Original Article
Impacts of phosphatase and tensin homology deleted on chromosome ten (PTEN)-inhibiting chitosan scaffold on growth and differentiation of neural stem cells

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Abstract: Objective: The aim of this study was to investigate growth and differentiation of neural stem cells (NSCs) on the phosphatase and tensin homology deleted on chromosome ten (PTEN)-inhibitor-adsorbed chitosan scaffold. Methods: NSCs were divide into the chitosan group and the control groups, and performed CCK-8 test on 1st, 3rd and 7th d to compare the proliferation between the 2 groups. The chitosan scaffold adsorbed PTEN inhibitor bpv (pic), and the empty scaffold was used as the control for co-culture of NSCs, immunofluorescence staining was performed on 7th d to detect the differentiation of NSCs on the scaffold. Results: The results of CCK-8 test showed no significant difference in the absorbance between the 2 groups. Immunofluorescence staining showed that the NSCs numbers of the bpv scaffold group were more than the empty scaffold group, among which the anti-glial fibrillary acidic protein (GFAP) positive cells were less than the empty scaffold group, while the anti-β-Tubulin III positive cells were more than the empty scaffold group, the two groups both showed rare anti-receptor-interacting protein (RIP) positive cells. Conclusions: Chitosan scaffold exhibited good compatibility to NSCs, the PTEN-inhibitor-adsorbed chitosan scaffold could promote the migration of NSCs towards the scaffold and their differentiation towards neurons.

Keywords: PTEN, tumor suppressor gene, neural stem cells, chitosan, neural tissue engineering

Introduction

An important reason for the disorders of central nervous regeneration was the sustained high expression of certain genes inside mature neurons, which had poor abilities of autologous growth, while control the overgrowth, and this was related with the post-injury regeneration of central nervous system (CNS) [1]. Phosphatase and tensin homology deleted on chromosome ten (PTEN) was originally discovered as a tumor suppressor gene, it was very lowly expressed during embryonic development, while highly expressed in adults, which could inhibit proliferation and development of cells that had already developed. PTEN was also highly expressed in adult CNS, many studies had confirmed that inactivated PTEN in maturely differentiated neurons could promote the growth of axons [2], ectopic axonal sprouting and synapse formation would also occur [3, 4], and promote the oligodendrocyte-mediated myelination [5]. Our previous study also found that, PTEN inhibitor bpv (pic) could promote neurons’ growing on the myelin membrane proteins, and promote NSCs to differentiate into neurons. Endogenous NSCs (eNSCs) were widely distributed in adult mammalian CNS [6, 7], and could migrate to injured lesions when stimulated by injuries, etc. [8], so it could treated as potential seed cells for nerve repairing [9]. NSCs also had PTEN expression, and its proliferation and differentiation were regulated by PTEN [10, 11].

The measures to improve the internal growth abilities of CNS itself mainly lied in two aspects, the first was to increase the numbers of seed cells in damaged sites, and promote their differentiation into neurons, and the second was to promote and guide axons to grow correctly. Applying PTEN inhibition into the study of spinal cord injuries could not only promote eNSCs to
differentiate into neurons, but also promote the growth of neurons, thus achieving good functional recovery of spinal cord. Chitosan was a degradable multi-channel bio-scaffold, had good biocompatibilities [12], it could not only provide suitable environments and limited space for the growth of seed cells, but also act as good carrier for biological active factors. In this study, we used chitosan scaffold to adsorb PTEN inhibitor bpv (pic), then co-cultured and observed growth and differentiation of NSCs, thus providing experimental evidence for the repairing of spinal cord injuries with this kind of scaffold.

