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The interaction of Schwann cells with chitosan membranes and fibers in vitro

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Abstract

The bridging of nerve gaps is still one of the major problems in peripheral nerve regeneration. A promising alternative for the repair of peripheral nerve injuries is the bioartificial nerve graft, comprised of a biomaterial pre-seeded with Schwann cells (SCs), which is an effective substrate for enhancing nerve regeneration. Interaction between cultured SCs and biomaterials is of importance. For the purposes of this study, culture systems of normal SCs were used. The biocompatibility of chitosan, including chitosan membranes and chitosan fibers, was evaluated in vitro. The growth of SCs was observed by light and scanning electron microscopy at regular intervals. SCs were identified by immunocytochemical staining and the viability of SCs was measured by MTT assay. The experimental results indicated that SCs could grow onto chitosan materials with two different shapes: spherical and long olivary. They contacted with the extensions. The long olivary cells inclined to encircle chitosan fibers up. It was also found that the cells on the chitosan fibers migrated faster than those on the chitosan membranes. There was a good biological compatibility between chitosan and SCs. Compared with the chitosan membranes, SCs migrated more easily onto the stereoframe of chitosan fibers. These studies contribute information necessary to enhancing our understanding of biocompatibility of chitosan.

Keywords: Schwann cell; Biocompatibility; Chitosan

1. Introduction

A major problem related to the treatment of peripheral nerve injury is the bridging of large gaps between the cut ends of the transected nerve. Three-dimensional materials have recently been used as scaffolds for tissue regeneration to take the place of traditional direct suture, autograft, and allograft techniques, which have some disadvantages, such as the limited availability of donor tissue and morbidity related to its sacrifice. The use of artificial nerve materials that create a favorable environment for nerve regeneration has become a strategy for repairing major nerve defects.

In recent years the possibility of using artificial materials such as fibronectin mats through which axons could regenerate has been explored [1,2]. Another possibility would be filaments composed of poly-glycolic acid [3–5]. Yet despite these advances and contributions in the field of tissue engineering, current results with nerve conduits have failed to equal nerve regeneration equivalent to autogenous grafts for large distances. The failure is proposed to be the result of a combination factors [6–8], such as the inability of neurotrophic factors produced by distal stumps to reach the proximal stump, the lack of a matrix bridge as a supporting structure, and the absence of Schwann Cells (SCs) [9–11]. Nevertheless, an ideal nerve guidance material remains a challenge.

Therefore, there is a need for a new material that can take the place of autografts and naturally degrade in the body [12,13]. This material could repair certain distant nerve injury and provoke very little or no immune rejection by the body. Chitosan is the fully or partially deacetylated form of chitin, which is the second most abundant form of polymerized carbon found in nature [14,15]. Chitosan is one of the most abundant polysaccharides with positive charges found in nature [16]. Natural polysaccharide usually contributes to cellular adhesion and inhibition of scar formation [17–24].

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Because SCs play a crucial role during nerve regeneration through the production of growth factors and the excretion of extracellular matrix [25,26], interests have been directed to introduce cultured SCs into nerve regeneration as a persistent source of neurotrophic factors.

A promising alternative for the repair of peripheral nerve injuries is the bioartificial nerve graft, comprised of biomaterials pre-seeded with SCs, which is an effective substrate for enhancing nerve regeneration. In this paper, we cultured SCs on the chitosan membranes and fibers in vitro. The growth properties of SCs on chitosan membranes and fibers were investigated. The study aimed to find out more about the biocompatibility between chitosan and peripheral nerve system.

2. Materials and methods

2.1. Materials

Chitosan (purchased from Nantong Water Products Institute, Jiangsu, China) was refined twice by dissolving it in dilute acetic acid solution. It was then filtered, precipitated with aqueous NaOH, and finally dried in vacuum at room temperature. The degree of deacetylation was 92.3% [27], and it has less than 1% ash, not more than 10 ppm heavy metal.

The chitosan fibers, 15 μm in diameter, were purchased from Shanghai Gao Chun biomaterial Company (Shanghai, China). All other reagents were of analytical grade and used without further purification.

2.2. Preparation of chitosan membranes

one gram of chitosan was dissolved in 50 ml 1% acetic acid solution and stirred. Then the solution was spread on glass dishes and dried at 50°C in a drying oven. After the membranes had dried, the membranes were immersed in 1% NaOH solution for 24 h to neutralize the acetic acid and washed with distilled water until neutral pH was obtained. The thickness of a dried membrane is 50 μm.

