar rats, eight weeks old, were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) and were housed on a 12 h light/dark cycle with free access to food and water. This study was done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). The rats were allocated to one of four groups. In the first group (n=3), the tubes (Clay Adams, id=1.2 mm; od=1.8 mm) were filled with collagen gel extracted from rat tail, while in the second group (n=3), the tubes were filled with collagen gel from bovine tendon. In the third group, the tubes were left empty and in the fourth, the rats were not operated.

Collagen extraction

Rat collagen was extracted according to Vidal [17]. Briefly, rat tail tendons were immersed in 2% acetic acid to extract the acid soluble collagen. After filtration, the acid soluble collagen was centrifuged (2,000 rpm) and 10% NaCl was added to the supernatant up to a final concentration of 5%, in order to reconstitute the collagen fibers. The reconstituted fibers were then submitted to differential dialysis to achieve the necessary degree of hydration and aggregation.

Bovine calcanear tendon collagen was extracted and separated using a patented technique (#P.I. 97015709, B.C. Vidal). Briefly, small fragments of defatted and cleaned bovine tendons were immersed in an aqueous solution of 5% acetic acid containing 0.01% HCl and 1 mg of pepsin/g tendon at 7–10ºC for 24 h. The solubilized collagen was filtered and the fibers were reconstituted by adding NaCl solution to a final concentration of 5%. The fibers obtained were then dialyzed against distilled water in 6 mm diameter tubes at 5ºC, with the water being changed every 24 h. A total of 1.5 liters of water was used for every 200 g of collagen gel.

Surgical procedures

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the left sciatic nerve was exposed and transected at the mid-thigh level. The proximal stump was introduced into the polyethylene tube and sutured to the tube end with an epineural stitch (9-0 nylon suture). In groups 1 and 2, the tube was filled with 10 µl of rat tail collagen and bovine tendon collagen, respectively. The distal stump was then sutured to the distal end of the tube, leaving a 6 mm gap. In group 3 (control), the tube was left empty. Three unoperated rats were used as controls. The musculature and the skin were closed with 6-0 silk sutures and the rats were maintained for six weeks on a 12 h light/dark cycle, at 23 ºC, with food and water *ad libitum*. No analgesic or antibiotics were given to the rats.

Specimen preparation, morphological analysis and counting of regenerated axons

Six weeks after tubulization, the rats were sacrificed with an overdose of chloral hydrate. The left sciatic nerve was exposed and the thigh region was filled with Karnovsky buffered solution (2% glutaraldehyde, 1% paraformaldehyde, 0.1 M, pH 7.4) for 10 min. The regenerated sciatic nerves were removed and the polyethylene tubes were bisected. The proximal half of the nerve was post-fixed with phosphate buffered osmium tetroxide (2%) and processed for Araldite embedding. Transverse, semi-thin sections (0.5 µm thick) were stained with toluidine blue and the total number of regenerated myelinated fibers (TMF) was counted at the tube midpoint. The results were expressed as the mean ± SD, and one-way ANOVA and the Newman-Keuls test were used for statistical analysis, with a value of $p<0.05$ indicating significance.

For ultrastructural analysis, ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Leo 906 transmission electron microscope operated at 60 KV.

The distal half of the sciatic nerves was processed and embedded in Paraplast, and longitudinal sections were stained for 15 min with toluidine blue in McIlvaine's buffered solution, pH 4.0. The sections were then washed in the same buffer, dehydrated, mounted and examined under polarized light. The collagen birefringence, and the toluidine blue metachromasy and dichroism, were assessed in the two experimental groups that received collagen in order to assess the implant supraorganization.

RESULTS

Normal and polarized light

Six weeks after tubulization, a tissue cable surrounded by fluid connected the proximal and distal stumps in the middle of the tube. Transverse semi-thin sections from the midpoint of the regenerated nerve showed a number of regenerated axons and a variable amount of collagen was detected in the center of the tube. The regenerated myelinated axons were organized into small bundles surrounded by perineural-like cells and fibroblasts.

