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Synthetic collagen fibers coated with a synthetic peptide containing the YIGSR sequence of laminin to promote peripheral nerve regeneration *in vivo*

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The usefulness of collagen fibers and the YIGSR sequence (Tyr-Ile-Gly-Ser-Arg) of laminin for nerve regeneration were examined *in vivo*. Type I collagen gel (G-group), Type I collagen fibers (F-group), Type I collagen fibers coated with laminin (L-group) or the YIGSR sequence (Y-group) were packed into silicone tubes, 15 mm long, and transplanted to the sciatic nerves of Wistar rats. Empty silicone tubes were used as the control. The animals were sacrificed 8 weeks after transplantation. Bridging of the nerve was confirmed in the F-(7/12), Y-(7/10) and L-group (6/10), but no bridging was observed in any of the animals of the G- and control group. Nerve regeneration among the space of collagen fibers was observed, and it was suggested that fibroblasts infiltrated the gap in the substance of the degenerated collagen fibers were followed by Schwann cells on the basis of immunocytochemistry. The number of myelinated axons per regenerated tissue in the tube (density), and total area of myelinated axons per measured regenerated tissue in the tube (% axon area) in each the L- and Y-group were significantly higher than that in the F-group ($P < 0.05$). These results suggest the possibility of obtaining adequate nerve regeneration with new artificial materials only.

1. Introduction

When end-to-end anastomosis of severed nerves is difficult, nerve grafts are necessary to repair them. Autogenous nerve grafts are clinically the most reliable grafts. However, although the grafts are usually taken from nerves whose loss of function will result in only minimal disability, amputation neuroma and paraesthesia of the donor site are troublesome, and thus nerve regeneration with artificial materials is preferred whenever it is possible. A number of tubulization techniques have been attempted to improve results of grafting. Among them, tubes made of polyglycolic acid (PGA) are the only type of tubes shown to serve as a nerve conduit that provide for an adequate nerve regeneration [1]. However, other recent reports showed disappointing results using the PGA fine mesh [2]. Consequently, the development of new materials and techniques for artificial conduits is desirable.

In the case of autogenous nerve grafts the grafted nerve works as a conduit for the regenerating neural tissue. Since Ide *et al*. [3] reported that when nerves subjected to freezing and thawing are grafted, the amputated nerves could sprout regenerating axons into the lumen formed by the basement membrane of Schwann cells, the role of the basement membrane in the regeneration of nerves has received much attention. The basement membrane is the thin extracellular matrix that surrounds all epithelia, adipose cells, and smooth, striated, and cardiac muscles. It is composed of Type IV collagen, laminin, fibronectin and proteoglycans. Among these substances, laminin, a complex trimmer glycoprotein, is the major component of the basement membrane and has a neurite-promoting activity, stimulates Schwann cell mitosis [4–6] and plays a critical role in the regeneration of peripheral nerves through the basement membrane tube [7]. Good results of tubulization using laminin-containing gel [8] or laminin-coated tubes [9] have been reported. However, the technique cannot be adopted for clinical use because the laminin used in these experiments was obtained from the tumorous products of rats [10]. Although laminin is very difficult to synthesize because of its large molecular weight, there have been many reports about the cell adhesion sites in laminin [11], and some of its functional sequences, such as YIGSR, IKVAV (Ile-Lys-Val-Ala-Val) and RGD (Arg-Gly-Asp), have been synthesized [12, 13]. Their activities have been examined only *in vitro*, and no reports about safety for clinical use or effects on nerve regeneration *in vivo* are found. In this study, we examined the usefulness of collagen fibers as scaffolds and the effect of a synthetic laminin peptide on nerve regeneration *in vivo*.