2.3. Preparation of glass coverslips, chitosan membranes and fibers

The round coverslips were coated with polylysine. The chitosan membranes and fibers were washed three times with distilled water and sterilized with routine hyperbaric methods. They were then equilibrated into the L15 basic culture medium (Gibco, Grand Island, NY) for 30 min.The membranes were cut into 8×8 mm size and the fibers were cut into 2 cm length. The chitosan membranes and fibers were then put onto the bare round coverslips embedded in the culture clusters (Costar). The bare round coverslips were uncoated with anything.

2.4. Tissue culture

Six neonatal Sprague–Dawley rats, 1–2 d old, were used as donors. The bilateral sciatic nerves were excised out under sterile condition. The epineurium and perineurium were then stripped off the sciatic nerves carefully. The nerves were dissected into discrete fascicles. They were then washed with L15 basic culture medium and sheared into 0.01 mm^3 nerve tissue fragments. The nerve fragments were planted onto the polylysine coated coverslips, chitosan membranes or chitosan fibers in the six-well tissue culture clusters. The polylysine coated coverslip group was used as a control. The clusters were incubated in L15 medium containing 15% fetal bovine serum and 100 u/ml penicillin and streptomycin, in a 37°C humidified incubator with 5% CO_2. Half of the medium in the clusters was replaced with fresh L15 medium every 2 days. The nerve tissue fragments were incubated for two weeks. All of the experimental procedures involving animals were conducted as per Institutional Animal Care guidelines.

2.5. Cell culture

The same rats were used as the above. The nerve tissue fragments were digested with 0.125% trypsin and 0.03% collagenase (Sigma, St. Louis, MO, USA) at 37°C for 10 min.The cells from the sciatic nerves were diluted to a density of 2×10^4 cells/cm^2. They were then added to 24-well tissue culture clusters embedded with bare coverslips, polylysine coated coverslips, chitosan membranes and chitosan fibers. The bare coverslip and polylysine coated coverslip groups were used as controls. The clusters were incubated in L15 medium containing 15% fetal bovine serum and 100 u/ml penicillin and streptomycin, in a 37°C humidified incubator with 5% CO_2. Half of the culture medium was replaced every 2 days.

2.6. Light microscopy

The growth of SCs on materials was observed under a inverted microscope (Olympus, Tokyo, Japan) every day. Photographs were taken at regular intervals.

2.7. Scanning electron microscopy

The arrangement of SCs on the chitosan membranes and fibers were studied using a scanning electron microscope (SEM). After 7 d incubation, the samples were washed with L15 basic culture medium and fixed in 4% glutaraldehyde solution. The samples were then postfixed with 1% OsO_4, dehydrated in graded acetone

and dried at critical point drier (Hitachi, Tokyo, Japan). After this, the samples were coated with gold in a JFC-1100 unit (Jeol Inc., Japan) and observed under a SEM (JEM-T300, Jeol Inc., Japan).

2.8. Immunocytochemistry

After 7 d incubation, the samples were washed three times with L15 basic culture medium and fixed with a fresh-prepared 4% paraformaldehyde solution in 0.01 M PBS (pH 7.2) for 30 min at room temperature. The fixed samples were incubated for 30 min in 1% goat serum in PBS at room temperature to block nonspecific binding. Then, they were incubated with mouse anti-S-100 antibody (1:800 dilution, Sigma) overnight at 4°C in a humidified chamber. After washed three times with PBS, the samples were labeled with alkaline phosphatase-labeled goat anti-mouse IgG (1:800 dilution, Sigma) for 4 h at room temperature. The samples were washed three times with PBS and then developed using NBT/BCIP as a substrate. Controls for the immunostaining included leaving out the primary antibody and using non-labeled secondary antibodies to confirm that non-specific binding was not present.

2.9. MTT assay

A modified 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide MTT assay, in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in cells, was used to assess viability of cells. After 1, 3, 7, 10 and 14 d incubation, the viability of every groups was assessed. Briefly, the cells were washed three times with L15 basic culture medium carefully. Then the culture medium of every wells were replaced with 100 μl L15 basic medium. 25 μl MTT in PBS (0.1 M, pH 7.2) was added to each well of 24-well plates (1 μg/ml final concentration). After 4 h incubation at 37°C, the reaction was stopped by adding 100 μl lysis buffer (20% SDS in 50% N,N-dimethylformamide, pH 4.7). After 20 h incubation at 37°C, the supernatant of all wells were respectively aspirated out to be measured photometrically at 570 nm (OD_{570}) with an ElX-800 Microelisa reader (Bio-Tek Inc., USA). A one-way ANOVA was used to compare the means of different groups, and statistical significance was accepted at the 0.05 confidence level.

