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Guided regeneration with resorbable conduits in experimental peripheral nerve injuries

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Abstract Guided tissue regeneration is a new approach in the reconstructive surgery of peripheral nerves. Artificial conduits can be constructed from biodegradable polymers. Lactic/caproic acid copolymers and polyphosphazenes are biocompatible materials with a slow resorption rate. Conduits made from either poly-[l-lactide-co-6-caprolactone] or poly-[bis-(ethylalanate)-phosphazene] were assessed for use as guides for nerve regeneration in experimental animals. Under general anesthesia and by using a microsurgery technique both sciatic nerves were exposed in 2 groups of 9 Wistar rats. On the right side, a 10 mm segment of the nerve was removed, and the defect was then repaired using a conduit. On the left side, the same defect was bridged using as an autograft the nerve segment, which had been removed from the right sciatic nerve. Histological and electron microscopy investigations were performed after 30, 90 and 180 days and showed the gradual degradation of both types of conduits without any evidence of local toxicity. The regeneration of the nerve fibers in the lumen was not significantly different from that shown by the autologous grafts. Likewise, no differences were found at 180 days in the functional recovery of the nerve (evoked muscle action potential). Both conduits were found to be effective for guided nerve regeneration. Poly-[l-lactide-co-6-caprolactone] tubes were easier to insert, while polyphosphazene conduits allowed the use of neurite-promoting factors.

Résumé La régénération tissulaire guidée est une nouvelle perspective dans la reconstruction des nerfs périphériques. Des conduits artificiels peuvent être obtenus avec des polymères biodégradables. Nous avons évalué dans un modèle expérimental deux types de polymères le

poly-[l-lactide-co-6-caprolactone] et le poly-[bis-(ethylalanate)-phosphazene]. Cette expérimentation a été, conduite sous anesthésie générale au niveau du nerf sciatique de 9 rats Wistar, un segment de 10 mm de nerf a été réséqué et du côté droit la reconstruction a été faite en utilisant une polymère du côté gauche, la réparation a été faite avec une autogreffe avec le segment réséqué du côté opposé. Une étude histologique et par microscopie électronique a été faite à 30, 90 et 180 jours. Il n'y a pas eu de toxicité locale des conduits en polymère. La régénération des fibres nerveuses dans les deux types de conduits ne fut pas différente de celle observée avec les autogreffes. Les deux types de conduits peuvent être considérés comme efficaces pour guider la régénération nerveuse. Le tube en polycaprolactone est plus maniable alors que celui en polyphosphazene peut contenir des facteurs favorisant la croissance axonale.

Introduction

Reconstructive surgical procedures are required following traumatic or iatrogenic damage to peripheral nerves or after the excision of primitive neoplasms [5,20,28]. While autologous grafting is the current surgical procedure of choice for this purpose [7,24] experimental [8,25,30] and clinical [21] investigations report satisfactory results with the use of artificial guides. The advantage of this procedure is the avoidance of sacrificing a segment of the donor nerve with subsequent loss of function and/or neuroma formation.

The ability of a divided nerve to regenerate through artificial conduits was discovered in the nineteenth century [10], and this is considered to be the first example of guided tissue regeneration (GTR). However, GTR is a new concept in surgical practice and is based on the assumption that different types of tissues have different abilities to penetrate into a wounded area during the reparative process. At the same time, GTR creates a "walled space" in which intrinsic growth factors are protected and contained in situ with resulting enhancement of their biological activity.

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GTR is widely employed in dental surgery [9] but experimental research has also demonstrated its effectiveness in orthopaedic [12] and peripheral nerve [3] surgery. In peripheral nerve surgery a wide variety of conduits made from biological or synthetic materials have been assessed. Among synthetic materials there are non-resorbable materials such as silicone [15] or expanded polytetrafluoroethylene (ePTFE) [29], and resorbable polymers such as polyglycolic acid [22] or polyglactin [24].

As a consequence of the unfavorable results observed by some authors with the use of non-resorbable conduits [23], interest is now being directed towards slowly absorbable materials. These last longer and thus produce a prolonged stability of the guide during the time required for nerve regeneration.

In previous studies we have had promising results by using resorbable nerve guides made from poly-[1-lactide-co-6-caprolactone] [11,25]. At the same time we observed that a group of polymers, the poly-[organo]-phosphazenes already assessed as carriers for controlled drug release systems [4] have good biocompatibility properties and a slow degradation rate. Therefore, they should also be considered as suitable materials with which to make conduits for guided nerve regeneration. The aim of the present paper is to report and to compare the results obtained by using these two types of guide.

Materials and methods

Co-polymers of lactic and caproic acid are a group of biodegradable materials with a slow rate of degradation. Accordingly, using a previously described procedure [26] conduits were prepared from poly-[1-lactide-co-6-caprolactone], which were 1.3 mm. in diameter with a wall thickness of 175 μ m and were 12 mm in length. These conduits were stored in an anhydrous environment until implanted.

