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Review
Peripheral nerve regeneration using non-tubular alginate gel crosslinked with covalent bonds

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We have developed a nerve regeneration material consisting of alginate gel crosslinked with covalent bonds. In the first part of this study, we attempted to analyze nerve regeneration through alginate gel in the early stages within 2 weeks. In the second part, we tried to regenerate cat peripheral nerve by using alginate tubular or non-tubular nerve regeneration devices, and compared their efficacies. Four days after surgery, regenerating axons grew without Schwann cell investment through the partially degraded alginate gel, being in direct contact with the alginate without a basal lamina covering. One to 2 weeks after surgery, regenerating axons were surrounded by common Schwann cells, forming small bundles, with some axons at the periphery being partly in direct contact with alginate. At the distal stump, numerous Schwann cells had migrated into the alginate 8–14 days after surgery. Remarkable restorations of the 50-mm gap in cat sciatic nerve were obtained after a long term by using tubular or non-tubular nerve regeneration material consisting mainly of alginate gel. However, there was no significant difference between both groups at electrophysiological and morphological evaluation. Although, nowadays, nerve regeneration materials being marketed mostly have a tubular structure, our results suggest that the tubular structure is not indispensable for peripheral nerve regeneration.

1. Introduction

When the peripheral nerve is injured due to traffic accidents, removal of malignant tumors, etc., the functions of movement, sensation, and the autonomic functions are lost. The neurectomized peripheral nerve having a gap of less than 5 mm was clinically neuroanastomosed. Presently, when the gap is than 5 mm, an autologous nerve graft using comparatively unimportant sensory nerve tissue is often performed. However, this technique has several drawbacks, including the sacrifice of the normal sensory nerve, with loss of sensation, scarring, and possible neuroma formation. When multiple thick nerve tissues such as the brachial plexus are amputated, several additional problems occur, such as the shortage of collecting nerves, extending of operative time, and so on.

To overcome these problems, attempts are made to develop various bioresorbable and non-resorbable materials as alternatives to autologous nerve tissue. In this article, we introduce the nerve regeneration material that can resolve the abovementioned problems.

2. Peripheral nerve regeneration mechanism

When the peripheral nerve is resected, a distal axon from the transected part degenerates and disappears (Wallerian degeneration), and only the Schwann cells remain. After processing the myelin sheath with the assistance of the macrophage, the Schwann cells proliferate and organize into ordered columns, which are called as "bands of Büngner." On the other hand, the

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regenerating axons sprout from the proximal axon end or adjacent Ranvier node within a few hours after injury. The regenerating axons extend along the surface of the Schwann cells or the inside of the basal laminae [1]. In case of little or very short nerve gap, regenerating axons extend in the Schwann cell columns and reach the target organ. If, however, the gap is too wide, extension of the regenerating axons is disturbed by scar tissue formed in the gap due to fibroblast proliferation and the axons cannot reach the distal stump. The regenerating axons that have no goal to extend, form a neuroma, and nerve functions can never be recovered.

3. Nerve regeneration through basal lamina scaffold

Ide *et al*. demonstrated that a number of regenerated axons were observed when an acellular peripheral nerve tissue prepared by freezing-thawing treatment was implanted into the gap [2]. This fact shows that extracellular matrix (ECM), including the Schwann cell basal laminae, can provide an effective pathway for the regenerating axon, and acellular biomaterials can be a scaffold for regenerating axons.

4. Nerve regeneration using the artificial matrix of alginate gel crosslinked with covalent bonds

4.1. Preparation of implanting materials

Favorable biomaterials for peripheral nerve regeneration should have the following characteristics: excellent biocompatibility such as low cytotoxicity and small foreign-body reaction, provision of a desirable microenvironment for axonal elongation and Schwann cell migration, appropriate biodegradability, and inhibition of fibroblast invasion and scar tissue formation. We attempted to examine alginate as the material satisfying these requirements. Alginate, which is a block co-polymer consisting of β-D-mannuronic acid and α-L-gluronic acid, is a well-established natural bioabsorbable acidic polysaccharide extracted from brown seaweed.