Although regeneration occurred when the gap was filled with collagen gel from either rat tail or bovine tendon, the number of regenerated fibers was significantly higher ($p<0.05$) for collagen implants derived from bovine tendon compared to rat tail and the empty tube (Fig. 1). Conversely, the amount of collagen remaining six weeks after surgery was much higher when the source was rat tail tendon. In this case, only the collagen present at the boundaries of the regenerated axons showed signs of degradation.

In this region, a number of blood vessels and groups of regenerated axons were observed, indicating that metabolism occurred centripetally (Fig. 2). The implants derived from bovine tendon, occurred as small patches along the tissue cable that were always invaded by fibroblasts and perineural cell projections containing a variable number of growing axons.

The supraorganization of the collagen implants was assessed by analyzing longitudinal sections of the regenerated nerve found inside the tube. The sections were stained with toluidine blue, pH 4.0, and observed with polarized light. Analysis of these nerve sections revealed an intrinsic supraorganization of the fibrils derived from bovine tendon that was not seen in fibrils derived from rat tail tendon. As shown in Figure 2, collagen from bovine tendon had the same orientation as that found in the perineurium and endoneurium, and the high birefringence of the bovine-derived collagen after staining with toluidine blue, reinforced the idea of its high molecular organization. In contrast, lower birefringence of rat tail collagen indicated that this macromolecule was not oriented. The dichroism seen in toluidine blue-stained longitudinal sections (Fig. 3) supported the conclusion that the bovine collagen remained oriented after implantation. These findings also agreed with the results obtained using polarized light and ultrastructural analysis .

Electron microscopy

Ultrastructural analysis of transverse sections obtained from the midpoint of the tube showed the presence of myelinated and unmyelinated axons that were usually organized as small bundles surrounded by perineural cells. These cells were distinguished from the endoneural fibroblasts by the presence of a basal lamina. The perineural cells contained a large number of lysosomes, indicating an effort to reestablish the nerve blood barrier.

The regenerated axons showed a normal morphology and different degrees of myelination, with a number of unmyelinated axons being surrounded by Schwann cell projections.

In implants of collagen derived from rat tail tendon, there was a clear demarcation between the nerve tissue and the collagen implant, indicating poor integration with the regenerating environment, and slow degradation by non-neuronal cells. In some cases, macrophages and fibroblasts were observed in the implant area and, at such sites, the collagen fibers were organized parallel to the tube wall.

There was no difference in the aggregation state of the bovine tendon and rat tail collagen, but there was a marked difference in the supraorganization of the two types of implants. When collagen was derived from rat tails, the fibrils were not organized along a specific axis, but were arranged in variable directions. Conversely, bovine tendon collagen showed a much higher supraorganization, with the fibrils being oriented parallel to the growing axons. Additionally, interaction of the implant with the nerve tissue was intense, with fibroblasts, perineural cells and macrophages invading the area.

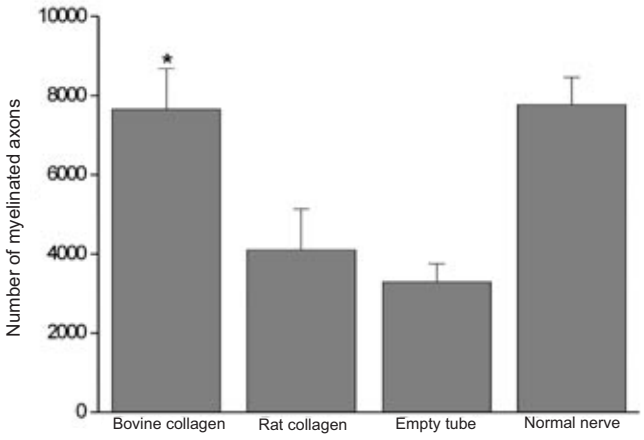

Figure 1. Number of regenerated myelinated fibers six weeks after tubulization of the left sciatic nerve in rats. Note the significantly higher number of axons when collagen from bovine tendon was implanted (n=3 for each experimental group; $p<0.05$, Newman-Keuls test).