Polyphosphazenes are a family of polymers in which a backbone of nitrogen and phosphorus holds lateral organic groups. Physicochemical properties as well as the degradation rate are a result of the chemical features of substituents on the lateral chains. It is also possible to incorporate drugs into the polymer and thus obtain their controlled release during polymer degradation [4].

Poly-[organo]-phosphazenes prepared from amino-acid substituents slowly degrade in water with the release of non-toxic products that are excreted or metabolized. Poly-[bis-(ethylalanate)-phosphazene] belongs to this group and is prepared according to a standard procedure of synthesis for this group of polymers as previously described [31]. The conduits were then made by depositing the polymer onto a rotating glass mandrel, they were 12 mm in length with an external diameter of 1.8 mm and a wall thickness of 500 μ m.

In vivo studies were carried out strictly following European and Italian laws on animal experimentation. The animal research protocol was approved by the scientific committee of the Rizzoli Orthopaedic Institute and the responsible public authorities as demanded by Italian law and according to EC regulations (D.L. 27 January 1992, no. 116).

Eighteen male Wistar rats weighing 350 g $\pm$ 50 g were used, which were divided into 2 groups. In group 1 (9 rats) poly-[1-lactide-co-6-caprolactone] conduits were implanted and in group 2 (9 rats) poly-[bis-(ethylalanate)-phosphazene] conduits were implanted.

Under ketamine (0.4 ml i.m.) and xylazine (0.2 ml i.m.) anesthesia the lower back of the animal was shaved and disinfected

with a solution of iodine. On the right side the sciatic nerve was exposed through a gluteal muscle incision, transected proximal to the tibio-peroneal bifurcation where a 10 mm segment was excised. The conduit was implanted between the 2 nerve stumps and was sutured to the epineurium with 2 10/0 nylon stitches. The entire procedure was performed with a microsurgery technique by using a Zeiss OPMiMDM operating microscope. On the opposite side, and with the same procedure, a 10 mm defect was created in the sciatic nerve and this was repaired by using the nerve segment excised from the right side as an autologous graft.

After careful hemostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 silk threads. The animals were housed in single cages and received unlimited food and water. No drugs were administered during the postoperative period. The rats were killed with an anesthetic overdose in groups of 3 at 30, 90 and 180 days postoperation.

Before being killed, the last group (180 days postoperation) underwent electric muscle action potential (EMAP) recordings. The sciatic nerve segment proximal to the chamber/graft was supra-maximally stimulated and the EMAP recorded by a needle electrode placed in the flexor digitorum superficialis muscle. Then, after being killed, the graft and the conduits were excised in continuity with the recipient nerve. Samples for optical microscopy were fixed in 10% formalin, then dehydrated in alcohol and embedded in paraffin. Serial longitudinal and transverse sections of 5–8 μ m were cut and stained with hematoxylin-eosin and mal-lory-azan. Samples for electron microscopy were fixed in 0.1 M cacodylate buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide in Veronal Buffer, dehydrated through a graded series of ethanol and embedded in Epon resin. Semi-thin sections (0.5–1.0 μ m) were stained with Toluidine blue. Ultra-thin sections stained with lead citrate and uranyl acetate were examined with a Philips 400 transmission electron microscope (TEM).

The number of myelinated axons in the middle part of the conduit and in the graft were counted in cross-sections stained with Toluidine blue, by using a transmission light microscope (Axioscope, Carl Zeiss, Jena, Germany) and by means of a computerized image analysis system with Kontron KS 300 software (Kontron Electronic GmbH, Eichling bei Munchen, Germany). Statistical significance was evaluated by means of the Student's *t*-test.

Results

Poly-[1-lactide-co-6-caprolactone]

At 30 days the conduit was recognizable and was easily separated from the surrounding tissues. The wall maintained its transparency and a bridge of tissue found in the lumen joined together the 2 nerve ends. Histologic examination revealed the presence of Schwann cells and axons in the regenerating tissue.

At 90 days the conduit had lost its transparency and was more adherent to the surrounding tissues. Optical and electron microscopy demonstrated the presence of signs of degradation of the wall together with a moderate foreign body reaction around the polymer. Myelinated and un-myelinated nerve fibers were present in the newly formed tissue that was lined by a thin perineural layer of collagen.

At 180 days the degradation of the conduit had progressed. In the regenerating tissue found in the lumen, myelinated and unmyelinated nerve fibers with a regular pattern were present (Fig. 1), (Table 1).

Table 1 Poly [L-lactide-co-6-caprolactone] and poly [bis(ethylalanate)phosphazene] conduits. Comparison of characteristics

	Poly [L-lactide-co-6-caprolactone]	Poly [bis(ethylalanate) phosphazene]
Handling	Wall easily pierced by suture threads	Possible breaking of wall by suture threads
Resorption	Almost complete after 180 days	Almost complete after 180 days
Tissue reaction	Foreign body giant cells	Foreign body giant cells. Fibrous tissue around fragments
E.M.A.P.	3.6 ± 0.2 mV ^a	3.1 ± 0.2 mV ^a
Myelinated axons in the conduit ^b	2359 ± 135 ^b	2275 ± 140 ^b
Possibility of neurite-promoting factors release	No	Yes

^a Autologous grafts: 2.8 ± 0.5 mV, *t*-test not significant.

