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Effect of carboxymethylated chitosan on the biosynthesis of NGF and activation of the Wnt/β-catenin signaling pathway in the proliferation of Schwann cells

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ABSTRACT

The proliferation of Schwann cells around injured peripheral nerves supports the process of Wallerian degeneration and is critical for axonal regeneration. In this publication, carboxymethylated chitosan (CMCS) was studied to determine its capacity (i) to induce proliferation and secretion of nerve growth factor (NGF) and (ii) to activate Wingless-type(Wnt) protein/β-catenin signaling pathways in rat Schwann cells. CMCS was found to induce Schwann cell proliferation and NGF synthesis in Schwann cell in a dose and time dependent manner. CMCS was shown to activate factors in the Wnt/β-catenin signaling pathway, including Dvl-1, β-catenin, Tcf4, Lef1, C-myc, and Cyclin D1 which are active in the proliferation of Schwann cells and biosynthesis of NGF of Schwann cell. Overall, this study suggests that CMCS can promote the proliferation of cultured Schwann cells and synthesis of NGF by activating the Wnt/β-catenin signaling pathway.

1. Introduction

Schwann cells play a critical supportive role in the repair of injured peripheral nerves. Schwann cells proliferate at the distal ends of these nerves, and thereby support the formation of bands of Büngner and axonal regrowth (Feneley et al., 1991). Schwann cells proliferation also occurs at the proximal end of the injured nerve, providing a guide for regenerating axons (Daniloff, 1991; Guenard et al., 1992; Williams et al., 1983). Schwann cells are a major source of neurotrophins, such as nerve growth factor (NGF), which play a critical role in response to nerve injury. There have been several reports indicating that Schwann cell proliferation is crucial for successful axonal elongation (Anton et al., 1994; Torigoe et al., 1996).

Wnt/β-catenin signaling plays a major role in the development of the nervous system and contributes to neuronal plasticity. Wnt signaling is known to play an essential role in neurogenesis and in the fate of neural progenitors (Lee et al., 2004; Gao et al., 2007). Moreover, it has been identified as a major regulator of neuronal circuit development, playing a role in neuron positioning, polarization, axon dendrite development, and synaptogenesis (Salinas and Zou, 2008). Wnt/β-catenin signaling has also been reported to be involved in Schwann cells proliferation (Azim and Butt, 2011; Narciso et al., 2009; Flaiz et al., 2008; Gess et al., 2008).

Chitosan, a natural cationic biopolymer, is derived from chitin by N-deacetylation (Aiba, 1993). Chitosan has been shown to have antibacterial and neuroprotective properties (Rabea et al., 2003; Cho et al., 2010a, b), while also being biocompatible and biodegradable (VandeVord et al., 2002; Khor and Lim., 2003). Carboxymethylated chitosan (CMCS), a soluble derivative of chitosan, possesses many of the same desirable physiochemical and biological features: biocompatibility (Zhu and Fang, 2005), promotion of cell proliferation (Krause et al., 1998; Chen et al., 2002; Kim, 2011), and enhancement of rat sciatic nerve repair in vitro (Wang et al., 2010). Our previous studies indicated that CMCS and chitosan could protect chondrocytes from apoptosis, significantly suppress the degeneration of cartilage in osteoarthritis and protect chondrocytes from IL-1beta induced catabolism (Chen et al., 2006; Liu et al., 2005; Lei et al., 2008). Recently, we have found that CMCS are able to promote the proliferation of cultured Schwann cells via the activation of MEK/ERK and PI3K/Akt signaling cascades in vitro (He et al., 2011). In this study, we have expanded our research to determine whether CMCS can induce the biosynthesis and secretion of NGF in cultured Schwan cells and activate Wnt/β-catenin signaling cascades involved in Schwann cells proliferation.

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2. Materials and methods

2.1. Materials

Newborn (3–5 days old) Sprague-Dawley (SD) rats (provided by the Center of Animal Experimentation of Wuhan University, Wuhan, China) were used as the source of Schwann cells for culture. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Paisley, UK). Carboxymethylated chitosan (CMCS, purity > 99%) was supplied by the Institute of Chemistry and Environmental Science of Wuhan University (China). We utilized the Cell Counting Kit-8 (CCK-8) offered by Company (Kumamoto, Japan). Primers were provided by Invitrogen (USA). Antibodies were obtained from Cell Signaling Technology (Beverly, MA) and included: anti-NGF, anti-β-catenin, anti-Tcf4, anti-Lef1, anti-c-Myc and anti-cyclin D1. Anti-S-100 and anti-β-actin antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Other high-purity reagents and materials were purchased locally.

2.2. Cell isolation and culture

All protocols were approved by the institutional ethics committee of the Wuhan University School of Medicine. Primary Schwann cells were isolated from Spragur-Dawley rats according the procedure described by Brockes et al. (1979). Schwan cells were collected and cultured from the sciatic nerve of 8–10 newborn SD rats with each experiment. Briefly, the sciatic nerves were freed from connective tissue by incubating them for 20 min at 37 °C in DMEM containing 0.25% trypsin and 0.1% collagenase type Cells collected from the SD rat nerves tissue were separated by centrifugation and seeded onto tissue culture flasks or 6-well plates pre-coated with poly-L-lysine where they were cultured in DMEM supplemented with 4 mM glutamine, 50 μg/ml penicillin-streptomycin and 10% FBS. The cells were kept in a humidified atmosphere containing 5% CO_2 at 37 °C. The Schwann cells cultures were purified through the removal of contaminating fibroblasts via collagenase differential cell detachment (Jin et al., 2008).

