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Development of the Tendon Chitosan Tube and its Application to Nerve Conduit

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Abstract:

On the inner surface of tendon chitosan tubes having a triangular shape and a hydroxyapatite coating (t-chitosan/HAp tube) laminin-1 and laminin peptides have been adsorbed in order to develop nerve growth conduits. The mechanical property, biocompatibility and efficacy of these tubes for nerve regeneration were examined. Step-1: bridge grafting (15mm) into the sciatic nerve of SD rats was carried out using either t-chitosan or t-chitosan/HAp tubes having either a circular or triangular cross-section (N=12 in each group). Specimens were taken after 2, 4, 6 and 8 weeks post-implantation (N=3 in each group) for histology determinations. Step-2: t-chitosan/HAp tubes having a triangular cross-section with adsorbed laminin-1, CDPG**YIGSR** or CSRARKQAAS**IKVAV**SAD, as well as control tubes without pre-adsorption were used for implantation (N=18 in each group). Isografting was also carried out (N=6). Histological evaluation was carried out similarly as in Step-1. Furthermore, evoked muscle and sensory nerve action potentials were recorded, and the percentage of myelinated axon area measured at 10mm distance of the distal anastomosed site in the experimental, control and isograft groups after 12 weeks (N=6 in each group). Although histological regeneration in both the YIGSR and laminin-1 treated t-chitosan/HAp tubes matches the isografts, the functional recovery is however delayed.

Key words: chitosan tube; nerve scaffold; laminin-1; laminin peptide; nerve regeneration.

1. INTRODUCTION

Laminin-1, a major component of basement membranes has multiple biological activities. Synthetic peptides from the active region of laminin-1 are found to promote cell adhesion, spreading, migration, and neurite outgrowth in vitro, i.e. (i) **IKVAV** near the carboxyl globule on the longer site of the alpha1 chain, and (ii) a pentapeptide **YIGSR** derived from the beta1 chain of laminin-1 being a minimum recognition sequence for a 67-kDa receptor. Tendon chitosan tubes onto which laminin-1 and laminin peptides are adsorbed have been developed for nerve conduit [1, 2]. In this study, the mechanical property, biocompatibility and efficacy as a nerve conduit of the hydroxy apatite-coated and laminin peptides adsorbed tubes were examined.

2. MATERIALS AND METHODS

The crab's tendons were treated in a 4 wt% NaOH aqueous solution at 100 °C for 4 h. Subsequently, the samples were treated in ethanol of 95 wt% at 95 °C

for 8 h. Furthermore, the samples were deacetylated in a 50 wt% NaOH aqueous solution at 100 °C for 8 h under a nitrogen atmosphere. This deacetylation process was repeated 3 times. The samples obtained were rinsed repeatedly with distilled water to remove any excess NaOH, and called "tendon chitosan". The FT-IR spectra of tendon chitosan showed that the absorption bands of the amide I and amide II groups decreased with the deacetylation treatment, while the amino group increased correspondingly, and both amide groups could not be detected at all after 5 times deacetylation treatment [1]. This means that the chitin changed perfectly to chitosan by the deacetyl reaction.

Hydroxy-apatite binding to t-chitosan was carried out following an alternate soaking method. The tubes were then thermally annealed in the triangular shape. The HAp-coated chitosan tubes having a triangular cross section were immersed in either laminin-1, YIGSR or IKVAV solution, in order to adsorb these peptides on the surface. The relationship between the strain and force where collapsing of the sample occurred was measured.

Experiment 1: The right sciatic nerve of SD rats was exposed and bridge grafting of 15 mm length into the nerve gap was then carried out. Circular and triangular tubes, as well as circular tube/HAp and triangular tube/HAp, were used for the implantations. Three rats in each experimental group were sacrificed after respectively 2, 4, 6 and 8 weeks of implantation. Specimens were taken from the middle 1/3 part of the grafted tube in each group for a transverse section. The surface area of the inner tube and regenerated tissue in the t-tube and t-tube/HAp was measured to compare the degree of collapse and effect of stenosis on the regenerating nerve tissue.

Experiment 2: Bridge grafting of 15 mm length into the right sciatic nerve of SD rats was carried out using chitosan tubes prepared as below. To compare the positive effect on nerve regeneration, the t-chitosan/HAp tubes having a triangular cross-section were adsorbed with laminin-1, **YIGSR** or **IKVAV**, as well as the t-chitosan/HAp tubes without pre-adsorption were used for the implantation. Isografts of 15 mm length were also used in six rats. Three rats in each experimental group were sacrificed after respectively 2, 4, 6 and 8 weeks of implantation. Specimens were taken from the middle 1/3 part of the grafted tube in each group for a transverse section, and the proximal 1/3 part for a longitudinal section. The surface area of occupied regenerating tissue or the length of the region where regenerating tissue attached to the inner surface of the tube was measured to compare the efficacy of laminin-1 and laminin peptides for nerve tissue extension by using the transverse section.