Alginate crosslinked with calcium ion has been historically used for wound dressing. However, this material has drawbacks such as high cytotoxicity and large foreign-body reaction originating from an excess amount of calcium ions. Therefore, in order to substitute the excess calcium ion with the covalent bonds, we attempted to crosslink alginate with ethylenediamine under the existence of water-soluble dehydrating agent in water. After washing, the porous alginate gel crosslinked with covalent bonds (hereinafter called alginate gel) was obtained by freeze-drying (Fig. 1) [3–5].

4.2. Investigation of nerve regeneration mechanism using alginate gel

We first investigated the positional relationship among the alginate gel, the Schwann cells, and the regenerating axons at early stages after surgery [6]. Two pieces of alginate gel that were longer than the gap were implanted into the 10-mm gap created in the rat sciatic nerve, by a simple method (Non-tubular method) [7, 8] with implantation only and not suturing (Fig. 2B), and the two nerve stumps were concatenated. The suturing manipulations between the nerve tissue and the material were not performed. The animals were euthanized at early stages of regeneration within 2 weeks and the implantation sites were excised for histopathological evaluations. For immunohistochemical evaluation, longitudinal sections were double stained with specific anti-S100 antibody for Schwann cells and anti-β-tubulin class III antibody for regenerating axons. For electron microscopic evaluation, transverse Epon sections cut from tissue embedded resin block were used.

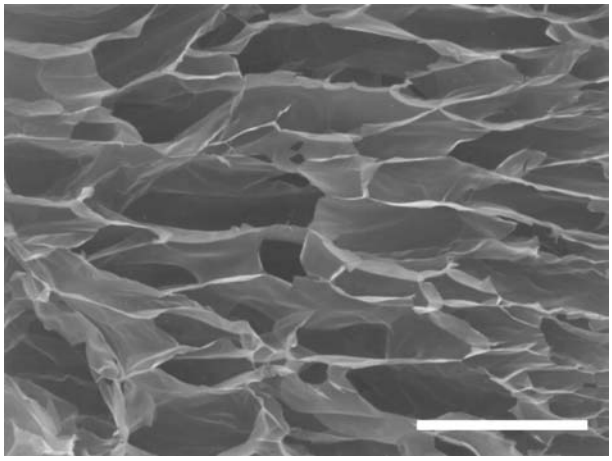

Figure 1 Scanning electron microscopic view of alginate gel. A number of pores are observed on the cross-sectional surface of the alginate gel. Scale bar: 1 mm.

4.2.1. Histopathologic observation at the site near the proximal stump

Generally, regenerating axons extended by using the support of the basal laminae at an early stage of regeneration, as mentioned above, whereas the naked axons without Schwann cell investment were observed in the alginate gel implantation group at 4 days after surgery. The naked axons grew through the partially degraded alginate gel, being in direct contact with the alginate gel without basal lamina covering. One to 2 weeks after surgery, the regenerating axons were surrounded by common Schwann cells, forming small fascicles. Some axons at the periphery were partly in direct contact with the alginate gel (Figs. 3 and 4) [6]. In addition, few fibroblasts were observed in the gap.

4.2.2. Histopathologic observation at the site near the distal stump

On the other hand, numerous Schwann cells originated from the distal stump were observed to infiltrate into the alginate gel 8–14 days after surgery (Fig. 5) [6].

Schwann cells provide the most effective scaffold for regenerating axons because they produce many kinds of neurotrophic factors, NGF, NT-3, BDNF, and so on [9], and express several adhesion molecules such as N-CAM and L-1 [10]. In previous studies, cultured

