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Survival and Differentiation of Neuroepithelial Stem Cells on Chitosan Bicomponent Fibers

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Abstract

Chitosan is a popular biomaterial used in tissue engineering. Fibers of chitosan could provide a favorable anatomical substrate for cell growth which provides a promising application for axonal regeneration during peripheral injury. Neuroepithelial stem cells (NEPs) are the most primitive neural stem cells with multipotential for neuronal and glia differentiation. To assess the biocompatibility between NEPs and chitosan fibers, and to explore whether the NEPs have the ability to differentiation on chitosan fibers, NEPs were harvested from the neural tube and seeded on chitosan fibers in *in vitro* culture. The biocompatibility of chitosan fibers was tested by MTT assays. The growth and survival were observed by light and scanning electronic microscope at different times in culture. And, the differentiation of NEPs was examined by immunocytochemical staining. The results indicated that NEPs could grow on the chitosan fibers and attach firmly to the surface of fibers. On chitosan fibers, NEPs could differentiate into neurons and glia. Our study demonstrated that chitasan fibers had a good biocompatibility with NEPs which affords a potential alternative for the repair of peripheral nerve injury.

Key Words: neuroepithelial stem cells, biocompatibility, differentiation, chitosan fiber

Introduction

In recent years, more patients have suffered from peripheral nerve injuries including acute trauma and nerve paralysis. Many recent studies have focused on the repair of peripheral nerve injury and have achieved great progresses (3, 5). Clinical nerve repair prefers microsurgery using autografts. However, nerve autografting is limited by the availability of nerve grafts and the permanent dysfunction at the site of graft harvest (7). Currently, a cell-based tissue engineering technique has been proved to be one of the most promising alternative therapies for nerve repair. This approach consists of an interactive triad of responsive seed cells, scaffold materials and bioactive molecules promoting regeneration (24).

In tissue engineering, the three-dimensional (3D) have frequently been used as scaffold materials which also influence cell migration, proliferation and differentiation through cell-cell and cell-substrate interactions (9). To mimic this 3D growth environment (24), a lot of biomaterials have been studied such as collagen, poly-glycolic acid (PGA), polylactic acid (PLA) and chitosan (2). Among these materials,

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chitosan is an abundantly common and naturally occurring polysaccharide biopolymer with positive charges (20). This natural polysaccharide usually contributes to cellular adhesion. Due to many advantages such as porosity, biodegradability and excellent biocompatibility, chitosan has been employed in a variety of applications in the biomedical field such as bone reconstruction (12), cell encapsulation (13), drug delivery (6) and nerve regeneration (8). Chitosan fibers remolded from chitosan were employed in this experiment.

Recent studies showed that many cells could attach chitosan, including marrow stroma cells, Schwann cells (SCs) and neural stem cells (1, 4, 10). These cells were also considered as alternative seed cells for tissue engineering. The Schwann cell is probably the best candidate due to its special role in peripheral regeneration. However, two times of surgery are needed for autograft of SCs and allograft of SCs may cause immunological rejection in the host (17). As for marrow stroma cells, they need to trans-differentiation into cells in the nervous system. Neural stem cells, which possess the potential to differentiate into neuron, glia and Schwann-like cells, were a better probable cell source for tissue engineering. Previously, Zahir and his colleagues (23) reported that chitosan coated with neural stem cells derived from subependyma of lateral ventricles had repaired spinal cord injury. More recently, Mothe reported that neural stem cells had differentiated into Schwann-like cells and generated peripheral myelin (11). Thus, the neural stem cells may have a great potential in treating damaged nerves. Neuroepithelial stem cells (NEPs) are multipotent neural stem cells originating from the embryonic neural plate or neural tube during early development. These cells demonstrate the ability to self-renew and are capable of differentiating into neurons and glia (19). Up to now, survival and differentiation of NEPs on chitosan fibers are unclear.

To determine the biocompatibility between NEPs and chitosan fibers, and to explore whether NEPs have the ability to differentiation on chitosan fibers, in this study MTT assay, scanning electron microscopy and immunostaining techniques were employed to address these questions.

Materials and Methods

Animals

In the handling and care of all animals, the International Guiding Principles for Animal Research were followed, and all procedures were performed in accordance with the China Animals (Scientific) Procedures Art.