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2. Materials and methods

2.1. Preparation of the grafted materials

Type I collagen from rat tail tendons was prepared by the method described by Christopher *et al*. [14]. All procedures were performed under aseptic conditions in a clean bench. The purified Type I collagen was allowed to gelate in silicone tubes, dehydrogenized by placing it in 70% alcohol and allowing to stand at room temperature overnight, elongated with a weight of 10 g overnight to make a collagen fiber and air dried. Collagen fibers made in this way had a diameter of 100–150 μm, and were cut into pieces 15 mm long. Eight collagen fibers were pulled into a silicone tube (15 mm long, 2.5 mm outer diameter and 1.5 mm internal diameter): this was the F-tube. Type I collagen gel (Cell matrix Type I -A collagen gel, Nitta gelatin, Osaka, Japan) -packed silicone tubes were also prepared (G-tube). Collagen fibers coated with laminin or a synthetic peptide containing the YIGSR sequence (Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH_2) from the cysteine-rich region of the B1 chain short arm of laminin (Sigma, St. Louis, MO) were prepared as follows. Calcium and magnesium free (CMF) Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride at 1 : 10 dilution (Gibco, Gaithersburg, MD) and HEPES buffer (0.08 N NaOH + HEPES 4.77 g/100 ml) were added to a 0.3% Type I collagen gel (Nitta gelatin) at a 1 : 1 : 8 ratio. Laminin (mouse laminin, BTI, Stoughton, MA) was added to this collagen solution (laminin 50 μ g ml^{-1}), into which the collagen fibers were dipped (laminin-coated fiber). EDAC (1-ethyl-3-(3-dimethylaminoprophyl)carbo-diimide) (Sigma) was added to the prepared collagen solution at a 1 : 2 ratio (EDAC 40 μ g ml^{-1}). The YIGSR sequence was added to the collagen solution (YIGSR 100 μ g ml^{-1}), and then the collagen fibers were dipped for coating with this solution (YIGSR-coated fiber). Laminin- or YIGSR-coated fibers were dried at room temperature in a clean bench, and eight of each fibers were pulled into silicone tubes 15 mm long; these were the L- and Y-tubes.

2.2. Experimental methods

Male Wistar rats weighing 200–250 g were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1} body weight). The right sciatic nerve was exposed and a section 10 mm in length was excised at the center of the thigh. Bridge grafting was then performed with 8–0 monofilament nylon along 13 mm using the G- (G-group), F- (F-group), L- (L-group) or Y-tube (Y-group) under a surgical microscope (Fig. 1). An empty silicone tube, 15 mm long, was grafted in the same manner as the control. Twelve rats in each the G- and F-group, and 10 rats in each the L-, Y-, and control group were operated on, and were sacrificed by intraperitoneal injection of a high dose of sodium pentobarbital, 8 weeks after transplantation.

The specimens were fixed first in 2.5% glutaraldehyde, and then in 2.0% osmium tetroxide; thereafter they were embedded in Epon 812 in the standard manner. Thin sections were prepared and stained with toluidine blue for light microscopy. Ultrathin sections were double-stained in uranyl acetate and lead citrate for transmission

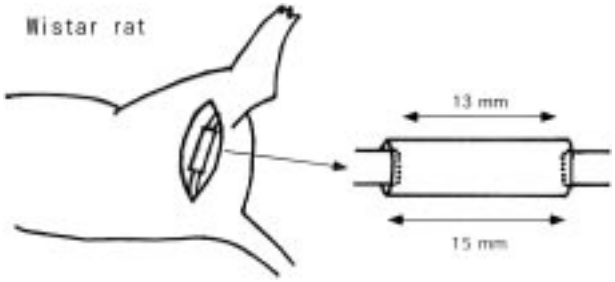


Figure 1 Experimental method. The silicone tubes were prepared (15 mm long, 1.5 mm internal diameter) and bridge grafting was performed using these tubes. F-tube: eight collagen fibers were pulled into a silicone tube. G-tube: type I collagen gel was packed into a silicone tube. L-tube: eight collagen fibers coated with laminin were pulled into a silicone tube. Y-tube: eight collagen fibers coated with the YIGSR sequence were pulled into a silicone tube. Empty silicone tube; control group.

