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Expression and purification recombinant human dentin sialoprotein in *Escherichia coli* and its effects on human dental pulp cells

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ABSTRACT

Dentin sialoprotein (DSP) is cleaved from dentin sialophosphoprotein (DSPP) and most abundant dentinal non-collagenous proteins in dentin. DSP is believed to participate in differentiation and mineralization of cells. In this study, we first constructed recombinant human DSP (rhDSP) in *Escherichia coli* (*E. coli*) and investigated its odontoblastic differentiation effects on human dental pulp cells (hDPCs). Cell adhesion activity was measured by crystal violet assay and cell proliferation activity was measured by MTT assay. To assess mineralization activity of rhDSP, Alizarin Red S staining was performed. In addition, the mRNA levels of collagen type I (Col I), alkaline phosphatase (ALP), and osteocalcin (OCN) were measured due to their use as mineralization markers for odontoblast-/osteoblast-like differentiation of hDPCs. The obtained rhDSP in *E. coli* was approximately identified by SDS–PAGE and Western blot. Initially, rhDSP significantly enhanced hDPCs adhesion activity and proliferation ($p < 0.05$). In Alizarin Red S staining, stained hDPCs increased in a time-dependent manner. This odontoblastic differentiation activity was also verified through mRNA levels of odontoblast-related markers. Here, we first demonstrated that rhDSP may be an important regulatory ECM in determining the hDPCs fate including cell adhesion, proliferation, and odontoblastic differentiation activity. These findings indicate that rhDSP can induce growth and differentiation on hDPCs, leading to improve tooth repair and regeneration.

Introduction

Dentin sialophosphoprotein (DSPP)[2] is a principal protein of dentin extracellular matrix in tooth [1]. DSPP is predominantly expressed by odontoblasts and widely used as a unique marker for odontoblast [2,3]. To date, full-length DSPP have never been isolated or identified in dentin. DSPP is mainly cleaved into dentine sialoprotein (DSP) and dentin phosphoprotein (DPP) [4]. DSP is the amino-terminal part of DSPP and primarily found in the dentin [5]. DSP is first identified as a 95 kDa glycoprotein, which that was 29.6% carbohydrate, of which 9% was sialic acid [6]. It has been reported that both porcine and bovine DSP are chondroitin sulfate-type proteoglycans [7,8]. Generally, DSP has similar characteristics with other sialoproteins such as osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein-1 (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE) due to similar amino acid composition. DSP has little or no effect on formation and growth of hydroxyapatite crystals *in vitro* [9]. However, DSP induces initial dentin mineralization [10] and enhances the mechanical properties of dentino-enamel junction (DEJ) *in vivo* [11]. Furthermore, recombinant DSP induces differentiation and mineralization on human dental periodontal ligamental stem cells and mouse dental papilla mesenchymal cells [12].

Human dental pulp cells (hDPCs) can proliferate and differentiated into dentin-forming odontoblasts, owing to contain progenitor/stem cells [13–15]. Subsequently, odontoblasts can form reparative or secondary dentin, and the human dental pulp healing and repair processes are successively performed through proliferation, chemotaxis, and differentiation of human dental pulp cells into odontoblasts to regenerate dentin formation [16]. Generally,

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[2] *Abbreviations used:* DSPP, dentin sialophosphoprotein; DSP, dentine sialoprotein; DPP, dentin phosphoprotein; OPN, osteopontin; BSP, bone sialoprotein; DMP-1, dentin matrix protein-1; MEPE, matrix extracellular phosphoglycoprotein; DEJ, dentino-enamel junction; hDPCs, human dental pulp cells; OCN, osteocalcin; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; TGF-β, transforming growth factor-beta; rhDSP, recombinant human DSP; Col I, collagen type I; BSA, bovine serum albumin; PBS, phosphate-buffered saline; C_T, cycle threshold; ON, osteonectin.

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osteocalcin (OCN), alkaline phosphatase (ALP), OPN, BSP, DMP-1, DSPP, and MEPE have been used as markers for odontoblast-/osteoblast-like differentiation of hDPCs [14,17]. Recently, various growth factors such as bone morphogenetic protein (BMP) and transforming growth factor-beta (TGF-β) have been used to confirm the synergistic odontoblast healing and repair effects on hDPCs [18,19].

In the present study, we constructed recombinant human DSP (rhDSP) in *Escherichia coli* (*E. coli*) and investigated the cell adhesion and proliferation activity of rhDSP on hDPCs. In addition, the odontoblastic differentiation activity of rhDSP on hDPCs was measured by using alizarin reds. Further, the mRNA levels of collagen type I (Col I), ALP, and OCN of rhDSP with hDPCs were measured.