Isolation and Differentiation of NEPs

NEPs were isolated from rostral parts of neural tubes of Wistar rat embryos at E11 (E11, day of gestation). The classical hematoxylin & eosin (HE) staining was performed to explore the histology of neural tube. Isolation and culturing of NEPs was performed as described by Sun *et al* with some modifications (16). Briefly, NEPs were dissociated from neural tube treated with 0.25% trypsin for 10 min at 37°C and washed in D-Hank's solution containing 0.004% DNase. Then, NEPs were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12-based medium (Life Technologies, Paisley, UK) containing 10% new-born calf serum at a density of 5×10^5 cells/ml and prepared for use. For differentiation assay, NEPs were cultured on polydlysine-coated twenty-four-well culture plates at 37°C in a humidified incubator with 5% CO_2 for an additional 5 days in culture. Half of the culture medium was replaced every 3 days.

Preparation of Chitosan Fibers

The chitosan fibers used in this experiment were purchased from Shanghai Gao Chun biomaterial Company (Shanghai, PRC). The diameter of the chitosan fiber is 15 μm. The preparation of chitosan fibers was modified as previously described (22). Briefly, the chitosan fibers were cut into 1.8 cm in length, washed three times with distilled water and sterilized with routine hyperbaric methods. The chitosan fibers were then equilibrated into the DMEM/F12 culture medium (Gibco, Grand Island, NY, USA). Subsequently, chitosan fibers were put onto the bare coverslips embedded in the twenty-four culture plate. Nothing was coated on the bare coverlips.

NEPs Culture on Chitosan Fibers

NEPs were suspended at a density of 5×10^5 cells/ml and then added to a twenty-four-well culture plate embedded with bare coverslips and chitosan fibers. The bare coverslips were used as a control. NEPs and chitosan fibers were incubated in DMEM/F12 culture medium containing 10% new-born calf serum at 37°C in a humidified incubator with 5% CO_2. Every 3 days, half of the culture medium was replaced.

Light Microscopy and Scanning Electron Microscopy

After being plated in a culture plate, NEPs were observed under an inverted microscope. Photographs were taken at regular intervals.

To examine the growth and the arrangement of NEPs on chitosan fibers, a scanning electron mic-

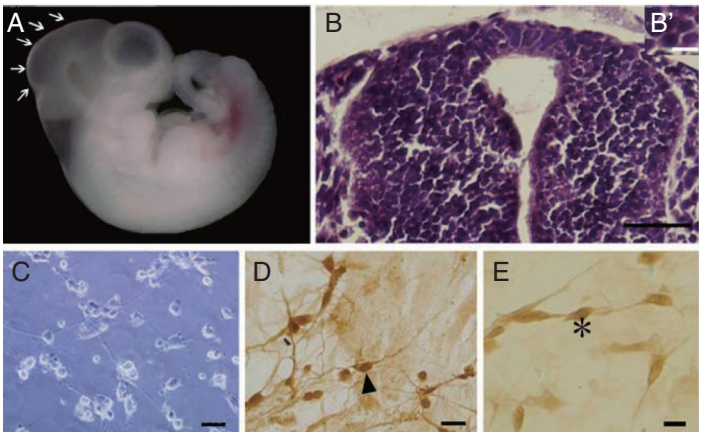

Fig. 1. Isolation and culture of NEPs. Rostral parts of neural tubes were dissected from rats of embryonic day 11 (A, arrows). HE staining showed NEPs in neural tube (B); B' was magnification of B; NEPs were detected. NEPs were cultured for 5 days *in vitro* (C). Immunocytochemical staining demonstrated that NEPs differentiated into NSE-positive cells (D, triangle) and GFAP-positive cells (E, asterisk) at day 5 in culture. Scale bars: B, 50 μm, C-E, 20 μm, B', 10 μm.

roscope was employed. After 3 days of incubation, NEPs and chitosan fibers were washed with DMEM/F12-based medium and fixed with 4% glutaraldehyde solution and then rinsed in cacodylate buffer three times (15 min each). This was followed by post-fixing with 1% OsO_4 in a 0.1 M sodium cacodylate buffer and rinsing in a cacodylate buffer three times (3 min each). The samples were then dehydrated in 35%, 50%, 70%, 80%, 95% and 100% ethanol successively for 10 min each and dried in a critical point drier (Bio-Rad CPD 750, Sydney, NSW, Australia). SEM images were observed under a scanning electron microscope (LEO Electronenmikroskopie GmbH Korporation, Germany).

Cytotoxicity Assays

Cell viability was determined by the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. This method measures mitochondrial activity based on the reductive cleavage of yellow tetrazolium salt to a purple formazan compound by the dehydrogenase activity of intact mitochondria.

