Nel-like Molecule 1 Contributes to the Odontoblastic Differentiation of Human Dental Pulp Cells

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Abstract

Introduction: Nel-like molecule-1 (Nell-1) with epidermal cell growth factor–like coding structures is a recently discovered secreted protein that can specifically induce osteoblast differentiation, bone formation, and bone regeneration. The expression and localization of Nell-1 during murine molar development suggest that this protein participates in odontoblastic differentiation. This study aims to assess the expression of Nell-1 in the pulp-dentin complex of human teeth and determine the effect of human recombinant Nell-1 (hrNell-1) on odontoblastic differentiation of cultured human dental pulp cells. **Methods:** Immunohistochemical assay was performed to detect the expression and location of Nell-1 in healthy human teeth. The activity of alkaline phosphatase in human dental pulp cells stimulated by hrNell-1 was examined. Moreover, the expression of related odontoblastic markers was evaluated using quantitative real-time polymerase chain reaction and Western blot. **Results:** Immunohistochemical results showed that Nell-1 was primarily expressed in the odontoblasts (odontoblastic bodies and processes), pulp fibroblasts, and endothelial cells of the blood vessels in human teeth. *In vitro* experiments showed that hrNell-1 could increase the activity of alkaline phosphatase and enhance the expression of important odontoblastic markers including osteopontin and dentin matrix protein 1 in cultured human dental pulp cells. **Conclusions:** Nell-1 plays a role in odontoblastic differentiation of human dental pulp cells and dentin formation. *(J Endod 2016;42:95–100)*

Key Words

Human dental pulp cell, Nel-like molecule-1, odontoblastic differentiation, pulp-dentin complex

Dentin is the mineralized tissue that constitutes the bulk of the tooth and encloses and protects the dental pulp. Given their close anatomic and functional connections, dentin and pulp are often referred to as the pulp-dentin complex (1). After tooth eruption, the pulp-dentin complex protects the tooth pulp and maintains the normal physiological function of the tooth during secondary and tertiary dentin formation. Under normal conditions, secondary dentin formation occurs throughout the life of teeth immediately after primary dentin formation (1, 2). Meanwhile, tertiary dentin can be produced by surviving odontoblasts or newly formed odontoblastlike cells differentiated from dental pulp stem cells to protect the dental pulp from further injury when suffering from external stimuli such as caries and attrition (2–4).

Recent studies have discovered several biological signal molecules that are involved in odontoblast differentiation and dentin formation such as member 10A of wingless-type mouse mammary tumor virus (MMTV) integration site family, silent mating type information regulation 2 homologue 1 (SIRT1), bone morphogenetic proteins, adseverin, LIM domain protein 1, osterix, and integrin αv (5–12). However, the precise mechanisms underlying odontoblast differentiation and dentin formation remain poorly understood. Other potential molecules might also be involved in the formation and function of the pulp-dentin complex.

Nel-like molecule-1 (Nell-1) is a newly identified secreted protein in craniosynostosis (13, 14). Nell-1 promotes osteoblast differentiation and mineralization by regulating the expression of related genes of osteoblast differentiation *in vitro* (15, 16). This protein induces bone formation and regeneration and has been successfully used in bone regeneration both in small and large animal models (14, 17). Odontoblasts and osteoblasts share several similarities in some aspects, including origin (both differentiating from mesenchymal cells) and protein profile. In addition, Nell-1 is directly regulated by Runt-related transcription factor 2 (Runx2), an important transcription factor that controls bone and tooth development (18, 19). Therefore, we hypothesize that Nell-1 is associated with odontoblast differentiation and dentin formation. We previously investigated the spatiotemporal expression of Nell-1 in murine molar development and found its immunoreactivities during odontoblast differentiation (20). However, the precise role of Nell-1 in odontoblast differentiation remains undetermined.

Accordingly, the present study explored the role of Nell-1 in odontoblastic differentiation through *in vivo* and *in vitro* experiments. The immunohistochemical localization of Nell-1 was investigated in the pulp-dentin complex of human teeth to preliminarily assess the expression and function of Nell-1 in odontoblast differentiation and dentin formation. The effect of Nell-1 was explored by measuring the activity of alkaline phosphatase (ALP) and the expression of odontoblastic markers under the stimulation of human recombinant Nell-1 (hrNell-1) in cultured human dental pulp cells (HDPCs).

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Materials and Methods

Tooth Sample Collection, Preparation, and Hematoxylin-Eosin Staining

This study was approved by the Institutional Review Board of School of Stomatology, Shandong University, Shandong, China. A written consent form was obtained from every patient included in the study. Fifteen fully developed and erupted healthy third molars (wisdom teeth) with closed apices were obtained from young adult patients who were scheduled for routine extraction. The healthy pulp status of teeth was determined by clinical history, radiographs, thermal tests, and histologic staining.

The obtained teeth were immediately placed in 0.1 mol/L phosphate-buffered saline (PBS) after extraction and then washed 3 times. One third of the apical root was removed using diamond burs. Then, the teeth were fixed with 4% paraformaldehyde in 0.1 mol/L PBS for 48 hours at 4°C, demineralized for 12 weeks in 10% EDTA, and embedded in paraffin. Serial longitudinal sections 5 μm in thickness were prepared and mounted on poly-L-lysine–coated glass slides.