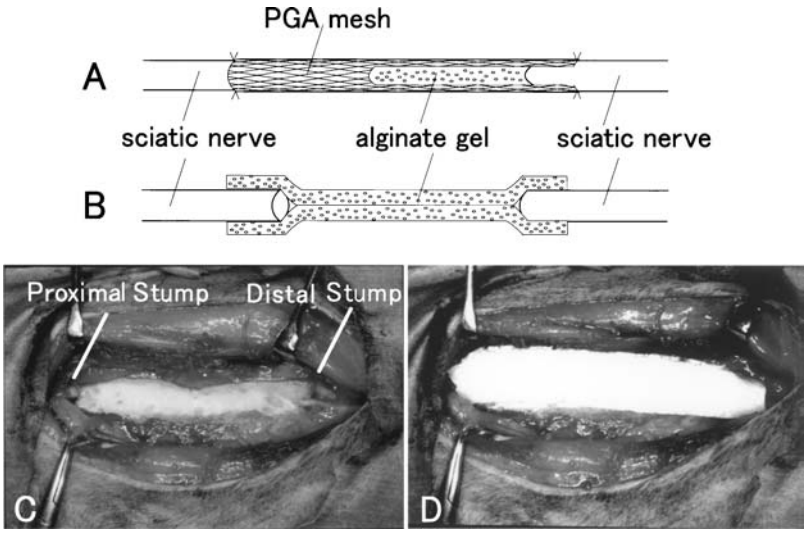

Figure 2 Illustration of tubular and non-tubular repair methods. (A) In the tubular method, an alginate covered with PGA conduit was implanted into the nerve gap and fixed to both nerve stumps by suturing. (B) In the non-tubular method, the nerve gap was simply filled with two pieces of sponge, which sandwiched the proximal and distal nerve stumps. (C) Intraoperative view of alginate gel implantation by non-tubular repair method. The photograph of the 50-mm gap in the cat peripheral nerve implanted with the first piece of alginate gel. (D) The photograph of the 50-mm gap in the cat peripheral nerve implanted with the second piece of alginate gel. (Partly reproduced from reference 7 with the permission of the authors and the publisher.)