Using six rats from each group, electro-physiological evaluations were carried out 12 weeks after implantation. To evaluate the motor functional recovery, the terminal latency quotient between the implanted and control side of the same rat was calculated. After recording of M-waves, specimens were harvested from the grafted tubes for histological examination. Furthermore, the nerves at 10 mm distance from the distal anastomosed site of the tube were harvested, and the percentage axon area was calculated.

3. RESULTS

The force was shown to be significantly higher in the triangular tube/HAp group when compared to the circular or triangular tube group. Furthermore, the force at collapsing in the circular tube/HAp group was higher when compared to the circular or triangular tube group.

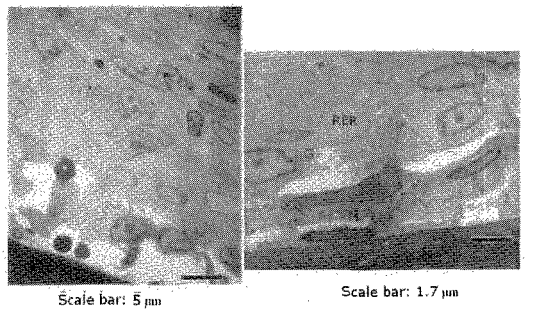
Experiment 1: The surface area of inner tube was

preserved in both the circular and triangular tube/HAp groups when comparing the initial weeks and beyond 6 weeks, but increased in the triangular tube group after six weeks. The area of regenerated tissue comprises of regenerated nerve tissue, newly formed vessels and fibrous layers encircling them. This area of regenerated tissue increased with time in all experimental groups, although in the circular tube group the increase is very small after 6-8 weeks. And the area of regenerated tissue in the tubes having a triangular configuration and a HAp-coating was significantly larger.

Experiment 2: Reactive inflammatory cell infiltration occurred during the initial 2-4 weeks after implantation. This tissue response decreased rapidly after 4 weeks, and instead regeneration of nerve tissue could be observed in the middle part of the tube after 6 weeks. TEM images of t-chitosan/HAp showed HAp nano-crystals scattered abundantly on the inner t-chitosan tube surface, with HAp aggregates aligning with the chitosan fibers. Macrophages attached to the chitosan tube surface phagocytized chitosan degraded parts containing nano-particles of HAp that may be a suitable size for phagocytosis by macrophages. Although chitosan tubes were biodegraded, including the loss of the triangular structure with time, scar tissue formation was not observed inside the tube.

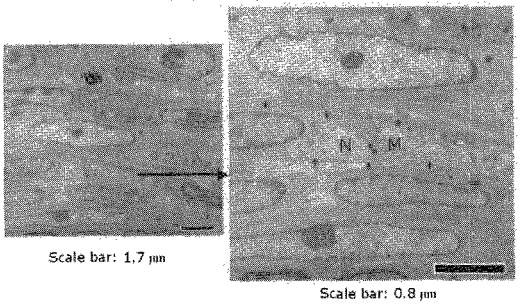
The regenerated nerve tissue elongated from the proximal end of the amputated nerve along the tube in the longitudinal section, being attached directly onto the inner tube surface of both the laminin and laminin peptide groups. The spindle cells lining along the inner surface of the t-chitosan/HAp tube possessed abundant organelles such as developed mitochondria (M), rough endoplasmic reticulum (RER), and Golgi apparatus (G) suggesting to be activated fibroblasts (Fig. 1).

Fig.1. TEM Images of the Proximal 1/3 Part of Grafted Tubes in the Laminin Group, p.o.4w (Longitudinal Section)



Among the cells having an oval nucleus located a more inside the tube and being separated by a thin layer of activated fibroblasts, both minor myelinated (M) and non-myelinated axons (N) were observed. Since some of these spindle cells aligned along the inner tube surface thereby folding regenerated axons elongating slender cytoplasm with basement membrane (asterisk), therefore suggesting to be Schwann cells associated with regenerated axons (Fig. 2).

Fig. 2. TEM Images of the Proximal 1/3 Part of Grafted Tubes in the Laminin Group, p.o.4w (Longitudinal Section)



Though the tube wall became fragmented with time, laminin-1 bound to the surface of t-chitosan/HAp tubes was stained positively with anti-laminin antibody at the tube surface remaining intact until 12 weeks after operation.