Material and methods

Construction of expression plasmid of rhDSP

The gene encoding DSP was amplified by PCR. Briefly, PCR primers were designed to recognize the human DSP: DSP upstream primers, 5′-CTAGATCTCAATAGAAATCAAGGGTC-3′; DSP downstream primers, 5′-TGGTACCATTATCCTTGCATGGACTTA-3′. PCR was performed in a 30 μl reaction sample containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.2 mM dNTPs, 1.25 U of Taq polymerase (iNtRON, Seoul, Korea), and 50 pmol each of the forward and reverse primers. The thermocycling parameters used in PCR were as follows: annealing, 1 min at 55 °C; extension, 1 min at 72 °C; and denaturation, 1 min at 94 °C. After 30 cycles, amplified PCR products were digested with *Bgl*II and *Kpn*I. After digestion, PCR product was ligated into the *Kpn*I sites of pBAD-HisA vector (Invitrogen, Carlsbad, CA, USA), producing a construct (pBAD-HisA-DSP).

Production and purification of rhDSP

For the expression of rhDSP, TOP10 cells were grown overnight in LB-Amp medium at 37 °C. When the cultures reached an A_{600} = 0.6, induction was initiated with 0.02% (w/v) L-arabinose as inducer. After 3 h, bacteria were pelleted by centrifugation, lysed, and sonicated. A soluble extract was prepared by centrifugation for 30 min at 14,000*g* in a refrigerated centrifuge, and the supernatant was transferred to a fresh tube.

The crude protein from the sonicated bacterial supernatant was purified through binding of the hexahistidine tag (located at the amino-terminal end of the protein) to the nickel–nitrilotriacetic acid resin column, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The degree of purification of the recombinant protein was examined under denaturing conditions, as determined by coomassie blue staining of 12% (v/v) SDS–PAGE.

Western blots were performed using a peroxidase conjugate of a monoclonal anti-polyhistidine antibody (sc-8036, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to confirm the expression of the rhDSP. The molecular sizes of the obtained protein were verified by comparison to the migration of pre-stained protein markers (Elpis Biotech, Daejeon, Korea) electrophoresed in parallel lanes.

Isolation of hDPCs

hDPCs were collected from the third molar teeth of adult patients with ages ranging from 19 to 25 years old at the Dental and Craniofacial Clinic of the School of Dentistry in Dankook University and used with the patients' informed consent [20]. Briefly, the pulp was separated from the crowns and roots, minced, and digested in a solution of 3 mg/ml type I collagenase and 4 mg/ml dispase for 1 h at 37 °C. Single-cell suspensions were then obtained by passing these cells through a 70 μm strainer.

hDPCs were cultured in α-minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37 °C. Confluent cells were detached with 0.25% trypsin–EDTA for 5 min, and aliquots were subcultured. hDPCs maintained for 3–4 passages were used for further cell proliferation and differentiation study.

Cell adhesion assay

Before cell seeding, rhDSP was coated on 24-well plates overnight at 4 °C. hDPCs were cultured at a density of 5×10^4 cells/well in 24-well plates and blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min and washed with PBS. After 50 min at 37 °C, adherent cells were washed twice with PBS, fixed with 3% paraformaldehyde (Sigma, St. Louis, MO, USA), and stained with 0.25% (w/v) crystal violet in 2% (v/v) ethanol/water. After extensive washing with distilled water, plates were allowed to dry. The A_{570} was read, and non-specific adhesion was determined in wells coated with 1% BSA as a negative control.

Cell proliferation assay

Cell proliferation activity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a yellow tetrazole) assay, which measures the number of viable cells, according to the manufacturer's instructions. hDPCs were cultured at a density of 1×10^3 cells/well in 24-well plates for 5 and 10 days. At the end of culture, 500 μl MTT solutions (5 mg/ml in PBS) were added to each well. After 4 h of incubation, the media was removed, and the formazan crystals were solubilized in 200 μl DMSO. The absorbance of each well was read at 540 nm using a microplate reader.

Cell differentiation assay

To assess odontoblastic differentiation activity of rhDSP, mineralization activity was measured by Alizarin Red S staining. hDPCs were incubated at a density of 1×10^3 cells/well for 7 and 14 days in differentiation-inducing media without or with rhDSP. Differentiation-inducing media contained 100 μmol/l/ml ascorbic acid, 2 mmol/l β-glycerophosphate, and 10 nmol/l dexamethasone. Then, hDPCs were washed with PBS and fixed in 95% methanol for 30 min. Next, cells were stained in Alizarin Red S solution overnight and washed three times and incubated in PBS for 15 min to eliminate nonspecific staining. Stained cells were photographed at 0, 7, and 14 days.

RNA extraction and cDNA synthesis

Total RNA was extracted using Easy-spin RNA Extraction kit (iNtRON, Seoul, Korea), according to the manufacturer's instruction. The purity of RNA was assessed by absorption at 260 and 280 nm (values of the ratio of A_{260}/A_{280} of 1.9–2.1 were considered acceptable) and by ethidium bromide staining of 18 S and 28 S RNA on gel electrophoresis. RNA concentrations were determined from the A_{260}. Two micrograms of total RNA were reverse-transcribed in a 20 μl sample of the reaction mixture containing 50 U of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 5 μm DTT, 40 U of RNaseOUT recombinant ribonuclease inhibitor, 0.5 μm of random hexanucleotide primers, and 500 μm of dNTP mixture. The reverse transcription reaction was conducted at 50 °C for 60 min. Subsequently, the reaction mixture was heated