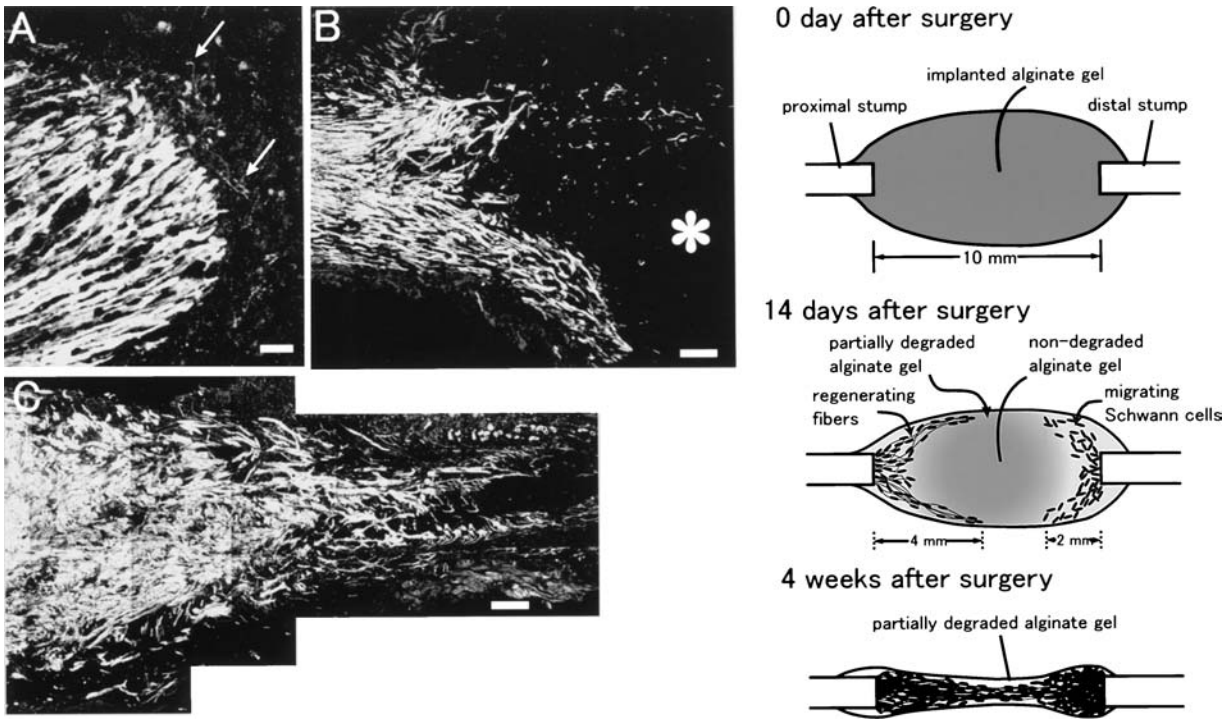

Figure 3 Confocal laser scanning micrographs of sprouting axons and migratory Schwann cells immunostained with anti-S100 (green) and anti-β-tubulin class III (red) antibodies. These pictures were taken from longitudinal sections including the proximal stump. (A) Four days after surgery. Double staining of the sprouting axons (red) and the Schwann cells (green) is shown. A small number of sprouting axons (arrows, red) are emerging from the proximal stump. (B) Fourteen days after surgery. Regenerating axons are divided into two groups lying in two directions along the periphery of the alginate gel (asterisk). (C) Four weeks after surgery. Regenerated fibers extend in a thick band toward the distal stump. The section is cut obliquely. Scale bars: 40 μm (A), 200 μm (B), and 400 μm (C). (Modified from reference 6 with the permission of the authors and the publisher.)

Schwann cells were implanted into the nerve gap to promote nerve regeneration [11, 12]. It is worth noting that Schwann cells can be observed at the site near the proximal stump as well as the distal stump by alginate gel implantation, because the regenerating axons establish the effective pathway at an early stage.

These results indicate that alginate gel provides a favorable microenvironment for regenerating axon extension and Schwann cell migration at an early stage of regeneration, and can act as a scaffold to promote peripheral nerve regeneration.

4.3. Reconstruction of the 50-mm gap in the cat sciatic nerve

In order to examine the necessity of the tubular structure of nerve regeneration material, we first prepared alginate nerve regeneration materials with and without

