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Nanohydroxyapatite increases BMP-2 expression *via* a p38 MAP kinase dependent pathway in periodontal ligament cells

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ABSTRACT

Objective: Bone morphogenetic protein (BMP)-2 promotes the osteoblastic differentiation of human periodontal ligament (PDL) cells, which play a pivotal role in periodontal regeneration. Recently, nano-sized hydroxyapatite (nano-HA) has been highlighted due to its advantageous features over micro-sized materials.

Design and results: We investigated the effect of nano-HA on BMP-2 expression in human PDL cells. Real time PCR analysis revealed that the expression of BMP-2 increased upon stimulation with nano-HA in dose- and time-dependent manners. An immunofluorescence assay demonstrated the synthesis of BMP-2 proteins. Concentrations of Ca^{2+} as well as phosphate (Pi) in culture supernatants were unchanged, suggesting that nano-HA functioned as a nanoparticle rather than as a possible source for releasing Ca^{2+} and/or Pi extracellularly, which were shown to also enhance the expression of BMP-2. Nano-HA-induced BMP-2 expression was dependent on the p38 MAP kinase pathway because increases in BMP-2 expression were inhibited by treatment with SB203580, a p38 inhibitor, and phosphorylation of p38 was detected by Western blotting.

Conclusions: This novel mechanism of nano-HA will be important for the rational design of future periodontal regeneration.

1. Introduction

During the process of periodontal tissue regeneration, periodontal ligament (PDL) cells are regarded to have the capacity to differentiate into cementoblasts or osteoblasts depending on the need, forming cementum or alveolar bone.[1] Recently, research has focused on the regeneration of periodontal tissue using growth factors, among which is bone morphogenetic protein (BMP)-2. BMP-2 has been studied extensively for its various biological functions, particularly during osteogenic differentiation.[2] It has been reported that periodontal ligament cells are negatively regulated for mineralization by expressing msh homeobox 2 (Msx-2)[3] and Twist[4]; however, BMP-2 can partly overcome this and is capable of inducing osteo/cementoblastic differentiation in PDL cells[5] and dental follicle cells, which are regarded as their precursor cells[6]. Previous reports showed that direct application of recombinant human BMP-2 (rhBMP-2) enhances the formation of not only alveolar bone, but also cementum and the insertion of periodontal ligament fibres in experimental animal studies.[7–9]

Hydroxyapatite, a synthetic calcium phosphate biomaterial, is commonly used as a bone graft material because of its

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high biocompatibility and osteoconductivity. However, it is not considered to be a favourable biomaterial due to its low resorption rate. Recently, nano-sized hydroxyapatite (nano-HA) has been highlighted due to its advantageous features over conventional micro-sized materials. Nano-HA has the potential to act as a carrier of therapeutic agents, enabling controlled drug release extracellularly or intracellularly, and at the same it has high absorbability in the body for the regeneration of hard tissue.[10] Moreover, it may be possible to form a three-dimensional scaffold suitable for periodontal tissue by combining it with synthetic absorbable biomaterials.[10] Also, nano-HA presents one of the most viable options for gene delivery systems.[11] Furthermore, unlike most metallic nanoparticles, which have all been shown to induce damage to DNA and decrease cell viability,[12] nano-HA belongs to a class of the safest nanomaterials. Recent research showed that nano-HA enhances adhesion and proliferation in human PDL cells.[13] However, still only limited data is available on the molecular mechanisms or pathway by which nano-HA acts on the cell functions of human PDL cells.

In this study, we demonstrated, for the first time, that nano-HA increases BMP-2 expression at gene and protein levels through activation of the p38 mitogen-activated protein (MAP) kinase pathway.

2. Materials and methods

2.1. Reagents

Hydroxyapatite-nanopowder ($\varphi < 200$ nm), brefeldin A, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PD98059 and SB203580 were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA).