electron microscopy (Hitachi H-600; Hitachi, Tokyo, Japan). In addition, light microphotographs of the thin sections were taken at a magnification of × 100 and enlarged to × 800 to measure the diameter of myelinated axons, the number of myelinated axons per regenerated tissue in the tube (density), and total area of myelinated axons per measured regenerated tissue in the tube (% axon area) (Fig. 2). Analysis was performed on a Macintosh model computer using the public domain NIH Image program (written by Wayne Rasband at the US. National Institute of Health and available from the Internet by anonymous ftp from Zippy.Nimh.Nih.Gov. or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, USA, part number PB93-504868). The differences between the experimental groups were determined for statistical significance by means of one-way analysis of variance (one-way ANOVA) and assessed by Fisher's protected least significant difference. The level of significance was $P < 0.05$.

The specimens used for immunocytochemical analysis were fixed in periodate-lysine-paraformaldehyde (PLP, pH 6.2) at 4 ℃ overnight, and then washed with PBS containing 10, 15 and 20% sucrose, each for 4 h at 4 ℃. The specimens were mounted using OCT embedding compound (Miles Scientific, Elkhart, IN) on a piece of cork and frozen in liquid nitrogen. Serial sections, 6 μm

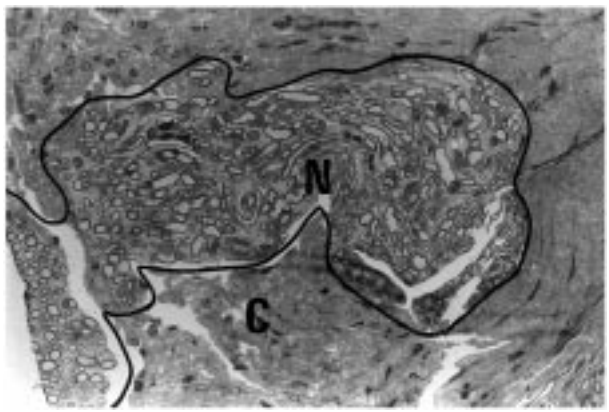


Figure 2 Analysis of the myelinated axons. Light micrographs of the grafted tube at the central part (F-group, toluidine blue, × 500). C: degenerated collagen fibers, N: regenerated neural tissue. Density: the number of myelinated axons per regenerated tissue in the tube, % axon area: total area of myelinated axons per measured regenerated tissue in the tube.

thick, were cut on a cryostat, air dried and stored at − 20 ℃ for a few weeks. Then, after being air-dried at room temperature for 15 min, the sections were stained by the indirect immunoperoxidase method using a rabbit monoclonal antilaminin antibody (Chemicon International, Inc., Temecula, CA) at a 1 : 50 or 1 : 100 dilution, rabbit anti-S-100 protein antibody (Chemicon International) at a 1 : 100 or 1 : 200 dilution, and rabbit monoclonal anti-GFAP antibody (Chemicon International) at a 1 : 50 or 1 : 100 dilution. Preparations were incubated with each antibody separately for 120 min at room temperature. Rabbit non-immune serum was used for the control. The sections were then incubated with biotinylated goat antirabbit antibody followed by streptavidin–peroxidase complex, fixed in 2.0% glutaraldehyde for 5 min at room temperature and the peroxides reaction was developed by diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA). They were treated with 1% OsO_4 for 90 min at room temperature, then dehydrated and embedded in Epon 812. Ultrathin sections for transmission electron microscopy were also prepared.

3. Results

3.1. Evaluation of the nerve fiber regrowth

One animal in each of the L- and Y-group became infected, and were therefore excluded from the final evaluation. Bridging of the nerve was confirmed in seven animals in each of the F- and Y-group and in six animals in the L-group, but no bridging was observed in any of the animals of the G- and control group. Specimens, 5 mm long, were harvested from the center of the graft; six specimens in each the F- and Y-group and five specimens in the L-group were used for light and electron microscopic observation, and the other specimens from each group were used for immunocytochemistry.