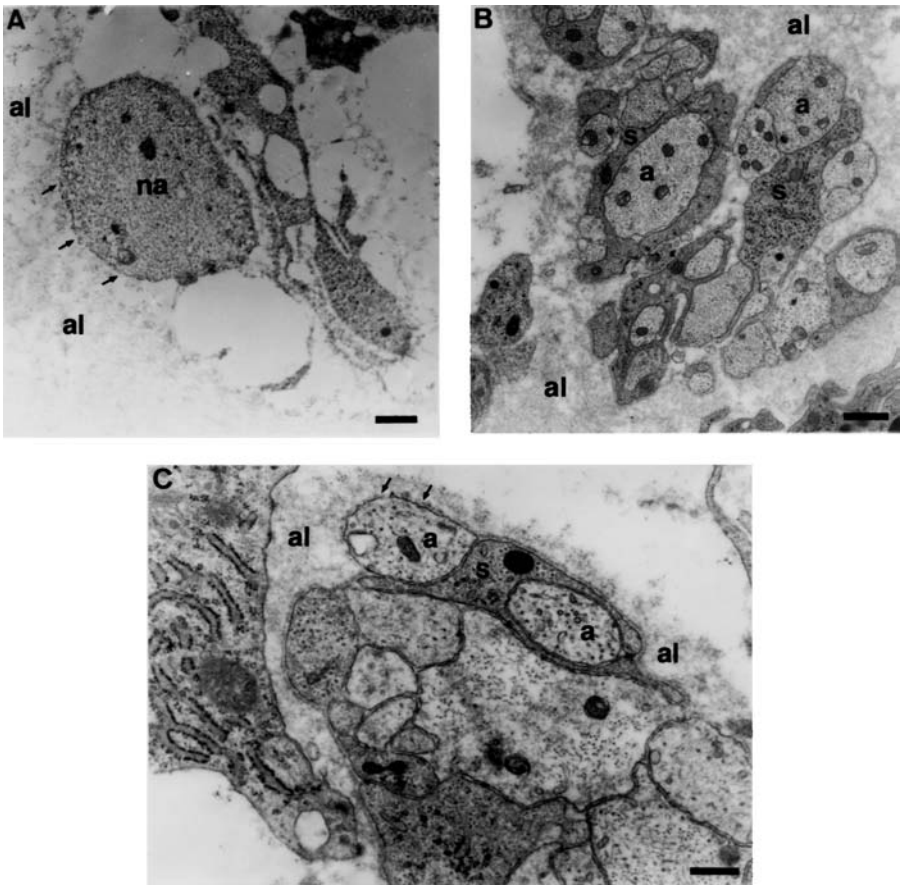

Figure 4 Electron micrographs of transverse Epon sections at a site near the proximal stump. (A) Four days after surgery. A naked axon (*na*) without basal lamina investment (arrows) is in direct contact with the fine lattice of alginate gel (*al*). (B) Seven days after surgery. Sprouting axons (*a*) extend individually or are fasciculated in small bundles. They are incompletely surrounded by Schwann cells (*s*). (C) Fourteen days after surgery. A part of the fasciculated regenerating axons is shown. Axons at the periphery of the bundle have basal laminae (arrows) and are in contact with alginate gel. Scale bars: 1 μm (A, B) and 500 nm (C). (Reproduced from reference 6 with the permission of the authors and the publisher.)

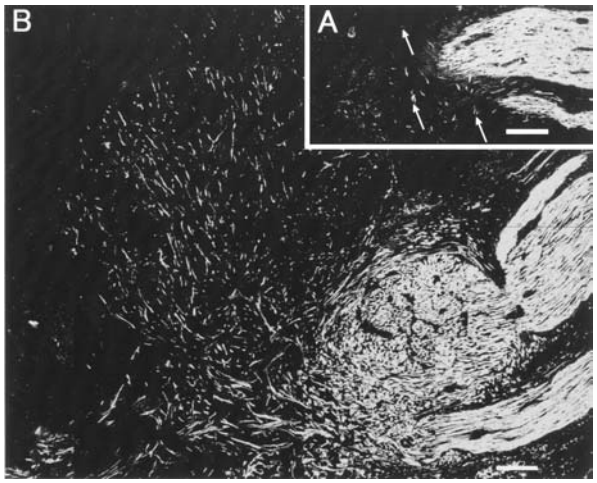

Figure 5 Confocal laser scanning micrographs of migratory Schwann cells from the distal stump. Schwann cells and axons were immunostained with anti-S100 (green) and anti-β-tubulin class III (red) antibodies, respectively, on longitudinal sections at the distal stump. (A) This micrograph shows a part of a longitudinal section 6 days after surgery. A few Schwann cells (arrows) have already migrated into the alginate gel. No staining with anti-β-tubulin class III antigen is detected in the distal stump, indicating that no axons survived at this stage. (B) Fourteen days after surgery. This photograph shows the entire distal stump. Numerous Schwann cells are migrating into the alginate gel from the distal stump. Scale bar: 200 μm. (Reproduced from reference 6 with the permission of the authors and the publisher.)

tubular structure. We attempted to compare their effectiveness by using the cat peripheral nerve regeneration model (Fig. 2) [7]. In the non-tubular experimental group, two pieces of alginate gel were implanted into the 50-mm gap created in the cat peripheral nerve, and the two nerve stumps were concatenated. The suturing manipulations were not performed. On the other hand, alginate gel covered by cylindrical polyglycolic acid mesh was fixed to both, the proximal and distal stumps, in the tubular experimental group.

Generally, the degree of peripheral nerve regeneration is determined by electrophysiological measurement, histopathological evaluation, walking pattern, tracer detective study for axoplasmic transport, and so on. Of these evaluations, some aspects of the electrophysiological measurements and histopathological evaluations are described below.