DISCUSSION

The suprastructural organization of the ECM is important for cell-ECM interactions, since this organization provides signals that are transmitted to the cells which make up the tissue [15].

Peripheral nerve transection disrupts contact and communication between the neuron and its target, but the gap between the stumps can be bridged by implanting a tubular prosthesis thereby allowing axonal regeneration and target reinnervation [6]. If the tube is left empty, the gap is eventually filled with a plasma like fluid that polymerizes to form a fibrin bridge connecting the stumps [5]. Non-neuronal cells migrate from proximal and distal stumps and precede axonal sprouting and regeneration [5,11,12]. However, if the gap between the stumps is filled with a substance such as collagen or laminin, the initial steps of regeneration can be accelerated, resulting in

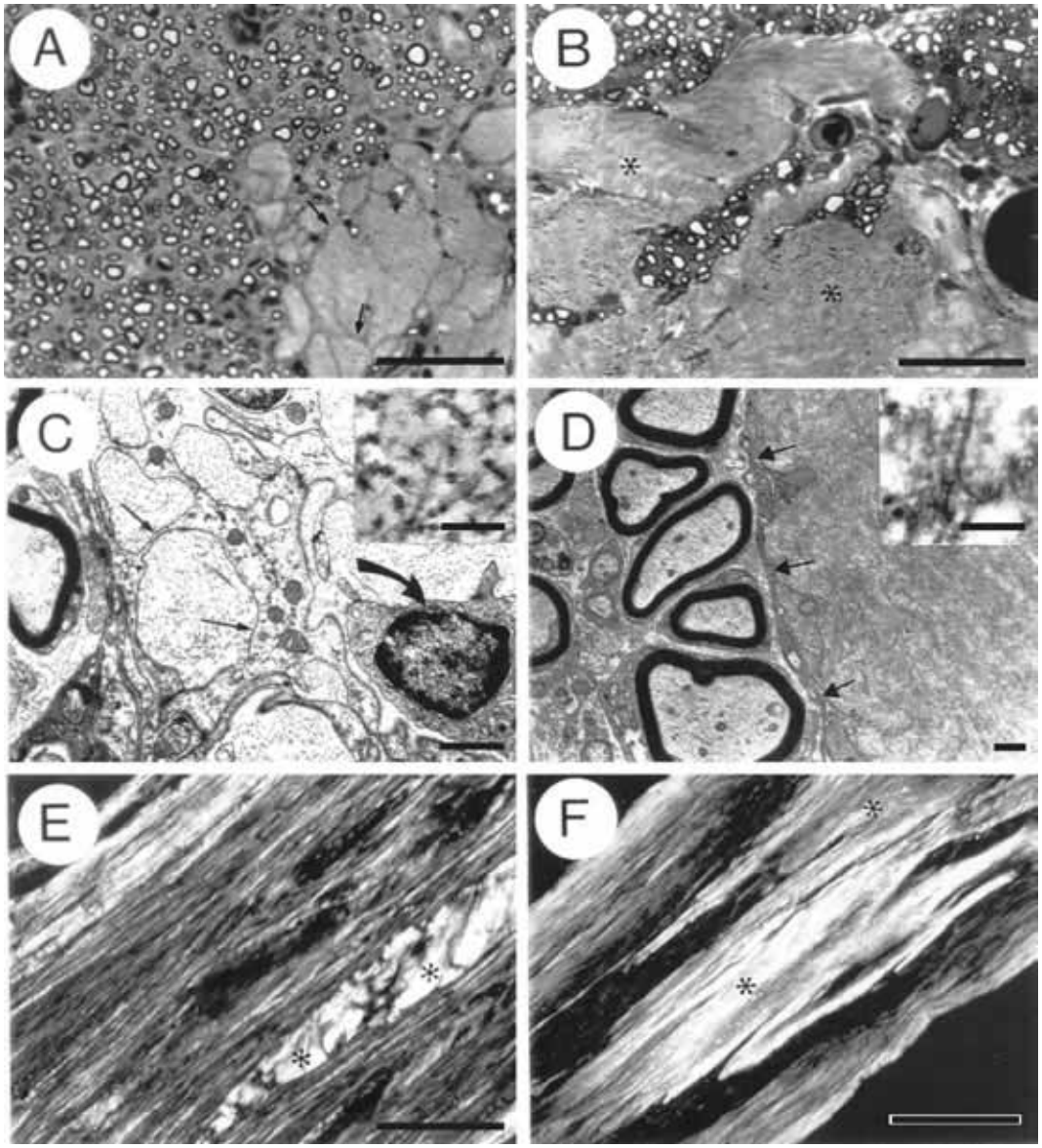

Figure 2. A. **Bovine tendon collagen**. Transverse, semi-thin section of the tissue cable at the midpoint of the tube, six weeks after surgery. Note the interaction between the regenerated nerve and the collagen implant, which was invaded by a number of cellular processes from fibroblasts and perineural cells (**arrows**). Scale = 100 μm. **B**. **Rat tail collagen**. Semi-thin section of a regenerated nerve six weeks after tubulization. Note the large amount of collagen remaining in the center of the tissue cable (*****). The poor coupling with the regenerating nerve is shown by the small number of cell projections into the implant area. Scale = 100 μm. **C**. **Bovine collagen implant**. Electron microscopy of the boundary between the implant and the regenerating fibers. Perineural cell projections penetrate the implant area and facilitate collagen degradation (**arrows**). A macrophage is seen in the implant area (**curved arrow**). Scale = 1 μm. The inset (**upper right**) shows the longitudinal arrangement of the collagen fibrils. Scale = 120 nm. **D**. **Rat tail collagen.