Hematoxylin-eosin staining was performed as follows. Tissue sections were deparaffinized in xylene, hydrated through gradient alcohol, and then washed with tap water. The sections were stained with hematoxylin and differentiated with hydrochloric acid alcohol solution. After the sections were stained with eosin, they were dehydrated with gradient alcohol. The sections were then immersed in xylene and coverslipped with resin.

Immunohistochemistry

Immunohistochemistry staining was performed in accordance with the instructions of the Power Vision Histostain Streptavidin-Peroxidase kit (Zhongshan, Beijing, China). First, endogenous peroxidase activity of the tissues was blocked with 3% H_2O_2 for 25 minutes. Antigen retrieval was performed with 0.1% (w/v) trypsin (Zhongshan) at 37°C for 10 minutes. After pretreatment with normal goat serum (Zhongshan) for 25 minutes to block nonspecific binding, the sections were incubated with Nell-1 antibody (1:50 dilution; Proteintech Group, Inc, Wuhan, China) at 4°C overnight. The sections were then incubated with biotinylated goat anti-rabbit immunoglobulin G and streptavidin-peroxidase conjugate (Zhongshan) at 37°C for 25 minutes. Diaminobenzidine solution (Zhongshan) was used to visualize localization for 2 minutes. Finally, the sections were lightly counterstained with hematoxylin. Negative controls were obtained by replacing the primary antibody with PBS.

HDPC Isolation and Culture

Third molars without dental, dental pulp, or periodontal diseases were collected from 18- to 25-year-old patients with informed consent in the Department of Oral and Maxillofacial Stomatology, Shandong University. Then, the tooth was washed 3 times with PBS containing 100 U/mL penicillin and 100 μg/mL streptomycin 3 times. The dental pulp was isolated, aseptically rinsed, shred, and digested with 5.0 mg/mL collagenase II (Invitrogen, Carlsbad, CA) for 20 minutes at 37°C. Loose tissues were inoculated in cell culture flasks with alpha-minimum essential media (Hyclone; GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT) containing 15% fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C atmosphere with 5% CO_2. The cell media were changed every 3 days until the cells grew around the tissues. The cells were subcultured at a ratio of 1:3 when the cell confluence reached about approximately 80%.

ALP Assay

HDPCs were seeded into 12-well plates and cultured with osteogenic medium containing 10% fetal bovine serum, 0.01 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO), 10 mmol/L β-glycerophosphate (Sigma-Aldrich), and 50 μg/mL ascorbic acid (Sigma-Aldrich). The cells were treated with hrNell-1 at concentrations of 0, 50, 100, and 150 ng/mL for 3 days. The cells were then treated with the minimum effective concentration of hrNell-1 and were continued to be cultured for 5 and 7 days. The total protein was collected, and the concentrations of the protein samples were tested using a BCA protein assay kit (Jiancheng Biotechnology, Nanjing, China). The activity of ALP was detected using an ALP detection kit (Jiancheng Biotechnology) in accordance with the manufacturer's instruction.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

HDPCs were planted into 6-well plates and cultured with 100 ng/mL hrNell-1 in triplicate for 0, 6, 12, and 24 hours. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect the changes in RNA levels of odontoblastic differentiation-related molecules. The total RNA in the HDPCs was extracted using TRIzol (TaKaRa, Otsu, Japan) in accordance with the manufacturer's instructions. The concentration and purity of the RNA samples were measured using a Gene Quant Pro UV/visible spectrophotometer (Biochrom Ltd, Cambridge, UK). Then, 1 μg total RNA was reverse transcribed into complementary DNA by using a complementary DNA synthesis kit (TaKaRa). Thereafter, qRT-PCR was performed with SYBR Green I (TaKaRa) and LightCycler 480 real-time PCR instruments (Roche, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The primers used are listed in Table 1.

Western Blot

The cell treatment for Western blot was the same as that for RNA extraction. The cells were washed 3 times with ice-cold PBS and then lysed in RIPA buffer (Beyotime, Shanghai, China) for 30 minutes. The buffer was added with 1 mmol/L phenylmethanesulfonyl fluoride in advance. The protein samples were centrifuged at 12,000*g*/min for 10 minutes, and their concentrations were measured using the BCA protein assay kit (Jiancheng Biotechnology). The samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked in 0.1% Tween 20 in Tris-buffered saline containing 5% nonfat dried milk for 1 hour at room temperature and then incubated overnight with the osteopontin (Opn) antibody (1:800 dilution) and dentin matrix protein 1 (Dmp-1) antibody (1:800 dilution) at 4°C. These antibodies were provided by Dr Larry Fisher of the National Institutes of Health/National Institute of Dental and Craniofacial Research

TABLE 1. Primers Used in Quantitative Real-time Polymerase Chain Reaction

Primer	Sequences (5′ to 3′)
Opn	Forward: CTCCATTGACTCGAACGACTC Reverse: CAGGTCTGCGAAACTTCTTAGAT
Dmp-1	Forward: AGGAAGTCTCGCATCTCAGAG Reverse: TGGAGTTGCTGTTTTCTGTAGAG
Ocn	Forward: AGGGCAGCGAGGTAGTGAAG Reverse: CTCCTGAAAGCCGATGTGGT
Runx2	Forward: TCCACACCATTAGGGACCATC Reverse: TGCTAATGCTTCGTGTTTCCA
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: TGGTGAAGACGCCAGTGGA

Dmp-1, dentin matrix protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ocn, osteocalcin; Opn, osteopontin; Runx2, runt-related transcription factor 2.