Under light microscopy, newly formed minifascicles were observed among the degenerating collagen fibers, and 6–10 layers of epineurium-like tissue were seen in contact with the inner surface of the silicone tube (Fig. 3). The neural tissue was usually found associated with newly formed vessels. Destruction of the collagen fibers had progressed in the periphery and a gap was formed in the substance of these degenerated fibers, into which regenerating neural tissues had penetrated. Many cells with a spindle-shaped or round nucleus had invaded in the gap formed in the substance of the degenerated collagen fibers from the periphery. However, the central portion of the collagen fibers remained hypocellular. There was no difference regarding the regenerating patterns of the neural tissue among the experimental groups.

Under electron microscopy, Schwann cells as well as many unmyelinated and myelinated axons were found among the degenerated collagen fibers (Fig. 4a). Fibroblasts were stretching out their slender cytoplasm to form several layers of septa among regenerating nerve tissues and fine collagen fibrils in each group (Fig. 4b). Minifascicles had been formed in the open space. Macrophages, which had many lysosomes and pinosomes containing debris of the phagocytized collagen fibers, were observed among the cells infiltrating into the disrupted collagen fibers. But they were few in number, especially at the central part of collagen fibers.

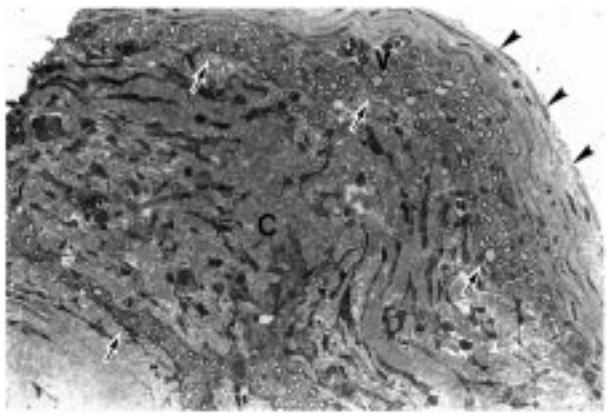
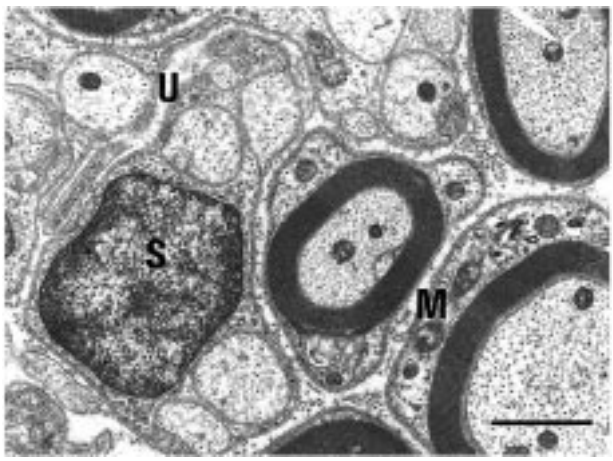
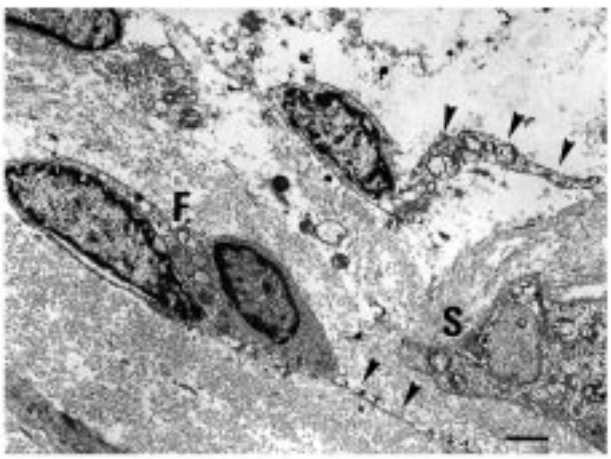


Figure 3 Light micrographs of the regenerating nerve (Y-group, toluidine blue, × 500). Minifascicle formation (arrow) in the spaces that exist among the degenerating collagen fibers (C). Six to 10 layers of epineurium-like tissue (arrow head) are seen. Neural tissues are usually associated with newly formed vessels (V). Destruction of the collagen fibers has progressed in the periphery. Many cells with a spindle-like or round nucleus have invaded in the gap formed in the substance of the degenerated collagen fibers.