In electrophysiological evaluation, compound muscle action potentials (CMAP) are indicators of recovery of the motor nerve function, and somatosensory evoked potentials (SEP) are indicators of recovery of the sensory nerve function. The electrophysiological measurements were performed weekly from 1 week after the surgery. Light and electron microscopic evaluations were performed by using the specimens from the nerve tissues at the midpoint and the distal part of

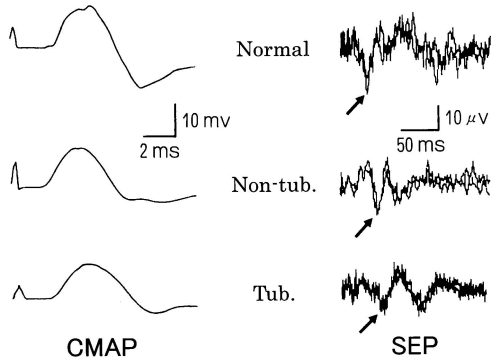

Figure 6 Electrophysiologic recording of compound muscle action potentials (CMAP) and somatosensory evoked potentials (SEP, average of 50 responses, arrows indicate peak latencies) 3 months after surgery. CMAP and SEP showed remarkable restoration in both the tubular and the non-tubular groups. Compared with normal nerve, the alginate-repaired nerves showed satisfactory recovery. (Modified from reference 7 with the permission of the authors and the publisher.)

the gap, comparing the morphologies with those of the contralateral normal nerve.

The earliest electrophysiologic restoration was observed 2 months after surgery, and short latencies equal to the contralateral normal nerve were observed in both CMAP and SEP at 3 months after surgery (Fig. 6) [7]. The macroscopic photographs at 8 months after surgery are shown in Fig. 7A–C. In both tubular and non-tubular experimental groups, thick nerve tissues identical to the contralateral normal sciatic nerve were reconstructed, and the implanted materials had disappeared completely. Thick axons identical to normal nerves were observed in the high-density in the gap part as well as in the distal part (Fig. 8A–F) [7]. Each evaluation showed no significant differences between the tubular and the non-tubular experimental groups.

The experimental results using a cat model show that the tubular structure is not necessary for peripheral nerve regeneration, and reconstruction of a wide nerve gap can even be achieved solely by alginate gel implantation. This fact is considered to be important for practical clinical treatment. If satisfactory nerve regeneration can be achieved solely by non-tubular alginate gel, a single standardized alginate gel can be applied to all traumatized peripheral nerves having varying thicknesses, shapes, and positions, especially in certain anatomic positions wherein it is difficult to perform suturing [13]. The tubular nerve regeneration material requires cumbersome microsurgical sutures under microscope, whereas the alginate gel exhibits its effectiveness by simple implantation into the nerve gap. Moreover, the latter is easy to use and has good operationality.

4.4. Peripheral nerve regeneration with other materials

The tubular structure of nerve regeneration material has historically been considered essential. The tubular nerve regeneration material is believed to function through the helpful factors for nerve regeneration