**Materials and methods**

**Animals**

SD pregnant rats (16 days) were provided by the Experimental Animal Center of Nantong University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Nantong University.
Isolation and single cell clone of NSCs

The cerebral cortex of SD embryonic rats (16 days, E16) was sampled under sterile conditions, pipetted and dispersed the cells after digested with trypsin, added DFNG medium (composed of DMEM/F12 (Invitrogen, California, USA), 1% N2 (Invitrogen, California, USA), 1% B27 (Invitrogen, California, USA), 20 nmol/L EGF (Sigma, ST. Louis, USA), and 20 nmol/L bFGF (Sigma, ST. Louis, USA), then seeded the cells with the concentration as $1 \times 10^6$ cells/ml in 25 cm flasks, cultured under conventional conditions (saturated humidity, 37°C, 5% CO$_2$), with half medium centrifugally changed every 3 d. Collected the cells that were cultured for 5~7 d, centrifuged at 1000 rpm for 5 min, discarded half amount of the supernatant, and pipetted the rest into single cell suspension, diluted the cells to 40/ml, then added 50 μL into 96-well plates together with 50 μL of DFNG, counted the cells 2 h later, chose the well that had only one cell for further culture; 5~7 d later, pipetted the medium to form single cell suspension, then seeded in 25 cm flasks, with half medium centrifugally changed every 3 d; performed passage once every 5 to 7 d, and the 3rd-generation cells were taken for the subsequent experiments.

Identification of NSCs

Placed NSCs subcultured for 5 days into 0.1% PLL (MW 30000-70000, Sigma, ST. Louis, USA) coated slides for wall-adherent growth for 2 h, then performed anti-Nestin immunofluorescence staining, with the primary antibody as mouse anti-Nestin monoclonal antibody (Santa Cruz, Texas, USA), and the secondary antibody as rhodamine-donkey anti-mouse IgG (Jackson Immuno Research Lab, West Grove, USA).

Preparation of chitosan scaffold

The porous chitosan scaffold was kindly gifted by the key laboratory of Neural Regeneration of Jiangsu Province, with the preparation methods as: dissolved the chitosan (Santa Cruz, Texas, USA) with 1% acetic acid solution, and made 2% chitosan solution, injected the solution into the mold which was arranged parallel by multi-nickel-chrome wire; pre-cooled at 4°C for 6 h, then frozen at -28°C overnight; electrically heated nickel-chrome wire, then drew the wire as soon as possible; placed the sample at -60°C for 24 h drying; deacidified with 0.1 M NaOH-methanol (1:1) and methanol-distilled water (1:1), rinsed in distilled water to neutral, cryodesiccated, then cut into 3 mm pieces, and sterilized with ethylene oxide for future use.

Biocompatibility evaluation of chitosan scaffold and NSCs

The experiment was divided into two groups: the chitosan group and the control group, with 5 wells in each group. The chitosan group was
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The experiment was divided into two groups: the bpv scaffold group, bpv (pic)-adsorbed chitosan scaffold was co-cultured with NSCs; the empty scaffold group, the empty chitosan scaffold was co-cultured with NSCs. NSCs were seeded into 0.1% PLL-coated 24-well plate with 2×10⁵ cells/ml, each group had 5 wells, and 1 ml was added into each well, as well as 3 corresponding chitosan scaffold (PLL coated) for the culture under conventional conditions; 2 h later, replaced 1 ml of differentiation solution (1% bovine serum-containing DFNG medium) for continuous culture, and replaced half amount of medium once every 2 d; 7 d later, used 4% paraformaldehyde to fix the chitosan scaffold, followed by frozen slicing, anti-β-tubulin III, GFAP, and RIP immunofluorescence staining, with the primary antibodies as mouse anti-β-tubulin III monoclonal antibody (Sigma, ST. Louis, USA), mouse anti-GFAP monoclonal antibody (Chemicon, USA), mouse anti-Rip monoclonal antibody (Santa Cruz, California, USA), and FITC-goat anti-mouse IgG (Sigma, ST Louis, USA), respectively; the positive cells in each slice were then counted.

Statistical analysis

The data of CCK-8 test and cell differentiation immunofluorescence staining were expressed as mean ± standard deviation, SPSS17.0 was used for two independent-sample t test, with \( P < 0.05 \) considered as statistically significant.

Results

Culture and identification of NSCs

Single NSC would split into 2 to 4 cells after ~2 d culture, exhibit 32–48 cell clones about 5 d later, and develop into large clone with hundreds of cells ~10 d later (Figure 1A-C). The clones from single cell could obtain a large number of subcloning after serial passages, which exhibited suspended spherical growth, and the cell balls exhibited anti-Nestin positive staining (Figure 1D).