(a)



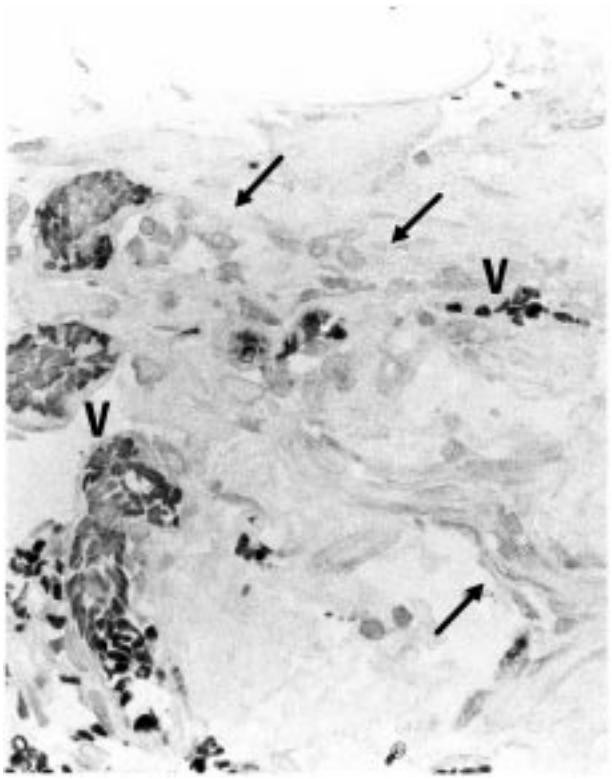
(b)

Figure 4 Electron micrographs of the regenerating nerve (Y-group, scale bar: 1 μm). (a) A Schwann cell with basement membrane (S) is holding many unmyelinated (U) and myelinated axons (M). (b) Fibroblasts (F) are stretching out their slender cytoplasm (arrow head) to form the septum among fine collagen fibrils. S: Schwann cell with unmyelinated axons.

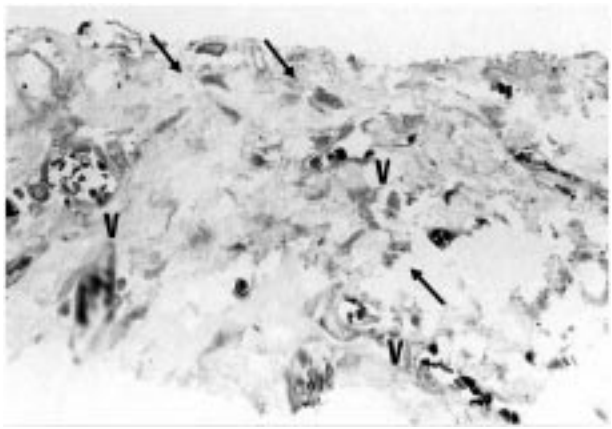
To identify the types of infiltrating cells in the gap of the degenerated substance of collagen fibers we immunostained tissue preparations with anti-S-100, anti-GFAP and anti-laminin antibodies.

Light microscopic observation: most of the cells with a spindle-shaped nucleus were not stained with these antibodies, whereas those with an elliptical nucleus were stained. With anti-S-100 and anti-GFAP antibodies the whole cytoplasm was stained. Many of the positively stained cells were observed around the vessels (Fig. 5a, b). Although the myelin sheath of the regenerated axons in the minifascicles was stained positively with antilaminin antibody, staining was not evident in the cytoplasm of these cells.

