Nephronectin Stimulates the Differentiation of MDPC-23 Cells into an Odontoblast-like Phenotype

Jia Tang, DDS, PhD, and Takashi Saito, DDS, PhD

Abstract

Introduction: The present study investigated the *in vitro* effects of nephronectin (Npnt) on the proliferation, differentiation, and mineralization of a rat odontoblast-like cell line (MDPC-23 cells). **Methods:** MDPC-23 cells were cultured on Npnt-coated polystyrene or in the presence of soluble Npnt. Cell proliferation was analyzed using a Cell Counting Kit-8 kit (Dojindo, Kumamoto, Japan). Alkaline phosphatase (ALP) activity was quantified using an ALP activity assay. A reverse-transcription polymerase chain reaction was performed to evaluate the messenger RNA (mRNA) expression level of odontogenic markers and integrin(s). Alizarin red staining was conducted to quantify the calcium deposition. **Results:** Soluble Npnt had no adverse effect on the proliferation of MDPC-23 cells, but it exhibited concentration-dependent inhibitory activity toward differentiation. In contrast, coated Npnt promoted cell proliferation dramatically and significantly up-regulated the mRNA expression of odontogenesis-related genes; moreover, mRNA expression of integrin α1, α3, α5, β1, and β5 was found to be augmented. MDPC-23 cells cultured on Npnt-coated polystyrene displayed markedly higher ALP activity as early as day 3 after inoculation. In addition, mineralization was accelerated on Npnt-coated polystyrene. **Conclusions:** Npnt in its immobilized form enhanced the proliferation of MDPC-23 cells and induced this odontoblastic precursor cell line to differentiate into a mineralizing phenotype. *(J Endod 2017;43:263–271)*

Key Words

Dentin, differentiation, nephronectin, odontoblast

Significance

Npnt is a novel RGD-containing ECM originally discovered in mouse embryonic kidney that is expressed in a variety of embryonic and adult tissues including tooth germ. The effects of Npnt in odontoblasts remain elusive. Our study showed Npnt in its coated form promoted the proliferation, differentiation, and mineralization in MDPC-23 cells, providing new insight into the functional roles of Npnt in the regulation of odontoblast activity.

Nephronectin (Npnt), first identified in mouse embryonic kidney as a potent $\alpha8\beta1$ ligand (1, 2), is an extracellular adhesion protein characterized by a central Arg-Gly-Asp (RGD) sequence, 5 epithelial growth factor–like repeats near the N-terminus, and a COOH-terminal domain with homology to meprin/A5-protein/PTPmu repeats. Although there are a number of $\alpha8\beta1$ ligands, such as fibronectin, vitronectin, tenascin C, and osteopontin (OPN) (3), only the binding of Npnt to $\alpha8\beta1$ is indispensable in nephrogenesis (1, 2, 4). A mutation study revealed that RGD in Npnt is responsible for effective binding with the integrin; the substitution of aspartic acid (D) with glutamic acid (E) dramatically attenuated cell spreading and survival activities (2). Recently, researchers have clarified another minimal binding moiety Phe-Glu-IIe (FEI) (11 residues downstream of RGD in Npnt), which binds $\alpha8\beta1$ synergistically with RGD (5). In addition to its presence in the kidney, Npnt was found to be distributed in other embryonic and adult tissues (eg, fetal ear, eye, heart, and lung and adult lung, brain, uterus, placenta, thyroid gland, and blood vessels) (6). Furthermore, a multitude of functions of Npnt have also been unveiled, including involvement in osteoblast differentiation (7–11), muscle cell fusion (12), piloerection (13), kidney development (1, 2, 14), forelimb formation (15), cardiovascular development (16), progression of malignant melanoma (17), acute tubular necrosis (18), and deterioration of diabetic glomerulosclerosis (19). The broad distribution and multiple functions of Npnt suggest that this extracellular protein has wider roles in tissue and organ development.