After 3, 5 and 9 days of incubation, the viability of the chitosan fibers group and the control group was assessed. Briefly, the assay medium containing 5 mg/mL MTT was added to each well of the plate at a final concentration of 1 μg/ml and incubated at 37°C for 4 h, then the supernatant was removed, the formazan product obtained was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) with stirring for 15 min on a microtiter plate shaker and absorbance at 570 nm was detected.

Immunocytochemical Staining

To determine the differentiation of NEPs, immunocytochemical staining was performed. Briefly, NEPs at day 5 in culture and NEPs on chitosan fibers at day 5 in culture were both fixed in a 4% fresh-prepared paraformaldehyde for 10 min at room temperature, then subsequently blocked with 10% normal goat serum for 60 min and incubated overnight with one of the following primary antibodies: rabbit anti-Neuron Specific Enolase (NSE), rabbit anti-glia fibrillary acidic protein (GFAP) and rabbit anti-S-100 (all diluted 1:200, Sigma). Secondary antibodies were biotinylated goat-anti-rabbit (diluted 1:200, Sigma). After being incubated in primary and secondary antibodies, and then incubated in avidin-biotin complex (vectastain ABC kit), the result was visualized with 3, 3-diaminobenzidine (Sigma). Selected cells with which primary antibodies were omitted while secondary antibodies were added were used as controls to verify the specificity of the staining patterns.

Statistical Analyses

All data were expressed as the mean ± the standard error of the mean (S.E.M.). A Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered significant.

Results Isolation and Differentiation of NEPs

NEPs were dissociated from E11 embryos (Fig. 1A) and plated in DMEM/F12 culture medium containing 10% new-calf serum. Neuroepitheliums located on the wall of neural tube were identified with HE histological staining (Fig. 1B). At 5 days *in vitro* culture, NEPs attached to the coverslips that were coated with polydlysine, and the adherent cells were

found to be connected to each other with long neurites (Fig. 1C). NSE-positive and GFAP-positive cells were both detected (Figs. 1D and 1E).

Light Microscopy

At 24 h in culture, many NEPs were clearly observed to grow along the chitosan fibers under the microscope. After 3 d incubation, many cells attached to the chitosan fibers in the culture medium (Fig. 2A). At day 5 *in vitro* culture, attached cells were densely distributed along chitosan fibers, encircling the fibers in all the surfaces. Some adherent cells connected with one another spiraling along the chitosan fibers (Fig. 2B), some cells attached to the fibers alone, whereas some cells grew in a mass on the surface of the fiber (Fig. 2C). Spherical and oval cells were both observed on the fibers. In the control group, NEPs were also found to grow in culture.

Scanning Electron Microscopy

To further investigate the growth properties of NEPs on chitosan fibers, scanning electron microscopy was employed. At 3d incubation, the NEPs were firmly attached to the fibers. Lots of microvilli were observed on their surface, a neuron-like cell extended long neurite (Fig. 2D). Many adherent cells connected to each other arranged longitudinally along the fibers (Fig. 2E). At 5d in culture, many cells grew together on the fibers including spherical and oval cells. A typical cell mass attached on the surface of a fiber was detected (Fig. 2F).

Immunocytochemical Staining

At day 5 *in vitro* culture, immunocytochemical staining showed that many NSE-positive cells were detected on the chitosan fibers. Positive cells which were stained deeply attached to the fibers and arranged alone, or a cell mass, along the fibers (Fig. 3A). Typical NSE-positive neurons could be found in which the neurite was evident (Fig. 3E). Similarly, GFAP-positive cells were detected which firmly adhered to the chitosan fibers and connected with each other (Figs. 3B and 3F). Furthermore, a few S-100-positive cells grew on the fibers (Figs. 3C and 3G). NSE- and GFAP-positive cells also could be detected in the control group (Figs. 3D and 3H).

Cytotoxicity Assays

Colorimetric MTT assay was performed after the NEPs were cultured 3, 5 and 9 days *in vitro*. The results indicated that there were no significant differences on cell viability between the chitosan fiber group and the control group ($P > 0.05$). At 3d in culture, the mean value of the cell viability of the chitosan fiber group was a little higher than that of the control group (Fig. 4).