Electron microscopic observation: the whole cytoplasm of the cells with an elliptic nucleus was stained sharply with anti-S-100 antibody, but the nucleus was not. These cells had a developed round bulging rough endoplasmic reticulum (RER) and mitochondria (Fig. 6a, b). With anti-GFAP antibody, these cells showed the same staining pattern as that observed with anti-S-100 antibody; that is intermediate filaments and RER were stained. While, although RER was stained with antilaminin antibody, the basement membrane of these cells was not (Fig. 6c). These observations suggest that the cells stained with these antibodies are immature Schwann cells which are actively producing laminin, but have no basement membrane, as yet.

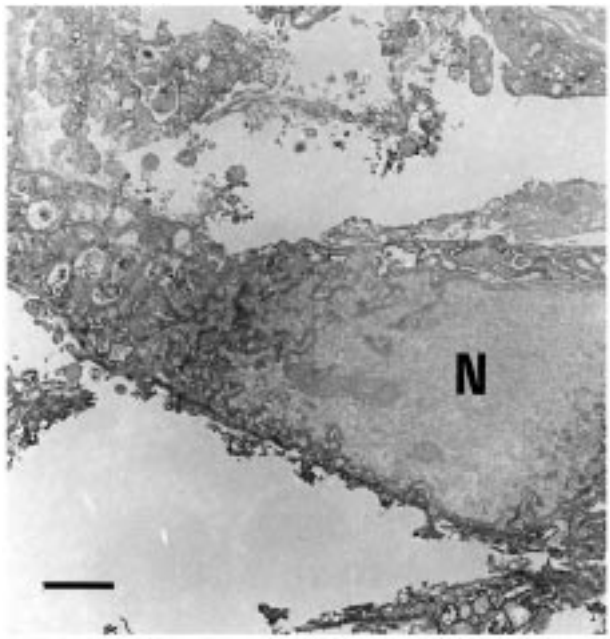


(a)

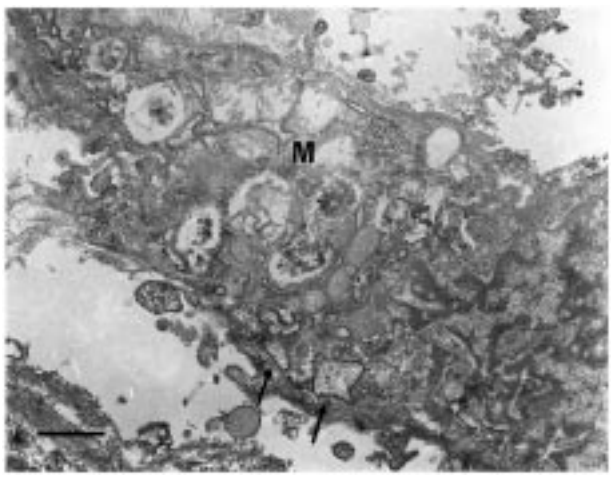


(b)

Figure 5 Immunocytochemistry: (light micrographs of the F-group, × 600). (a) (anti-S-100 antibody) and (b) (anti-GFAP antibody). Many of the positively stained cells with a round or oval nucleus (arrow) are observed around the vessels (V).