Traditional therapy of exposed teeth by direct pulp capping often involves the direct delivery of calcium hydroxide to the exposed site to promote wound healing. However, methods based on this approach suffer from undesirable side effects, including high alkalinity, porous dentin formation, and poor adhesion to dentin, which could lead to unsatisfactory clinical prognosis over time. In order to improve the success rate of pulp capping treatment and the life quality for patients, efforts have been ongoing attempting to identify novel biocompatible pulp capping reagents. Materials capable of inducing odontoblast proliferation and differentiation are particularly

From the Division of Clinical Cariology, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan.

Address requests for reprints to Dr Jia Tang, Division of Clinical Cariology, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu 060-0293, Hokkaido, Japan. E-mail address: tangjia@hoku-iryo-u.ac.jp

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2016.10.028

promising. As an RGD-containing cell adhesion protein, Npnt is capable of mediating adhesion and affecting the proliferation of a variety of cell types, such as mouse vascular endothelial cells (SVEC4-10), human fibrosarcoma cells (HT-1080) (4), and human neuroglioma cells (H4) (5). Moreover, it was reported that Npnt is expressed in the outer enamel epithelium, stellate reticulum, and the inner enamel epithelium of mouse tooth germ (2). Tooth development is a reciprocal process involving a mutual interaction between the epithelium and mesenchyme (ie, dental papillae). Given the expression of Npnt in the inner enamel epithelium, under which odontoblasts are located, it is of interest to investigate how Npnt interacts with odontoblasts (dentin-producing cells). Elucidation of the role of Npnt in odontoblasts could be useful for direct pulp capping; furthermore, we speculated that Npnt might affect the behavior of odontoblasts. Thus, the objective of this experiment was to investigate the effect of Npnt on the proliferation, differentiation, and mineralization of odontoblast-like cells. To that end, we coated non-tissue culture polystyrene dishes with Npnt or directly added soluble Npnt to cell culture medium and evaluated the efficacy of Npnt in enhancing rat odontoblast-like cell activity in terms of cell proliferation, alkaline phosphatase (ALP) activity, odontogenic as well as integrin(s) gene expression, and alizarin red staining.

Materials and Methods

Cell Culture

MDPC-23 cells (20), a rat odontoblast-like cell line, was used. The cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C, 5% CO_2 in a humidified atmosphere. The medium was changed every other day. The cells used in this study were obtained from passages 20 to 30. Recombinant mouse Npnt was purchased from R&D Systems (Minneapolis, MN) (catalog number: 4298-NP), reconstituted in phosphate-buffered saline (PBS, Gibco) at a stock concentration of 100 μg/mL, aliquoted, and then stored at −30°C until use. The control group is defined as cells that were treated only with vehicle (PBS).

Npnt Coating on Non-tissue Culture Polystyrene

Non-tissue culture polystyrene plates (96-well plate, catalog no. 351177; 12-well plate, catalog no. 351143; Falcon, Corning, NY) coated with Npnt were prepared by soaking the plates in 10 μg/mL Npnt (dissolved in PBS, 50 μL/well for the 96-well plate and 500 μL/well for the 12-well plate). After coating for 2 hours or overnight, the solution was aspirated, and the wells were washed twice with PBS (100 μL/well for the 96-well plate each time and 1 mL/well for the 12-well plate each time). The cells were rinsed with PBS, trypsinized, and seeded into the 96-well plate (500/well, 100 μL culture media/well) and the 12-well plate (2.5×10^4/well, 2 mL culture media/well). The cells were cultured in DMEM supplemented with 5% FBS for the experiment. Mineralization reagent (10 mmol/L β-glycerophosphate and 50 μg/mL ascorbic acid; Wako, Osaka, Japan) was added from day 5 on. Cells seeded in wells coated with PBS served as the control.