** Ultrastructural view of a regenerated nerve after six weeks in the presence of a rat collagen-derived implant. Note the cellular layer delimiting the boundary between the nerve tissue and the implant area (**arrows**). Scale = 1 μm. The inset (**upper right**) shows that the collagen fibrils had no major orientation. The birefringence images of newly formed structures revealed different levels of supra-organization, depending on the source of the collagen implant. Scale = 120 nm. **E**. Longitudinal section of a regenerated nerve plus bovine collagen seen under polarized light microscopy. Note that the remaining collagen patches (bright, birefringent structures) have the same orientation as the epineural and perineural collagen, which is parallel to the nerve fibers. Scale = 100 μm. **F**. Longitudinal, toluidine blue stained section of a regenerated nerve plus rat collagen seen under polarized light microscopy. Note the large amount of collagen remaining six weeks after surgery that did not have characteristic orientation of the fibrils (low level of birefringence). The birefringence was enhanced by the oriented binding of chromophores to the substrate and appeared as bright areas. Bar = 100 μm.

an increased number of regenerated axons [4,10,19]. This beneficial outcome may be diminished or even lost if the implant is not aligned longitudinally [10].

Ceballos *et al*. [1] reported that the use of a magnetically oriented collagen gel from bovine dermal tissue significantly improved the peripheral nerve regeneration in 4-6 mm gaps in mice. Although these authors [1] investigated the importance of aligned ECM molecules in axonal growth, they used a collagen implant that was artificially organized inside the prosthesis. So far, there have been no reports on the use of naturally oriented collagen fibers or on the suprastructural properties of this substance following extrusion. Such properties may be important during the initial steps of regeneration, when there is cell migration and axonal sprouting, and during the degradation and absorption of the implanted substance.