3. Results

3.1. Light microscopy

Observations with the inverted microscope showed that after 24 h incubation, SCs emerged from the explanted nerve tissue fragments and migrated onto the polylysine coated coverslips, chitosan membranes and chitosan fibers, superior to those on the bare coverslips. After 7 d incubation, a large number of cells migrated onto the materials and the nerve tissue fragments became smaller. The adherent SCs connected to each other and extended along the chitosan materials. SCs were observed to grow in multilayer fashion on chitosan fibers and grew between the neighboring fibers. The migrating cells had two shapes: spherical and long olivary. The spherical cells lined in a string of beads and the long olivary cells contacted end to end. For the polylysine coated coverslip (Fig. 1) and chitosan membrane (Fig. 2), the cells took on radiating vortex end to end arrangement. The cells on the chitosan fibers migrated spiralling along the fibers end to end and the long olivary cells encircled the fibers in three-dimensional fashion (Fig. 3). After 14 d, we observed that in comparison with the adherence of SCs onto chitosan membranes, SCs on chitosan fibers were more densely distributed and longitudinally arranged, forming cell chains (Fig. 4).

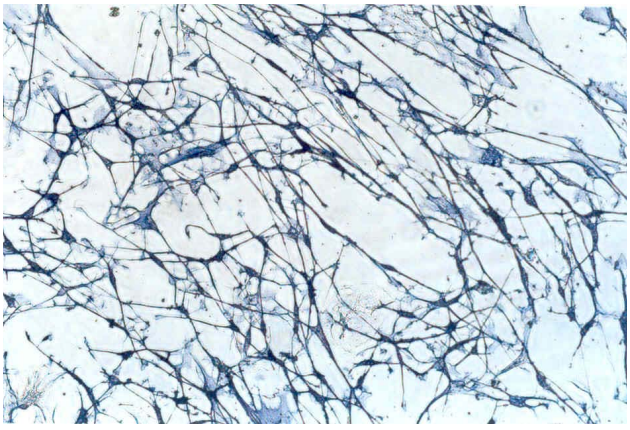

Fig 1. Schwann cells cultured on the coverslip for 7 d were stained for S-100 (Light micrograph, × 60).

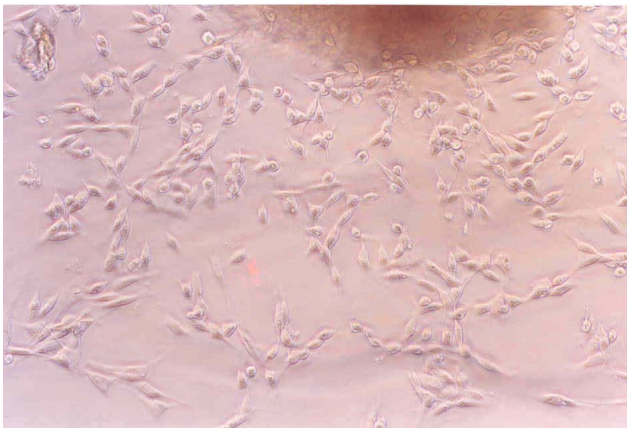

Fig 2. Schwann cells cultured on the chitosan membrane for 7 d (Light micrograph, × 60).

Fig. 3. Schwann cells cultured on the chitosan fiber for 7 d (Light micrograph, × 60).

Fig 4. Schwann cells cultured on the chitosan fiber for 14 d, forming cellular chain (Light micrograph, × 60).

3.2. Scanning electron microscopy

The SCs migrating onto the chitosan membranes and fibers had microvilli on their surface in abundance. The spherical cells interacted with the chitosan membrane and fiber by targeting small surfaces, whereas, the olivary cells interacted at large surfaces. SCs reached the minute foot plates to attach the membranes and fibers tightly (Figs. 5 and 6).