The length of the region where regenerated tissue attached to the inner surface of the tube in the laminin and both laminin peptide groups was large compared to the control after 2-4 weeks. It increased rapidly after 6 weeks in the control, though, and no significant difference in all groups was observed after 6-8 weeks. These results suggest that migration of Schwann cells

and bridging of the regenerated axons inside the tube occur more rapidly in the laminin-1 and laminin peptide coated tubes than in the control. Although the area of regenerated nerve tissue remained small in all groups 2-4 weeks post-implantation, a rapid increase in the laminin and both laminin peptide groups until 6-8 weeks was obtained. Only a small increase in regenerated tissue area in the control group was observed. These results suggest that nerve tissue growth inside the tube occurs more rapidly after accomplishment of bridging through the tube by the regenerated tissue in the laminin and laminin peptide groups than in the control group.

M-waves were recorded in all six rats in each experimental, control and isograft group. The terminal latency quotient in all experimental groups was found higher than in the isografts, and in the control higher than in the YIGSR group (Fig. 3). These results indicate that the functional recovery was fastest in the isografts, followed by the YIGSR, laminin and IKVAV and control group.

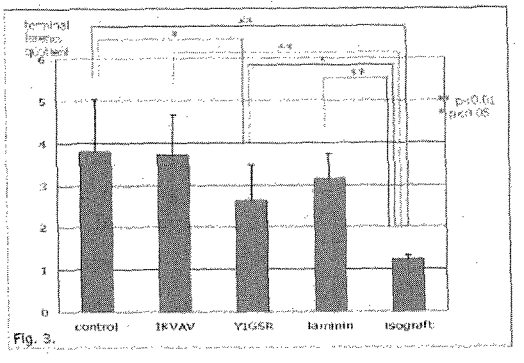


Fig. 3.

The percentage of axon area in the laminin and YIGSR group matched the isografts, and was found larger than in the control (Fig. 4). The percentage of axon area in the IKVAV group tended to be larger than in the control as well. Suggesting that the regeneration process of the nerves passing through the laminin-1-adsorbed and YIGSR-adsorbed tubes, and to a lesser extent followed by the IKVAV-adsorbed tubes, are histological compatible with the isografts.

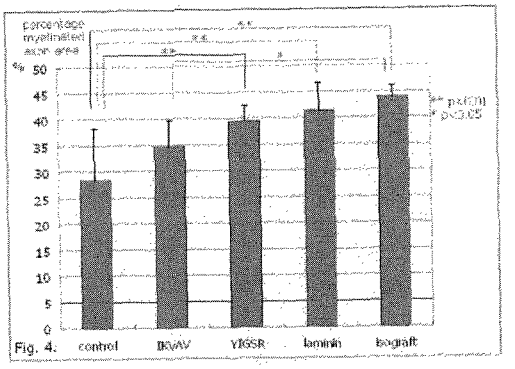


Fig. 4.

DISCUSSION:

The mechanical strength of tubes having a triangular shape is higher than having a circular shape. In addition, body fluids containing humoral factors are able to permeate the tube and even gas exchange may be possible due to minute distances between the chitosan fibers constructing the wall of t-chitosan/HAp tube, as well as the chitosan itself having a water-absorbing property. Because the distance from the wall to the tube's core is shorter in a tube having a triangular cross-sectional shape compared to a circular tube, this may also add to a faster nutrition exchange of regenerating nerve tissue. In addition, HAp on the t-chitosan tubes is able to adsorb laminin-1 and laminin peptides. Therefore, the t-chitosan/HAp tube with a triangular shape is suitable for nerve regeneration.Laminin-1 and laminin peptides adsorbed on the surfaces through hydrophobic interactions being strong enough to cause a 'stable' adsorption to the tube surfaces. Laminin-1 bound to the surface of t-chitosan/HAp tubes was stained positively with anti-laminin antibody until 12 weeks after operation. Furthermore, the area of regenerated tissue in the laminin and both laminin peptide groups increased rapidly until 6-8 weeks, suggesting that not only laminin-1 but also laminin peptides adsorbed on the tube surface enhance nerve tissue growth inside the tube. It is therefore anticipated that both adsorbed laminin-1 and

laminin peptides on the t-chitosan/HAp tube wall will not be desorbed at least until 8 weeks or longer periods after implantation.

In conclusion, the effect of laminin peptides adsorbed on the inner surface of a t-chitosan/HAp tube to enhance growth of regenerating axons has been found to compare with intact laminin-1. Histological regeneration in the YIGSR and laminin-1 treated t-chitosan/HAp tubes compares to isografting; the functional recovery however delays when compared to isografting.

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