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Effect of chitooligosaccharide on neuronal differentiation of PC-12 cells

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Abstract

Chitosan is now being widely used biomaterial in the tissue engineering field, and has great potential as a candidate material for preparing nerve guidance conduits due to its various favorable properties, especially that of good nerve cell affinity. Chitosan can be degraded in vivo into chitooligosaccharide. We have investigated the in vitro effects of chitooligosaccharide on neuronal differentiation of PC-12 cells to see what effects chitooligosaccharide have on certain functions in the regenerating neurons. The morphologic observation and assessment using the specific reagent of tetrazolium salt WST-8 indicated that neurite outgrowths from PC-12 cells and the viability of PC-12 cells were enhanced by treatment of chitooligosaccharide. The real-time quantitative RT-PCR and Western blot analysis revealed showed that chitooligosaccharide could upregulate the expression of neurofilament-H mRNA or protein and N-cadherin protein in PC-12 cells. The maximum effect of 0.1 mg/ml chitooligosaccharide was obtained after 2 week culture. All the data suggest that chitooligosaccharide possesses good nerve cell affinity by supporting nerve cell adhesion and promoting neuronal differentiation and neurite outgrowth.

Keywords: Chitosan; Chitooligosaccharide; Nerve cell affinity; Neuronal differentiation; PC-12 cells

1. Introduction

Chitosan is made up of D-glucosamine units linked by β (1–4) glycosidic bonds and is derived from deacetylation of chitin, the main component of the exoskeleton of crustaceans. It has a number of biomedical applications, including those in tissue engineering, due to its various favorable properties; especially its specific interactions with the extra cellular matrix and growth factors (Freier et al., 2005; Zheng et al., 2007). Many studies on peripheral nerve regeneration have also reported that chitosan-based nerve guidance conduits were used to bridge an extended nerve gap with considerable success because the conduit not only acted as a supporting scaffold to guide axonal growth, but also enhanced both the survival and neurite outgrowth of neurons (Wang et al., 2005; Christina et al., 2006; Isamu et al., 2003; Gianluca and Valeria, 2006; Bini et al., 2005; Yannas and Hill, 2004).

Chitosan is degraded in vivo by an enzymatic hydrolysis of lysozyme normally produced by macrophages (Ohara et al., 2004). The biodegradation products of chitosan are (low MW) chitooligosaccharides with different polymerization degrees (from 2 to 10) (Mendis et al., 2007).

After implantation of chitosan conduits, undegraded chitosan and chitooligosaccharide produced from degraded chitosan are likely to coexist around the regenerating neurons for a considerable period of time. Despite previous reports that chitosan material exhibited nerve cell affinity in vitro (Yuan et al., 2004; Yang et al., 2004; Freier et al., 2005; Gong et al., 2000), the issue of whether chitooligosaccharides, also affect certain functions in the regenerating neurons is worth exploring. Therefore we have chosen rat pheochromocytoma PC-12 cells to examine the neurobiological changes induced by chitooligosaccharide under in vitro conditions.

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2. Materials and methods

2.1. Preparation and purification of chitooligosaccharide

Chitosan, obtained from Nantong Xincheng Biochemical Company (Jiangsu, China), was refined twice by dissolving it in 10 g/L acetic acid solution. It was filtered, precipitated with 50 g/L NaOH, and finally dried in a vacuum at room temperature. Its degree of deacetylation was 92.3% according to titration analysis and its average MW was 2.5×10^5 according to viscosity measurements (Jia and Shen, 2002).

Crude chitooligosaccharide prepared by our previously reported method (Shao et al., 2003), was dissolved in a minimum volume of distilled water, and partially purified on a Sephadex-25 column (column size: 1.6 × 85 cm bed volume). The sample was placed on the column top and eluted with distilled water. The eluted chitooligosaccharide solution was lyophilised under vacuum (35–45 mTorr) to obtain chitooligosaccharide powder.

Its number average MW was determined by end-group analysis (Yang et al., 2006). The MW value showed that the chitooligosaccharide preparation was a mixture with variable molecular length, with an average polymerization of ~7.