3.3. Immunocytochemistry

From the results of immunochemistry S-100 staining, it was evident that the major cells migrating from the nerve tissues were S-100-positive. Not only the spherical cells but also the olivary cells were stained deep blue and their nucleuses were unstained (Figs. 1 and 7). Histologically, there were no obvious differences between the chitosan membrane group and the fiber group.

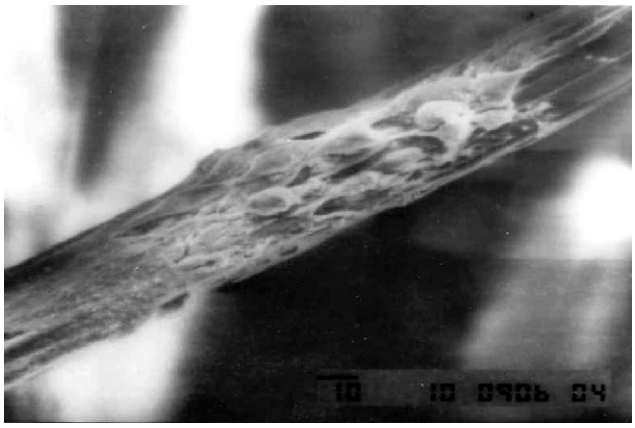

Fig 5. SEM of Schwann cells cultured on the chitosan fiber for 7 d, exhibiting a round cell body with abundant microvilli (Bar = 10 μm).

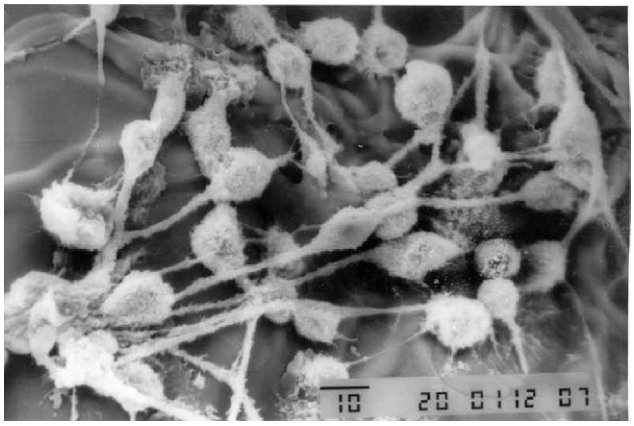

Fig 6. SEM of Schwann cells cultured on the chitosan membrane for 7 d, exhibiting a round cell body with abundant microvilli (Bar = 10 μm).

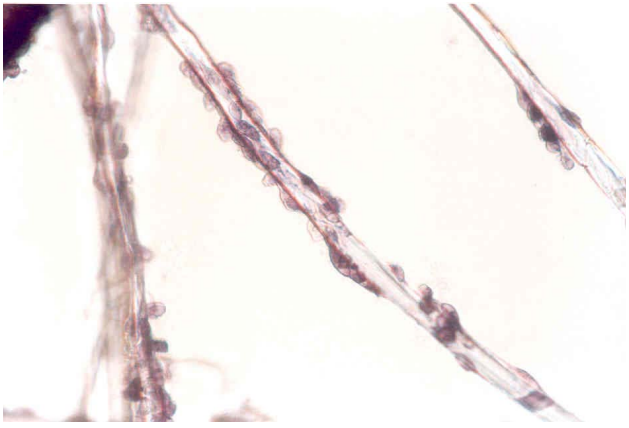

Fig 7. Schwann cells cultured on the chitosan fiber for 7 d were stained for S-100 (Light micrograph, × 30).

3.4. MTT assay

After SCs were cultured 1, 3, 7, 10 and 14 d, MTT assay was performed and the results indicated that there

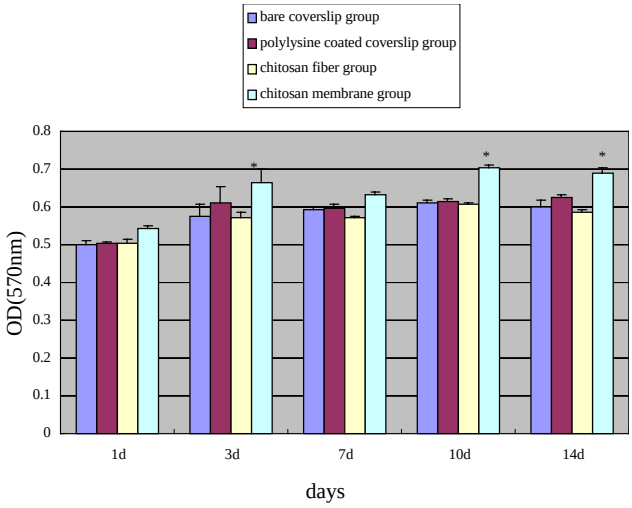

Fig. 8. Effects of the coverslip and chitosan materials on viabilities of the SCs cultured after 1, 3, 7, 10 and 14 d, $n=6$. Errors bars represent means $\pm$ SE. $^{*}P<0.01$ vs coverslip group.