Impacts of chitosan scaffold on proliferation of NSCs

CCK-8 test showed, the absorbance values at 425 nm on 1st, 3rd and 7th d were: chitosan

Figure 4. Statistics of cells attached onto scaffolds of the 2 group (vs empty scaffold group, \( P < 0.05 \)).
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**β-Tubulin III**  
**GFAP**  
**RIP**

**Figure 5.** Immunofluorescence staining of chitosan scaffold. Scale bar = 20 μm.

**Figure 6.** Statistics of NSCs differentiation situations (*vs empty scaffold group, \(P < 0.05\)).

Group: 0.267 ± 0.012, 0.444 ± 0.019 and 0.787 ± 0.031; control group: 0.237 ± 0.022, 0.467 ± 0.024 and 0.772 ± 0.021, respectively. There was no significant difference between the two groups (\(P > 0.05\), Figure 2).

**Delayed-release effects of bpv (pic)-adsorbed chitosan scaffold**

759 nm was the maximal absorption wavelength of saline soaking liquid of chitosan scaffold. The absorbance values measured on 1st, 3rd, 5th, 7th and 9th d were 0.0046 ± 0.0011, 0.0092 ± 0.0013, 0.0094 ± 0.0021, 0.0082 ± 0.0019 and 0.0066 ± 0.0011, respectively, while those of the empty scaffold were close to 0 at each time point (Figure 3).

**Impact of bpv (pic)-adsorbed chitosan scaffold on differentiation of NSCs**

After chitosan scaffold was co-cultured with NSCs for 7 days, the immunofluorescence staining showed: the cell numbers on the bpv scaffold group were more than the empty scaffold group (\(P < 0.05\)) (Figure 4); among which the anti-GFAP positive cells were lower than the empty scaffold group, while the anti-β-Tubulin III positive cells were more than the empty scaffold group (\(P < 0.05\)), and these 2 groups showed rare anti-RIP positive cells (Figures 5, 6).

**Discussion**

CNS in adults existed the phenomenon of lifelong neurogenesis in some parts, indicating that CNS had the regeneration potential [11], but a lot of factors would lead to the regeneration disorders of CNS. A large number of stud-
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ies were focused on neurotrophins’ deficiencies, microenvironmental nerve regeneration inhibitors and glial scar-caused spatial separation, etc., these were peripheral factors towards the growth of CNS. It was noticeable that the deficiency of autologous growth abilities was the important internal reason for the regeneration difficulties of CNS [1]. The measures to improve the internal growth abilities of CNS itself mainly lied in two aspects, the first was to increase the numbers of seed cells in damaged sites, and promote their differentiation into neurons, and the second was to promote and guide axons to grow correctly.

PIP3 was an important intracellular second messenger, and the key regulator of cell survival, the main biological effects of PTEN were achieved through PI3K/AKT/PTEN/mTOR pathway, which negatively regulated growth, survival and proliferation of cells [13], the balance between PTEN and PI3K was also involved in the exercise of CNS's normal functions [14], PTEN also played an important role in regulating neuronal differentiation and synapse formation in CNS. Christie used siRNA to knockout PTEN suppression mRNA, and observed significant regeneration of axons in peripheral nerves [15]; Zukor used shRNA to knockout PTEN, and observed significant regeneration of corticospinal tract in animal model of spinal cord injury [16]. Meanwhile, Walker also found PTEN inhibitor could protect oligodendrocytes, reduce demyelination, inhibit neuronal apoptosis after spinal cord injuries [5]. These all indicated that the inactivation of PTEN might promote the repairing of damaged nerve cells.

Researches on gene functions could be realized through a variety of ways, such as gene knockdown, RNA interference, and chemical inhibitors, etc. But the long-term silence of tumor suppressor genes might lead to tumorigenesis, and the long-term inactivation of PTEN might also lead to hypertrophy and abnormal polarity of nerve cells [11]. Chemical inhibitors would directly act on the proteins expressed, while the roles were short, and would not affect the normal functions of PTEN for a long period, so there was no carcinogenic risk to worry about bpv (pic) was a compound that could alter the structures, thus inactivate the cysteine residue inside the protein tyrosine phosphatase (PTPs) catalytic region, and PTEN had the activities of lipid phosphatase and protein phosphatase at the same time, and exhibited similar while larger catalytic active structure than PTPs [17]. Therefore, bpv (pic) had strong affinities with PTEN, and could inhibit the activities of PTEN’s phosphatase, so we used bpv (pic) for the short-term PTEN inhibition study.