2.3. S-100 immunofluorescence staining

To characterize the purified collections, cells were (i) cultured on glass slides before being fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with 0.1% Triton-X 100 for 5 min, and then subjected to 5 min phosphate buffer saline (PBS) washes three times each, and (ii) blocked with 5% bovine serum album (BSA) in PBS for 30 min at room temperature and washed again in PBS. The slides were incubated with rabbit anti-S-100 (1:100) at room temperature for 2 h and were further incubated with goat anti-rabbit IgG (1:5000) for 60 min at room temperature. Labeled cells were visualized under a fluorescence microscope using excitation and emission wavelengths of 488 and 533 nm, respectively. The images were digitally recorded and were processed with Image-Pro Plus (Media Cybernetics, USA).

2.4. CCK-8 assay

Purified Schwann cells (1×10^5/well) were cultured in flat-bottom 96-well microliter plates with the DMEM containing 0.1% FBS. 24 h afterward, their media were replaced with DMEM containing CMCS or PBS (as a control group) and treated for 24 h. The concentration of CMCS in the media ranged from 1 to 10,000 μg/ml. in order to perform quantitative analysis of cell proliferation, 10 μl of a WST-8 (Cell Counting Kit-8, CCK-8, Dojindo, Japan) solution was added to each well. After the given treatment period, absorbance at 450 nm was monitored with a microplate reader (EL × 800 Absorbance Microplate Reader, USA). The adjusted degree of cell proliferation was calculated by normalizing the optical densities (OD) to those of control cells incubated with PBS. The proportion of proliferating Schwann cells was calculated as follows: proliferating cell (%) = [(Ae − Ab)/(Ac − Ab)] × 100%, where Ae, Ab and Ac refer to the A450 values of the experimental, blank and control groups, respectively. All assays were performed in triplicate.

2.5. BrdU incorporation assay

The DNA synthesis rate was measured with BrdU (5-Bromo-2-deoxyUridine) incorporation by Immunofluorescence. The seeding amount of cells was adjusted to attain a density of 70–80% confluence. 24 h after CMCS treatment, the cells were serum starved overnight, treated with 200 μM BrdU and incubated for about 16 h. The cells were then washed with PBS, fixed in freshly prepared 4%(v/v) paraformaldehyde at RT for 10 min, and permeabilized with 0.5%(v/v) Triton X-100, followed by incubation with DNase I (0.5U/μl) for 30 min at 37 °C. The cells were incubated with primary antibody against BrdU overnight at 4 °C followed b Cy3-conjugated secondary antibody for 1 h at RT. Finally, nuclei were counter-stained with Hoechst 33342 for 15 min, rinsed with PBS for three times and visualized under fluorescent microscopy. Each assay was performed in quadruplet and repeated three times.

2.6. Reverse transcriptase-polymerase chain reaction

Total cellular mRNA was extracted from the cultured Schwann cells that had been induced by CMCS using TRIzol reagent according to the manufacturer's instructions. The mRNA was reverse-transcribed to cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Primer sequences used in this context are listed in Table 1. Polymerase chain reaction (PCR) amplification was performed with following parameters: 30 amplification cycles for NGF and Dvl-1 (94 °C for 45 s followed by 60 °C for 45 s, then 72 °C for 1 min); 30 amplification cycles for β-catenin and cyclin D1 (94 °C for 45 s followed by 62 °C for 45 s, then 72 °C for 1 min); 26 cycles for c-myc and β-actin (94 °C for 45 s followed by 55 °C for 45 s, then 72 °C for 1 min). The PCR products (5 μl) were subjected to electrophoresis in 2% agarose gels, and detected by staining with ethidium bromide. The relative intensity of the products was quantified using the Bio-Image Visage 110 system (BioRad, Hercules, CA, USA).

Table 1
Sequences of specific primers: F = forward primer; R = reverse primer.

Gene	Primer sequence	Amplification size (bp)
NGF F	5′-TCCAGGTGCATAGCGTAATG-3′	376
NGF R	5′-CTCCGGTGAGTCCTGTTGAA-3′	
Dvl-1 F	5′-CGCCGGGAGGCCCGCAAGTA-3	327
Dvl-1R	5′-AGCGGGTCGACAAGAAGCGAT-3′	
β-Catenin F	5′-CTGGAAGAAATTCGAGCTG-3′	476
β-Catenin R	5′-ACATACAGTCCTGGATGATGA-3′	
c-myc F	5′-GTGGCCTCTAAGATGAAGGAGA-3′	260
c-myc R	5′-AGAACGTCGACCCCAAGGCCAC-3′	
Cyclin D1 F	5′-CATTGACCTCAACTACATGGT-3′	488
Cyclin D1 R	5′-TGGTGTCTTCACCACCATGG-3′	
β-Actin F	5′-CCTGACCGAGCGTGGCTACAGC-3′	205
β-Actin R	5′-AGCCTCAGGGCATCGGAAC-3′	