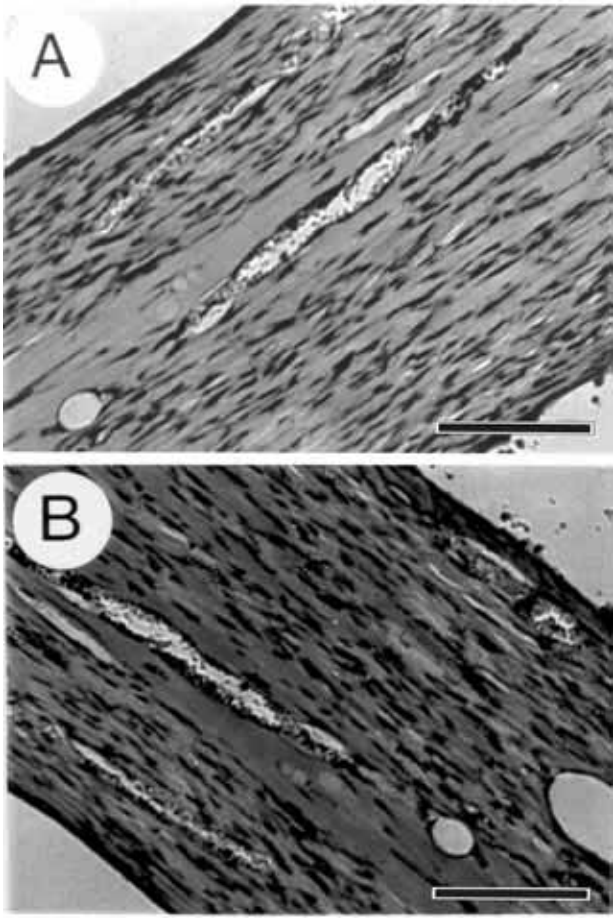

Figure 3. Bovine collagen implant metachromasy seen after toluidine blue staining (pH=4.0). Note the association of the implant with extracellular glycosaminoglycans. The specimen was placed parallel (**A**) and perpendicular (**B**) to the analyzer. Bar = 100 μm.

In the present study, we used collagen type I obtained from two different sources and with distinct physico-chemical characteristics. Rat tail collagen is more soluble since the aggregation state and cross-linking between the fibers is not as high as in bovine tendon. Rat tail collagen maintains its fibril structure, including the telopeptides, and is therefore similar to clinically used collagen, such as employed by Ceballos *et al*. [11]. This conserved structure probably influences the extent of aggregation when the collagen is reconstituted and hydrated, and results in aggregation without supraorganization of the fibrils after extrusion, as seen with polarized light and electron microscopy.

In contrast, bovine tendon collagen is highly compacted and is not as easily solubilized as that of rat tendon [16,20]. As a result, extraction of this collagen requires enzymatic treatment of the tendon with pepsin in order to unravel the fibrils, mainly by removing the C-terminal telopeptide. This moderate proteolysis alters the self-assembly and supraorganization of this collagen, and allows molecular orientation of the native fibrils formed after extrusion [17]. This arrangement was well observed at the ultrastructural level since the collagen fibrils derived from bovine tendon displayed a much higher longitudinal organization than those derived from rat tail.

The light microscopy results and the number of regenerated fibers showed that bovine tendon collagen enhanced nerve regeneration with increased coupling between the implant and the regenerated nerve. In addition, the presence of cellular processes invading the collagen residues indicated implant degradation.

The results obtained with polarized light microscopy agreed with those observed with semi-thin sections. When the implant was derived from bovine tendon, the collagen birefringence reflected the high molecular order. In contrast, implants of rat tendon collagen characteristically contained fibers with no particular orientation.

The results described here indicate that molecular order of bovine tendon collagen positively influenced the nerve regeneration six weeks after sciatic nerve tubulization. This supraorganization probably facilitated the diffusion of trophic factors, the recognition of collagen by non-neuronal cells, the migration of cells into the implant, and the stimulation of collagen degradation.

These results also reinforce the importance of the ECM supraorganization as a stimulating factor for nerve regeneration after tubulization. In this regard, the use of substances that show self-assembly in an ordinate pattern when placed in the tube may contribute to regeneration.

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