Cell Viability Assay in the Presence of Soluble Npnt

Cells were seeded into a 96-well plate at 1×10^3 cells per well. After incubation for 24 hours, various concentrations of Npnt (1, 10, 100, and 1000 ng/mL) were added into the serum-free culture medium, and the cells were incubated for another 24 hours. Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added to each well to a volume of 10% (10 μL/well) followed by incubation for 1 hour 45 minutes at 37°C, 5% CO_2 in a humidified atmosphere. The optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

Cell Proliferation Assay

For soluble Npnt, cells were inoculated into a 96-well plate (tissue culture treated, code: 3860-096; Iwaki, Tokyo, Japan) at an initial number of 0.5×10^3 cells per well. After incubation for 24 hours, various concentrations of Npnt (1, 10, and 100 ng/mL) in DMEM supplemented with 1% FBS were added to the cells (day 0). CCK-8 reagent (10 μL) was added to each well at prescribed time points (days 1, 2, 3, and 4) and incubated for 1 hour 45 minutes; then, the absorbance was read at 450 nm (Bio-Rad).

For the Npnt-coated well, cells were seeded into a 96-well plate (0.5×10^3/well) (non-tissue culture polystyrene, catalog no. 351177; Falcon). The absorbance of the medium was read on days 1, 2, 4, and 6 after adding the CCK-8 reagent as described previously.

Cell Morphology Observation of the Npnt-coated and Control Groups

Photographs of the cells were taken using light microscopy (Olympus) after 2 and 3 days of culture in an Npnt-coated 12-well plate (catalog no. 351143, Falcon) and control wells. For fluorescence staining, MDPC-23 cells were seeded into a 24-well plate (non-treated tissue culture polystyrene, 1820-024; Iwaki) (5×10^3/well). On day 3, F-actin and the nucleus were visualized by staining with Alexa Fluor 568 phalloidin (A12380; Invitrogen, Carlsbad, CA) and 4',6-diamidino-2-phenylindole (D9542; Sigma-Aldrich, St Louis, MO), respectively. Briefly, cells were fixed with 4% formaldehyde (16%, methanol-free, 28906; Thermo Fisher Scientific, Waltham, MA) for 15 minutes and permeabilized with Triton X-100 (0.1%, v/v, in PBS) (T8787-100 mL, Sigma-Aldrich) for 5 minutes. Alexa Fluor 568 phalloidin was reconstituted in 1.5 mL methanol to generate the stock solution (200 U/mL). The stock solution was subsequently diluted to 10 U/mL and added into a 24-well plate (200 μL/well). Tween 20 (0.05%, v/v) (Kanto Chemical, Tokyo, Japan) in PBS was used to wash the cells after 1 hour of incubation in room temperature. Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole (300 nmol/L in PBS) (Sigma-Aldrich) for 5 minutes and washed thoroughly with Tween 20 in PBS. Fluorescence photographs were taken using EVOS FLoid Cell Imaging Station (Advanced Microscopy Group, Mill Creek, WA).

ALP Activity

For soluble Npnt, cells were seeded into 35-mm tissue culture dishes (5×10^4/dish) (catalog no. 353001, Falcon) and incubated for 5 days in DMEM supplemented with 10% FBS. On day 5, 10 mmol/L β-glycerophosphate and 50 μg/mL ascorbic acid were added to serum-free DMEM (IM), and various concentrations (1, 10, and 100 ng/mL) of Npnt were also added to the IM. On day 6, the cells were removed from the culture plate using Triton-X 100 (0.1%, w/w, in distilled water) (Sigma-Aldrich) and sonicated (Bioruptor; Diagenode, Seraing, Belgium) for 10 minutes on ice. The lysates were centrifuged for 15 minutes at 10,483*g* at 4°C (Hitachi Koki, Tokyo, Japan). The resulting supernatant was diluted and assayed for ALP activity (300× dilution) (Wako) and BCA protein quantification (3× dilution) (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Absorbance was read at 405 nm and 570 nm for the ALP assay and the protein assay, respectively.

For the Npnt-coated group, cells were seeded (2.5×10^4/well) into a non-tissue culture 12-well plate treated with Npnt and cultured in DMEM supplemented with 5% FBS. IM was added on day 5. Samples were collected on days 3 and 7 using the method described previously. The supernatant was diluted and used for the ALP activity assay (300× dilution for samples from days 3 and 7) and the BCA protein quantification assay (no dilution for samples from day 3 and 2× dilution for