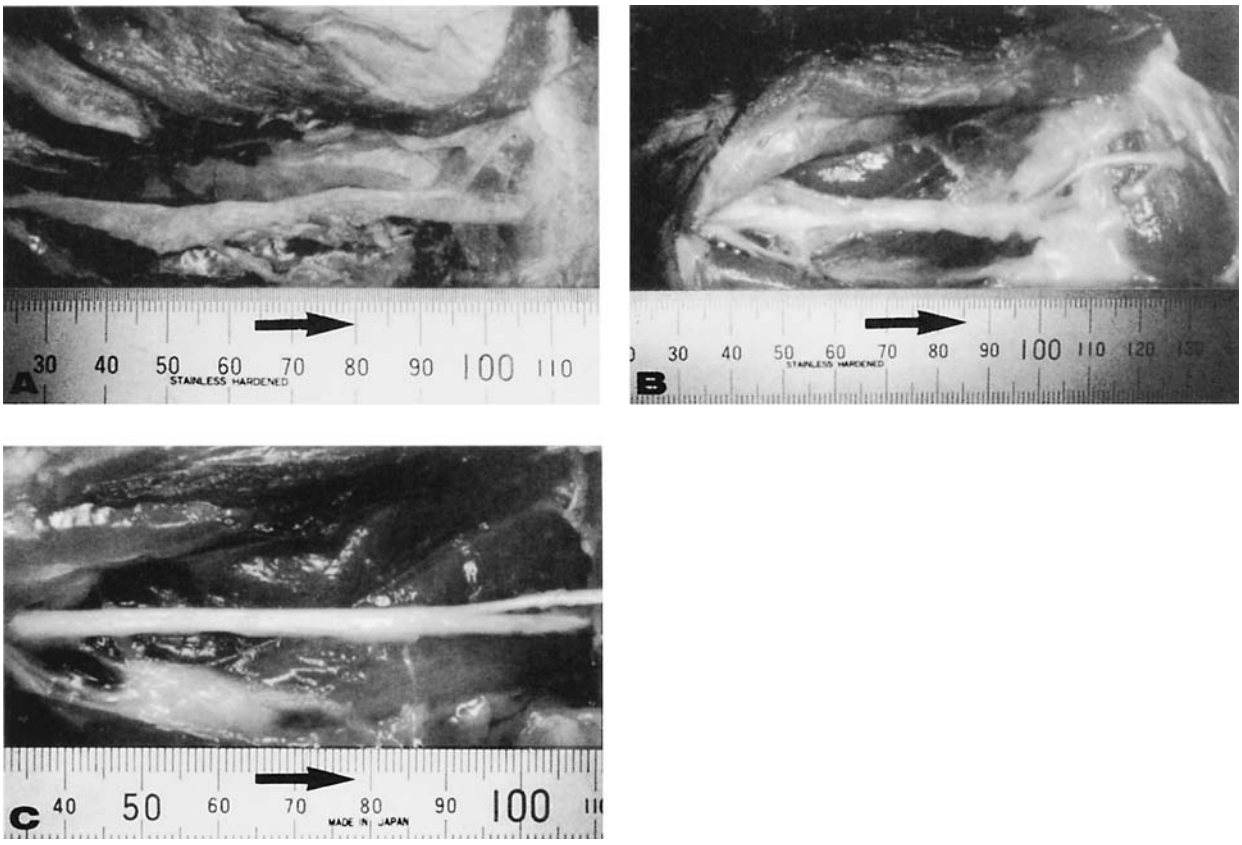

Figure 7 Anatomic appearance of tubular (A) and non-tubular (B) repaired nerve 8 months after surgery. In both groups, the regenerated nerves bridging the gap are observed. At the midpoint of the gap, regenerated nerve tissues appear thinner and wider than the normal sciatic nerve (C). The peroneal and tibial nerves show appearances similar to normal nerves (arrows toward distal position). (Reproduced from reference 7 with the permission of the authors and the publisher.)