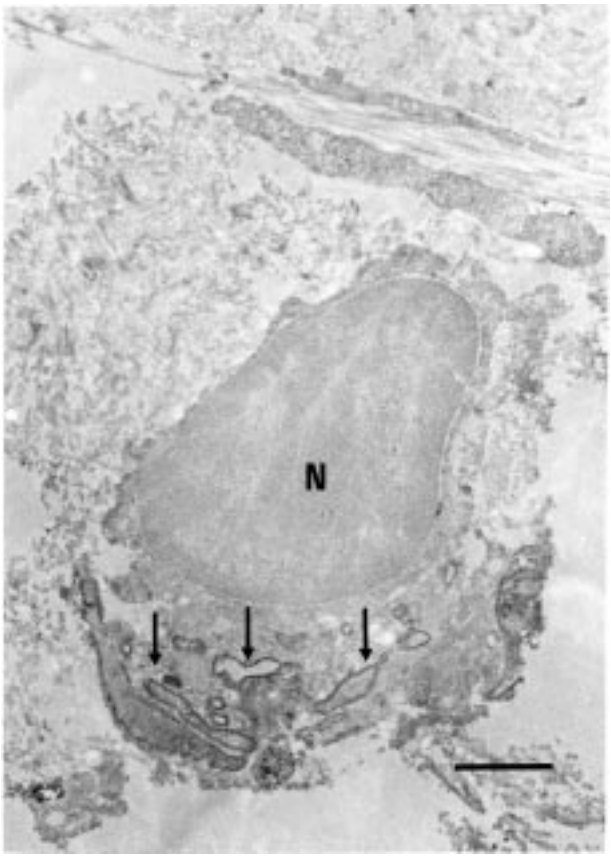


(a)



(b)

Figure 6 Immunocytochemistry: (electron micrographs of the F-group). (a) (anti-S-100 antibody, scale bar: 2 μm). The whole cytoplasm of the cell is stained, but its nucleus (N) is not. (b) (anti-S-100 antibody, scale bar: 1 μm). Magnification of the cytoplasm of the same cell as Fig. 6a. Developed bulging RER (arrow) and mitochondria (M) are observed. (c) (anti-laminin antibody, scale bar: 1 μm). Although RER is stained sharply (arrow), the basement membrane of these cells is not. N: nucleus.



(c)

Figure 6 (Continued)

3.2. Analysis of myelinated axons

Two infected animals, one in each the L- and Y-group, were excluded from the final analysis. Measurements were performed in six rats in each of the F- and Y-group, and in five animals in the L-group because they showed obviously regenerated axons under light microscopy (Fig. 7). There was no significant difference in the diameter of the axons among these experimental groups. The density of axons in each of the L- and Y-group was significantly higher than that in the F-group ($P < 0.05$). The % axon area in each of the L-and Y-group was also significantly higher than that in the F-group ($P < 0.05$).

4. Discussion

Numerous sheathing materials have been tried for tubulization to maximize regeneration and functional recovery after nerve repair. In this study we adopted the silicone tube to avoid the influence of the tube material on the regenerating nerve tissue, and to evaluate effects of Type I collagen and a synthetic laminin peptide on nerve regeneration. As observed in the control group, cells did not attach to the surface of the silicone tubes suggesting their poor affinity for silicone. Even though cultured cells exhibit strong affinity for petri dishes coated with Type I collagen gel, silicone tubes packed with Type I collagen gel did not promote nerve regeneration. On the other hand, Type I collagen fibers did serve as scaffoldings for the regenerating axons. This finding suggests that the growth cones of regenerating axons or Schwann cells from the nerve stumps may need to keep in contact with a hard surface for outgrowth or migration.

The promoting effect of laminin on nerve regeneration has been demonstrated both *in vitro* [6] and *in vivo* [9]. In this study too, laminin coated on the collagen fibers promoted the regeneration of nerve tissue. However, laminin available for research purpose is mainly extracted and purified from the Engelbreth–Holm–Swarm (EHS) tumor, and human type laminin extracted from placenta is available in limited quantities. Furthermore, synthesis of the whole laminin molecule is difficult because its molecular weight is too large (about 900 kD). Therefore, it is difficult to use laminin clinically, and the development of synthetic laminin peptides is inevitable. The RGD sequence (Arg-Gly-Asp) is a versatile cell attachment site for fibronectin and various other proteins, including the basement membrane glycoproteins entactin and laminin. Furthermore, it is known that synthetic peptides containing the RGD sequence are active in mediating cell adhesion, spreading, migration, and neural outgrowth *in vitro* [13]. We can find some trials using the synthesized RGD sequence to develop biomaterials with cell adhesion peptides [15]. *In vivo*, however, regenerating axons do not show affinity for fibronectin but for laminin [16], which suggests that growth cones of regenerating axons or Schwann cells may not recognize the RGD sequence