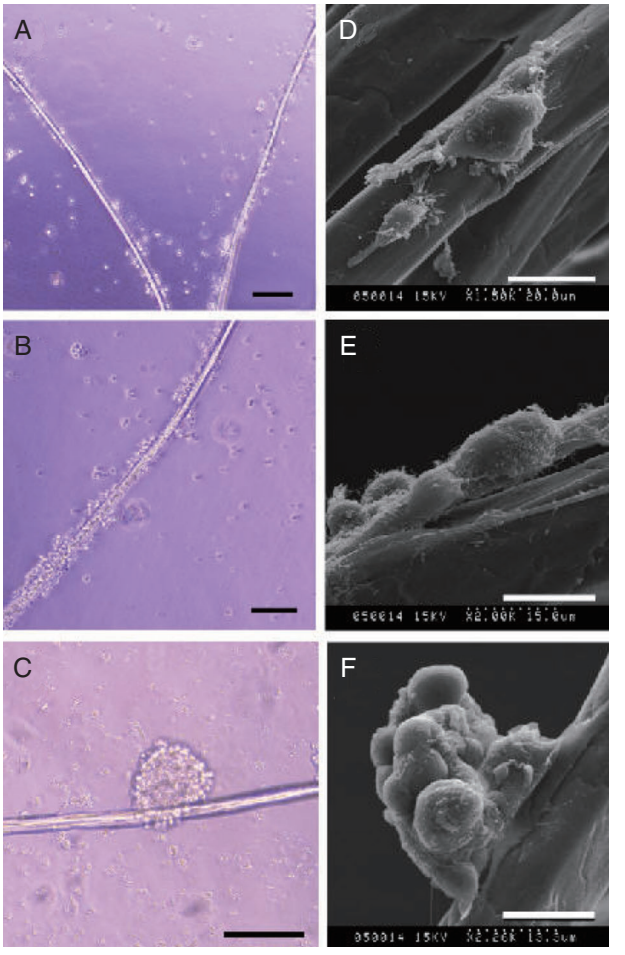

Fig. 2. Growth and survival of NEPs cultured on chitosan fibers. NEPs were cultured on chitosan fibers and observed by light microscope (A, B, C) and scanning electronic microscope (D, E, F) at 3 days (A, D) and 5 days (B, C, E, F) *in vitro* culture. Seeded NEPs grew along the chitosan fibers (A). Many cells are densely distributed on the chitosan fibers, encircling the fibers (B), or even a cell mass attached to the chitosan fibers (C). Under SEM, NEPs were detected firmly attached to the fibers with lots of microvilli (D), whereas many adherent cells connected to each other arranged longitudinally along the fibers (E). A cell mass grew on the surface of the chitosan fiber (F). Scale bars: A-C, 100 μm; D, 8 μm; E, 6 μm; F, 6.65 μm.

Discussion

In the present study, chitosan fibers showed a good biocompatibility with NEPs. In these biomaterials, the bicomponent fibers partly mimic Bunger bands and is helpful for axon regeneration. Further-

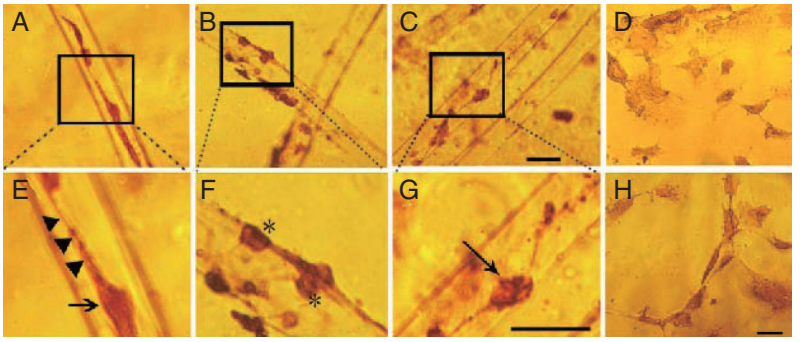

Fig. 3. Differentiation of NEPs on chitosan fibers in culture. E, F and G were magnifications of A, B and C, respectively. NEPs were seeded on the chitosan fibers. At 5 days *in vitro* culture, immunostaining with antibodies against NSE, GFAP and S-100 was performed. NSE-positive cells (E, arrows), GFAP-positive cells (F, asterisks) and S-100-positive cells (G, arrows) were detected. A typical neuron that extended long neurite on the chitosan fiber is shown in E (triangles). In the control group, D and H were immunostaining with NSE and GFAP, respectively. Scale bars: A-C, 25 μm; E-G, 25 μm; D-H, 25 μm.