2.2. PC-12 cell culture and treatments

PC-12 cells were obtained from Shanghai Cell Bank, Chinese Academy of Sciences, and routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% fetal bovine serum and 15% horse serum (Invitrogen Carlsbad, CA, USA).

Exponentially growing PC-12 cells were seeded onto 6-well plates in fresh culture medium containing chitooligosaccharide at concentrations of 0.01, 0.10 and 1.0 mg/ml, containing either 50 ng/ml NGF (Sigma, St. Louis, MO, USA) or no additive. The 2 latter served as positive or negative controls, respectively. After 2 weeks, PC-12 cells were observed under an inverted light microscope.

2.3. Cell viability assay

PC-12 cells were seeded in triplicate in 96-well plates (5×10^3 cells/well), and treated with 3 different concentrations of chitooligosaccharide or 50 ng/ml NGF, with or without any additive. After incubation for 1, 4, 7 and 10 days, a cell counting kit-8 consisting of tetrazolium salt WST-8 (CCK-8, Dojindo, Japan) was used to analyze the cell viability in different culture mediums according to the manual. Absorbance at 450 nm was measured with a microplate reader (ELx800, Bio-Tech Instruments, Winooski, VT, USA).

2.4. Cell proliferation assay

PC-12 cells were seeded in triplicate in 96-well plates (5×10^3 cells/well) and treated as described in Section 2.3. After incubation for 3, 4, and 5 days, BrdU (5-bromo-2′-deoxyuridine) labeling solution (Cell Proliferation ELISA BrdU, Colorimetric, Roche, Nutley, NJ, USA) was added to each well, plates were then incubated for 3 h. The cellular DNA was denatured, and Anti-BrdU-POD bound to the BrdU incorporated in newly synthesized DNA of proliferating cells. The immune complexes were detected by the subsequent peroxidase-substrate reactions. Absorbance at 450 nm was measured with a microplate reader (ELx800), and was expressed as a percentage of the value obtained relative to normalised control cells.

2.5. Real-time quantitative RT-PCR

Gene specific primers and probes were designed according to the whole sequences of NF-H (GeneBank No. NM_012607.1) and GAPDH (as an internal control, GeneBank No. BC059110.1), respectively (Table 1). The details for preparing standard plasmid and real-time PCR have previously been described (Liu et al., 2006). Briefly, the PC-12 cells were collected in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted and reverse transcriptized (Omniscript RT kit from Qiagen). The mRNA quantity of NF-H or GAPDH was automatically calculated based on the calibration curves generated by serially diluted NF-H or GAPDH plasmids.

2.6. Western blot analysis

PC-12 cells were cultured in different conditional mediums for 2 weeks and then lysed in cell/tissue protein extraction reagent (Biocolors, Shanghai, China) with protease inhibitor on ice. Whole-cell lysates, whose protein concentrations had been measured by a BCA Protein Assay Reagent kit (Biocolors), were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The membranes were blotted with 5% non-fat dry milk in TBST buffer and incubated with the primary antibodies: anti-N-cadherin (Abcam, Cambridge, MA, USA) or anti-β-actin (Sigma). After incubation with the second antibody goat anti-mouse-IRDye (1:10 000) or donkey anti-goat-IRDye (1:10 000), the membrane was washed with PBS/T and scanned with an Odyssey Infrared imager (Licor, Lincoln, NE, USA). The data were analyzed with the software attached to the imager. The antibody used was specific for recognizing an intracellular domain that commonly existed in most known N-cadherins. β-Actin was used as an internal control for normalizing the loaded protein.

Table 1
Oligonucleotide primers and probes.

Target gene	Sequence (5′–3′)	Position
NF-H-sense	aaggaaaccgtcatt gtagaggaa	nt1405–nt1428
NF-H-antisense	ggagacgtagttgctgcttctt	nt1544–nt1523
NF-H- probe	FAM-cttctgcctccttct tcttcctcccctt -TAMRA	nt1487–nt1514
GAPDH-sense	ccttcattgacctcaactacatg	nt177–nt199
GAPDH-antisense	cttctccatggtggtggaaac	nt 428–nt 413
GAPDH-probe	FAM-cccatcaccatcttccaggagc-TAMRA	nt 287–nt 308