^b Autologous grafts: 2290 ± 115 axons, *t*-test not significant

Fig. 1 Poly [L-lactide-co-6-caprolactone] conduit. Cross section. Myelinated and unmyelinated nerve fibers are demonstrated. 180 days control. T.E.M., 3100 $\times$

Fig. 2 Poly [bis(ethylalanate)phosphazene] conduit. Cross section. Evidence of regenerated myelinated and un-myelinated fibers. 180 days control. T.E.M., 3100 $\times$

Polyphosphazene conduits

These conduits were fairly easy to insert by using an operating microscope. At 30 days they were still intact, but it was difficult to separate them from the surrounding tissues. Histology demonstrated a cord of regenerating nerve fibers in the lumen of the conduit. A sheath of

connective tissue separated the nerve cord from the inner surface of the conduit wall.

At 60 and 180 days progressive signs of reabsorption of the conduit were present. The wall fragments were surrounded by a capsule of connective tissue containing foreign body giant cells around the polymer. No signs of marked inflammatory reaction or infection were found. A progressive increase in the number of myelinated and unmyelinated axons (Fig. 2) with a more regular fascicular pattern was found (Table 1).

Autologous grafts

Nerve grafts could be easily recognized at 30 days. At 60 and 180 days the connective tissue reaction made mobilization of the nerve more difficult. Optical and electron microscopy demonstrated myelinated and unmyelinated regenerating axons, and Schwann cells were also found to be present. Endoneural connective tissue with signs of fibrosis in the graft was also seen. At 180 days the number of myelinated fibers in the middle part of the graft was 2290 ± 115 . EMAP recordings at 180 days showed an amplitude of 2.8 ± 0.5 mV.

Discussion

To obtain nerve guided regeneration, sufficient time for slow degradation of the conduit and stability of the wall is essential in order to ensure adequate growth and reorganization of the nerve trunk. Results obtained with bio-absorbable conduits are not always satisfactory. Gibson [13] observed poor nerve regeneration and a marked inflammatory reaction with Polyglactin 910 conduits, while Keeley [17] obtained poorer results with glycolide trimethylene carbonate conduits than with autologous grafts.

Our conduits demonstrated an initial stability followed by progressive and slow degradation, which was enough to allow reorganization of the nerve trunk in its lumen. The reaction of the surrounding tissues showed only foreign body giant cells in poly-[L-lactide-co-6-caprolactone] conduits but a fibrous capsule around the polymer fragments in polyphosphazene conduits. No signs of local or general toxicity were observed with ei-

ther material. Both conduits allowed regeneration of nerve fibers in their lumen to occur. The difference in the number of axons counted in the conduits and in the nerve graft after 180 days was not statistically significant. In addition, the EMAP did not demonstrate any significant differences between the 2 conduits and between either conduit and a nerve graft. Therefore, the conduits permit nerve regeneration, which is no less than that achieved by the current elective surgical procedure.

The two materials have different characteristics that need to be appreciated before either one is used (Table 1). Lactic acid polymers and copolymers have a well-assessed biocompatibility [32] with the degradation process without the release of toxic products [2]. The elastic and adhesive properties of the poly-[l-lactide-co-caprolactone] give this conduit excellent mechanical characteristics and they are easy to handle under an operating microscope. The 175 μm wall thickness allows easy suturing of the epineurium. The physico-chemical features of polyphosphazene do not permit a reduction in the thickness of the wall below 500 μm . This made it harder to insert the suture threads under the operating microscope.

The main interest in using polyphosphazenes for conduits is related to their biocompatibility and to the possibility of their entrapping capability, which promotes axonal growth in the polymeric mesh that is released during wall degradation [1,19].

In fact nerve conduits can be divided into "inert", and "active" on the basis of their behavior. "Inert" conduits solely assure a structure in which the processes of nerve regeneration can develop, while active conduits release various factors by diffusion that may enhance regeneration.

The experimental use of promoting factors in association with nerve conduits has been widely studied [16,27]. Among these substances there are nerve growth factors [6], insulin-like growth factors [14] and laminin [18] when neurotrophic factors are introduced into the lumen of the conduit there is an early peak in the resulting activity followed by a progressive decrease. The entrapment of a promoting factor, in the polymer mesh and its release during degradation, as seen with amino acid substituted polyphosphazenes, allows a prolonged bioavailability of the factor during the regeneration process. Poly-(organo)-phosphazene conduits therefore give new possibilities when compared with the commonly used nerve guides and poly-[l-lactide-co-6-caprolactone] conduits. Further studies are in progress to assess the role of activated polyphosphazene conduits in guided nerve regeneration.

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