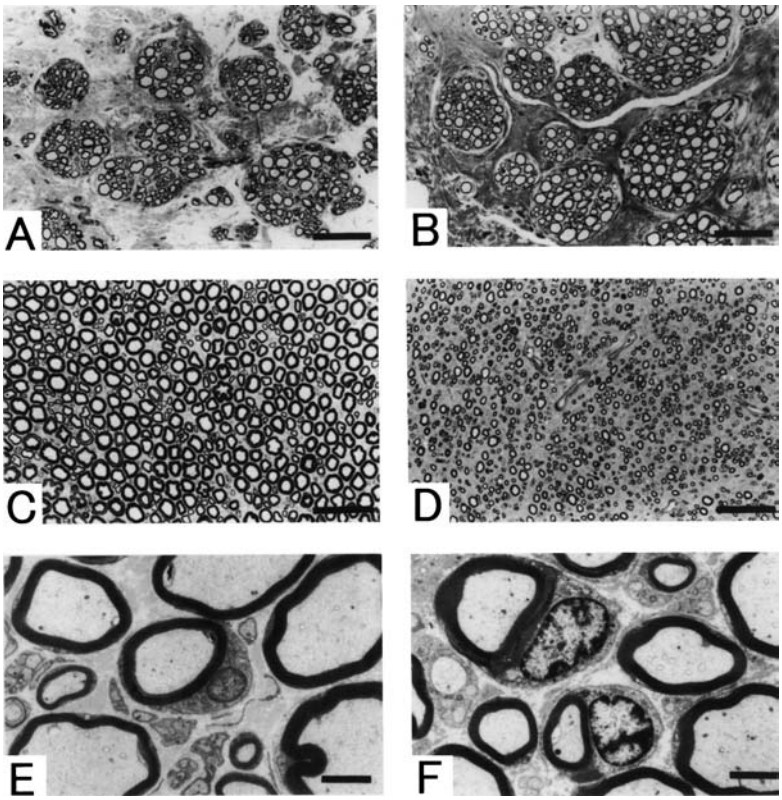

Figure 8 Histologic appearances 8 months after surgery. A large number of well-myelinated axons were observed both at the midpoint of the gap (A, tubular; B, non-tubular) and in the distal tibial nerve (D, non-tubular). The regenerated myelinated fibers were smaller in diameter and thinner in myelination compared to the normal nerve (C), and were organized as small fascicular structures by the surrounding perineurial cells. Numerous unmyelinated fibers were also observed by electron microscopy (E, tubular; F, non-tubular). No obvious difference was found between the two groups. Neither residual fragments of alginate nor inflammatory cells were found in the specimens. Scale bars: 50 μm, toluidine blue stain (A–D), 2 μm, double-stained with uranyl acetate and lead nitrate (E, F). (Modified from reference 6 with the permission of the authors and the publisher.)

contained in it [9], by inhibiting the invasion of fibroblasts resulting in scar tissue formation, and by navigating the axon extension. Lundborg *et al*. reported that a rat sciatic nerve gap wider than 10 mm could not be reconstructed by the silicon guidance channel [14].

Regenerated axons were inferior to normal nerve in axon diameter, axonal number, and thickness of myelin sheath, even when the nervous tissue could be connected in cases with a gap of less than 10 mm. These results are considered to be induced by the biological fact that the tubular nerve guide made from impermeable material could not supply enough nutrients and oxygen from the outside to the regenerating nerve tissue. To overcome this drawback, Aebischer *et al*. tried to improve the material permeability and permselectivity [15]. Moreover, several improvements have been attempted with regard to the surface roughness inside the tube [16], electrical charge [17], delivery of the neurotrophic factors, and so on. When the nonresorbable material is implanted, its removal is necessary after the recovery of the nerve functions. In order to solve these problems, application of bioresorbable materials derived from animals, including collagen, has been attempted for nerve regeneration [18, 19]. This ECM functions not only as a scaffold promoting axonal extension, but also as a scaffold stimulating fibroblast proliferation, resulting in the filling up of the nerve gap with scar tissue. In the case of a wide nerve gap, scar tissue serves as a physical barrier and inhibits the axons from extending [6]. Furthermore, these materials derived from animals have not only the antigenicity, but also the potential risk of infection by unknown pathogens such as prions, which are common to man and animals. In order to solve this problem, Mackinnon *et al*. examined the nerve regeneration material made from synthetic polyglycolic acid with a cylindrical shape [20]. Although various nerve regeneration materials have been evaluated by using animal models as described above, reconstruction of a gap wider than 25 mm has been considered impossible [21].

We have demonstrated that alginate gel has several excellent features as it is not only effective for the regeneration of a 50-mm gap in the cat peripheral nerve, but is also easy to handle during surgical implantation. Furthermore, it involves no risk of infection since it is prepared with raw material derived from a plant. It is suggested that the alginate gel has a favorable biocompatibility for regenerating axon extension and Schwann cell migration, as revealed by microscopic evaluation in the early stages.Therefore, it can be considered that alginate gel is a potent material for promoting peripheral nerve regeneration, and that the non-tubular method is a promising approach for the repair of the peripheral nerve.

Acknowledgments

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