NSCs had the abilities of self-renewal and differentiation, so they were the top seed cells in nerve repairing. CNS in adult mammalian had widely distributed endogenous neural stem cells (eNSCs), which were in relatively resting state under normal conditions, while activated and proliferated only in such specific factors as CNS damages and abnormal cellular regulations [18]. eNSCs did not exist the inhibition rejection reactions, so they could be used as good seed cells in repairing the damaged CNS. Several studies also showed that, PTEN would inhibit cell migration [19, 20]. Therefore, PTEN inhibitor would be likely to promote the migration of active eNSCs towards injured lesions [8], so as to provide more autologous cells for repairing the damages of CNS. In this study, we found that the anti-GFAP positive cells and anti-β-Tubulin III positive cells on the bpv scaffold group were significantly more than the empty scaffold group, and exhibited, while the empty scaffold group only exhibited a small amount of anti-GFAP positive cells, therefore, it could be considered as that after PTEN was inhibited by bpv (pic), more NSCs migrated inside the scaffold and differentiated into these cells.

The differentiation of NSCs was a very complex process, which was not completely controlled till now. NSCs also exhibited the expression of PTEN, and their proliferation and differentiation were regulated by PTEN [10, 11], knocking down PTEN could promote self-renewal of neurons [18, 21]. In our experiments, there appeared anti-β-Tubulin III positive cells on the bpv scaffold, while the empty scaffold group only appeared anti-GFAP positive cells, indicating that with bpv (pic)’s action, more NSCs differentiated into neurons, which was clearly in favor of repairing the damaged nerves. As for the mechanisms related, certain study considered that it was because GSK-3β/β-catenin regulated NSCs to differentiate into neurons [22], after PTEN was suppressed, the phosphorylation of GSK-3β was increased, therefore β-catenin was upregulated, and the signal level that resulted in the differentiation of NSCs into neurons was maintained [23, 24].
Besides suitable seed cells, repairing the damaged CNS also required suitable growth environments, as well as the limited space for axonal stretching. Chitosan was a degradable bioscaffold with good multiple channels and good biocompatibilities. CCK-8 test showed that the proliferation of NSCs was not affected by the soaking solution of chitosan, indicating that chitosan had good biocompatibilities. The absorbance measurement of chitosan soaking solution showed that the bpv (pic)-adsorbed composite scaffold still had high absorbance after 9-day soaking, indicating that chitosan-repairing bpv (pic) had good delayed-release effect [25]. After co-incubation with NSCs, the numbers of anti β-Tubulin III positive cells of the bpv scaffold group were significantly more than the empty scaffold group, which showed chitosan could absorb bpv (pic), and acted as its delayed-release carrier, and NSCs could more grow in chitosan scaffold and more differentiate into neurons.

Under in vivo environments, there existed such molecules as Nogo-66, MAG and OMgp around nerves, which could inhibit the growth of neuronal axons [26]. By activating PTEN, these molecules could suppress the expression of Akt, and interfered with PIP3/Akt/mTOR pathway, thus inhibiting nerve regeneration [13, 27]. Our previous experiments also showed, that after PTEN was inhibited, the neuronal growth cultured in in vitro inhibitory microenvironment was enhanced.

So our assumption was to graft the bpv (pic)-adsorbed chitosan scaffold into injured lesions of spinal cord, using it to inhibit PTEN, and to attract eNSCs to migrate towards the injured lesions, meanwhile, promoted the migrated NSCs to differentiate into neurons, and promoted the growth after neuronal differentiation, thereby promoting the repairing of damaged spinal cord. This would be continued in future animal experiments, thus providing new explorations for the treatment of spinal cord injuries.

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Disclosure of conflict of interest

None.

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References

[12] Aranaz I, Gutiérrez MC, Ferrer ML and del Monte F. Preparation of chitosan nanocompos-
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[27] Shi GD, QuYang YP, Shi JG, Liu Y, Yuan W and Jia LS. PTEN deletion prevents ischemic brain injury by activating the mTOR signaling pathway. Biochem Biophys Res Commun 2011; 404: 941-945.