2.2. Cell culture

Human PDL cells were obtained from periodontal ligaments of three fully erupted lower third molar teeth from females and males (aged between 19 and 29) without clinical signs of inflammation in the periodontal tissues at Tohoku University Hospital after obtaining informed consent. Periodontal ligaments were dissected from the middle third of the root with a sharp blade, and cultured in α-Minimum Essential Medium (MEM) (Gibco BRL, Rockville, MD, USA) with 10% heat-inactivated foetal bovine serum (FBS) and antibodies, with a medium change every 3 days until confluent cell monolayers were formed. After reaching confluency, cells were passaged with 0.25% trypsin–0.1% EDTA. PDL cells were used as confluent monolayers for experiments at subculture levels 3–10. Experimental procedures were approved (approval number: 20–19) by the Ethical Review Board of Tohoku University Graduate School of Dentistry (Sendai, Japan).

2.3. Treatment with nano-HA

Nano-HA suspended in culture medium at a concentration of 20 mg/ml was sonicated for several seconds, and sterilized under ultraviolet light. The nano-HA suspension was added into a confluent monolayer of PDL cells cultured on a 6-cm diameter culture dish containing 3 ml of culture medium with 5% FBS and 50 μg/ml ascorbic acid, and was then cultured for the indicated times.

2.4. Reverse transcription and real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was extracted using RNeasy® (QIAGEN Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions, and was treated with DNase (DNA-free™, Ambion Inc., Austin, TX, USA). Total RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit® (Roche Diagnostic Co., Indianapolis, IN, USA) to synthesize cDNA. Primer sequences for each human gene encoding BMP-2, -3, -4, -7, inhibitor of DNA binding/differentiation-1 (Id-1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows (forward/reverse): *BMP-2* (5′-CGAAATTCCCCGTGAC-3′/5′-AGTTACTAGCAATGGCCT-3′); *BMP-3* (5′-AATTCCAGCTATTTCACACCC-3′/5′-CACTTAAACCAAAGCGAATGC-3′); *BMP-4* (5′-ACTACATGCGGGATCT-3′/5′-AGATCGCCTCGTTCTCA-3′); *BMP-7* (5′-CGGTTTATCCTGATGCGTA-3′/5′-GGAGCAGACATTTGCTCTTT-3′); *Id-1* (5′-ACATGAACGGCTGTTACT-3′/5′-GGGTTCCAACTTCGGATT-3′); *GAPDH* (5′-TGAACCATGAGAAGTATGACAACA-3′/5′-TCTTCTGGGTGGCAGTG-3′). For real-time PCR, the amplification profile was 40 cycles at 95/60; 55/30; and 72/30 [temperature (°C)/time (s)]. PCR was performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBR Green Supermix® (Bio-Rad). After amplification, one cycle of linear temperature gradient from 55 to 95 °C at a transition rate of 0.5 °C/30 s was performed to assess the specificity of the PCR products. Relative expression levels of transcripts were shown after normalization to the corresponding sample expression level of GAPDH.

2.5. Immunofluorescence assay

Confluent monolayer cells on a poly-L-lysine-coated glass bottom dish (Matsunami glass LTD, Osaka, Japan) were fixed with 2% paraformaldehyde for 15 min followed by incubation with phosphate buffered saline (PBS) containing 0.25% Triton X-100 (PBS-T) for 10 min to permeabilize the cells, and were then incubated in PBS-T containing 5% (w/v) normal donkey serum (Abcam Inc., Cambridge, MA, USA) for 30 min to block non-specific binding. Next, cells were incubated with a 1:500 dilution of rabbit polyclonal antibody to BMP-2 (Abcam Inc.) for 2 h followed by a 1:1000 dilution of Alexa Fluor® 488 conjugated-goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. After incubating with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 1 min to identify nuclei, BMP-2 staining was evaluated by immunofluorescence microscopy fitted with the mirror units of U-MWIBA2 with 460–495/510–550 and U-MWU2 with 330–385/420 [excitation (nm)/emission (nm)]. All procedures were performed at room temperature. Immunostaining without the first antibody was used as a negative control.

2.6. Measurement of [Ca^{2+}] and [Pi]

Supernatants from cell cultures were harvested by centrifugation. The concentration of Ca^{2+} and inorganic phosphate (Pi)