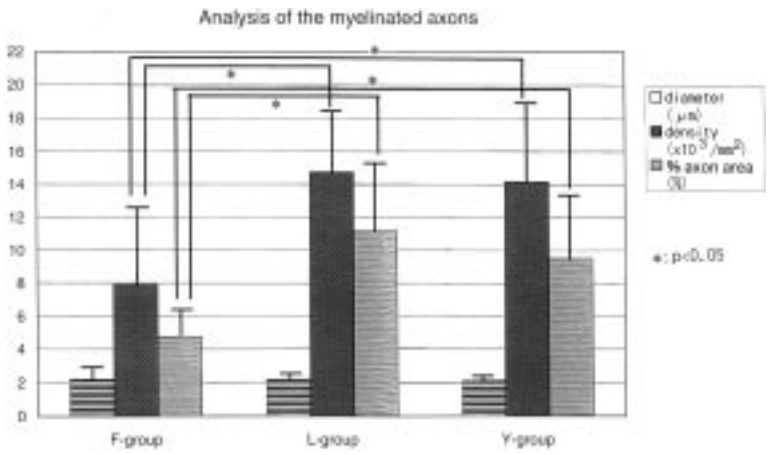


Figure 7 Analysis of the myelinated axons. There is no significant difference in the diameter of the axons among experimental groups. The density of axons in each of the L- and Y-group is significantly higher than that in the F-group ($P < 0.05$). The percentage axon area in each of the L- and Y-group is also significantly higher than that in the F-group ($P < 0.05$).

but attachment sites specific to laminin. The YIGSR [4] and IKVAV sequences [11] which are two of the cell binding domains of laminin, have been synthesized. In this study we employed the YIGSR sequence, which had been found to support cell attachment, 67 kD receptor-binding and migration. Although the previous study reported that the YIGSR sequence promoted neither neural outgrowth nor cell growth *in vitro* [2], we can show its promoting effect on nerve regeneration equal to that of laminin *in vivo*. It is considered that the YIGSR sequence does not affect neural cells directly, but acts on the growth cone or Schwann cells promoting their attachment and migration. Furthermore, because of its lower molecular weight, it is possible to attain a higher molecular density of the YIGSR sequence on the surface of the collagen fibers than that of laminin, and this may further contribute to its effect on nerve regeneration.

In this study we could observe nerve regeneration among the space of collagen fibers. This fact may suggest that although collagen fibers serve as a scaffolding of the growth cone or Schwann cells, if they are swollen by wound fluids they will obstruct further nerve regeneration from both stumps after bridging formation. This problem can be settled by increasing the number of intermolecular crosslinkage, although absorption of the degenerating collagen fibers might be delayed. And further, it may be desirable for getting better results to graft tubes with numerous pores such as the Type I collagen film as conduit instead of silicone tubes; which wall is expected to pass oxygen or be penetrated by vessels from the outer side.

Many cells infiltrated into the gap formed in the substance of the degenerated collagen fibers, in which even some groups of regenerating axons were found scattered. Most of the cells with a spindle-shaped nucleus were not stained with anti-S-100, anti-GFAP or anti-laminin antibody, and appeared to be fibroblasts based on their morphological characteristics. While, the cells with elliptical nuclei, which were fewer in number, were positively stained with these antibodies, suggesting that they were Schwann cells. Based on these histological findings, it is considered that initially fibroblasts infiltrate the gap in the substance of the degenerated collagen fibers, and that they are followed by Schwann cells waiting for elongation of the growth cone. Moreover, because macrophages did not always appear ahead of cellular infiltration and were few in number, the fibroblasts in the gap may play roles in absorption of the degenerated collagen fibers by releasing collagenase and other enzymes [17].

Thus, on the basis of the present findings, it is possible to expect early bridging of an amputated nerve by using the Type I collagen fibers coated with YIGSR sequence as a scaffold, and better results may be obtained by combining it with some other neurotrophic factors.

5. Conclusion

This study has demonstrated that Type I collagen fibers can serve as a scaffolding of the growth cone of the regenerating nerve or Schwann cells, and that the synthetic YIGSR sequence of laminin promotes nerve regeneration *in vivo*. These results suggest nerve regeneration can be attained by using such artificial materials only.

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