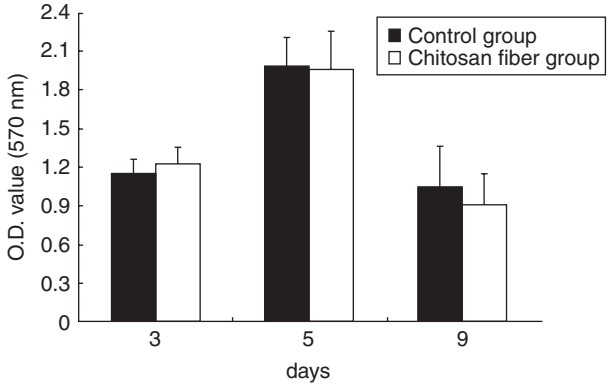

Fig. 4. Evaluation of biocompatibility of chitosan fiber by cytotoxic test *in vitro*. NEPs were seeded on chitosan fibers; MTT assays were performed to examine cell viability at 3d, 5d, and 9d *in vitro* culture. Errors bars represent means ± SE. NEPs viability all showed no difference between the chitosan fiber group and the control group. $P > 0.05$ *vs*. control group.

more, NEPs not only survived well on fibers but also differentiated into neurons and glia which distributed on the surface of the fibers. In particular, a few S-100 positive cells were detected on fiber. This study indicated that chitosan seeded with NEPs was a promising application in peripheral nerve tissue engineering.

To explore the biocompatibility of chitosan fiber with NEPs, several experiments were performed including cytotoxicity assays, cell culture and scanning electron microscopy. Cytotoxicity assays mainly measure cell viability which is widely used for biocompatibility. Our results indicated that chitosan fibers had no cytotoxic to NEPs. NEPs viability in the chitosan fiber group showed no difference compared to that in the control group. When seeded on chitosan fibers, NEPs survived well along the fibers. Under a scanning electron microscope, seeded cells were found to be firmly attached to the fibers (Figs. 2D-2F). Moreover, typical neuron-like cells were detected with long neuritis surrounding the fibers (Fig. 3A). So, chitosan fibers showed a good biocompatibility with NEPs and provided a suitable substrate material for cell growth. Previous studies reported that chitosan was a well-known biodegradable polysaccharide which was widely used in biomedical research and application due to its abundance, easy accessibility, non-toxicity and excellent biocompatibility (15). In particular, chitosan could be prepared in the form of film and fiber. Chitosan fibers partly imitate the structure of Bunger bands in which cell bridges were formed to guide axonal regeneration during peripheral injury (22). Furthermore, chitosan fibers could provide a favorable anatomical space for the growth of nerve fibers. Chitosan fibers model the structure and arrangement of nerve fibers and are helpful for attachment of cultured cells. A promising application of chitosan fiber in nerve regeneration will be investigated in the future.

This study used NEPs originated from neural plate and neural tube as a kind of seed cell. These cells could differentiate into neurons and glia in brain transplantation. Our previous study reported multipotential differentiation of NEPs in *in vitro* culture (16). NEPs demonstrated several characteristics as a donor material for neural regeneration such as easy availability, fine cyto-activity and low immunogenicity overcoming disadvantages of other neural cells (18, 21). In this report, when seeded on chitosan fibers in *in vitro* culture, NEPs differentiated into neurons, astrocytes and a few Schwann-like cells expressing S-100, a marker of Schwann cell (Fig. 3C). It was also suggested that neural stem cells

had the plasticity to differentiate into Schwann-like cells and were capable of myelinating axons in the demyelinated and dysmyelinated spinal cord (11). Thus, NEPs may be a suitable substitution for the traditional seed cells in peripheral tissue engineering. The potential of multiple-linage differentiation did not show differences between the chitosan fiber group and the control group. Chitisan fibers probably do not affect the multiple-linage differentiation of NEPs.

It is well known that bioactive molecules also play an important role in tissue engineering (14). This experiment only examined the chitosan fiber material and NEPs without using bioactive molecules. If chitosan fibers cohered with extracellular molecular and growth factors such as nerve growth factor and glia cell derived neurotrophic factor, and then the fibers were seeded with NEPs, seeded cells would probably show better survival, differentiation and migration. Thus, it is the effects of these factors in nerve tissue engineering is worthy of further investigation.

In conclusion, our study demonstrated that chitosan fibers showed a good biocompatibility with NEPs. When seeded on chitosan fibers, NEPs attached to the surface of the fibers and differentiated into neurons and glia. Moreover, chitosan fibers have a favorable anatomical morphology for the regeneration of nerve fiber so that these chitosan bicomponent fibers pre-seeded with NEPs will afford a promising treatment for peripheral nerve injury.

Acknowledgments

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