was no significance on SCs viability among bare coverslips, polylysine coated coverslips and chitosan fibers. There was a significance on SCs viability between chitosan membrane group and bare coverslip group after 3, 7 and 14 d. The SCs viability of every chitosan membrane group was found to be a little higher than that of the corresponding groups (Fig. 8).

4. Discussion

Among various biomaterials, chitosan becomes one of the most widely studied polymers. There have been no reports comparing chitosan membranes with chitosan fibers. This study uses several tests to investigate the biocompatibility of chitosan further. In a quantitative measure of cell viability (MTT) of this study, which is chiefly chosen method of the cytotoxic test in vitro on evaluation of biocompatibility of biomaterials at present, the results on the cultured SCs, a kind of normal neuroglial cell in the peripheral nerve system, explain whether chitosan membranes or fibers is cytotoxic to peripheral nerve tissue or not, so that it affords a promising application of chitosan to peripheral nerve reconstruction in future. The results showed that chitosan membranes or fibers have almost no cytotoxic on SCs growth. When suspensions of dissociated SCs were applied onto the chitosan materials, the flat chitosan membranes may provide more surface area for the settlement of SCs than the thin chitosan fibers. The SCs viability was comparatively higher for chitosan membranes compared to chitosan fibers and polylysine coated coverslips.

In order to examine the biocompatibility between nerve tissues and chitosan membranes or fibers, it is important to co-culture the nerve tissues with chitosan. We cultured the normal mammal nerve tissues on chitosan membranes and fibers. In vitro experiments showed that chitosan membranes and fibers were also useful for growth of nerve cells. When the nerve tissues were co-cultured with chitosan, SCs showed normal adhesion, survival, migration and proliferation. When peripheral nerve is injured, SCs will proliferate, forming cell bridge at the injured position [28,29]. In this experiment, the major finding of the present work is the better encircling growth with chitosan fibers than chitosan membranes. Although it imitates only in part the effects of Bunger bands, in which SCs columns were formed to guide axonal growth throughout the process of nerve regeneration [30,31], it offers some advantages. The remodeling of chitosan fibers that resembled precise anatomical nerve shape should play a key role to facilitate SCs migration and axonal growth in future transplantation [32]. This study indicated the potential of the chitosan pre-seeded with SCs to guide and direct regenerating axons.

In this experiment, SCs on the chitosan had two shapes: spherical and olivary. The olivary cells could encircle the fibers. After 14 d, it was observed that overlapping SCs form bungner to encircle the chitosan fibers and SCs attached to the fibers migrated faster than those attached to membranes. The two possibilities of the different shapes of SCs are as follows: The SCs of two different shapes may be the same variety of cells, yet behave differently and have respective functions, such as the olivary cells might have a role in the forming of myelin sheath. The second possibility is that the olivary cells attached to the chitosan fibers could be due to the surface topography of the fibers, which can affect the cell shape. Detailed explanation needs further study.

It is well known that SCs play a pivotal role in the regeneration of the peripheral nervous system. They can secrete bioactive molecules such as nerve growth factor, brain-derived neurotrophic factor, laminin, fibronectin and collagen IV among others. Chitosan is very hydrophilic and can take advantage of its expansion with water and poly-porous to construct artificial extracellular matrix suitable for the growth of neural cells. The molecular structure of chitosan is similar to that of glycosaminoglycan, which resides in the basal membrane and extracellular matrix. It is suitable for the cohesion of chitosan and extracellular adhesive molecular, such as laminin, fibronectin and collagen IV, which promote cells to adhere, migrate and differentiate [33].These factors provide a good microenvironment for nerve to regenerate.

5. Conclusion

In summary, chitosan membranes and fibers have excellent neuroglial cell affinity. They are promising

materials for nerve repair. They could be improved by incorporation with neurotrophic factors and neural cells.

Acknowledgements

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