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Asporin in compressed periodontal ligament cells inhibits bone formation

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ABSTRACT

Objective: During orthodontic tooth movement, bone resorption and inhibition of bone formation occur on the compressed side, thereby preventing ankylosis. Periodontal ligament (PDL) cells control bone metabolism and inhibition of bone formation on the compressed side by secreting bone-formation inhibitory factors such as asporin (ASPN) or sclerostin (encoded by *SOST*). The aim of this study was to identify the inhibitory factors of bone formation in PDL cells.
Design: In vitro, the changes in expression of ASPN and *SOST* and subsequent protein release in human PDL (hPDL) cells were assessed by semi-quantitative polymerase chain reaction (PCR), real-time PCR, and immunofluorescence in hPDL cells subjected to centrifugal force using a centrifuge (45, 90, 135, and $160 \times g$). *In vivo*, we applied a compressive force using the Waldo method in rats, and examined the distribution of ASPN or sclerostin by immunohistochemistry.
Results: In vitro, hPDL cells subjected to $90 \times g$ for 24 h demonstrated upregulated *ASPN* and downregulated *SOST* expressions, which were confirmed by immunofluorescent staining. In addition, the formation of mineralized tissue by human osteoblasts was significantly inhibited by the addition of medium from hPDL cells cultured during compressive force as well as the addition of equivalent amounts of ASPN peptide. *In vivo*, asporin-positive immunoreactive PDL cells and osteoclasts were found on the compressed side, whereas few sclerostin-positive PDL cells were observed.
Conclusions: PDL cells subjected to an optimal compressive force induce the expression and release of ASPN, which inhibits bone formation during orthodontic tooth movement on the compressed side.
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1. Introduction

During the application of orthodontic force to teeth, bone resorption is dominant on the compressed side, whereas bone formation is dominant on the tension side. Upon application of mechanical forces, the root shifts toward the alveolar wall, resulting in reduced vasculature to the area (Vandevska-Radunovic, 1999). In this manner, a cell-free or hyalinized zone is formed temporally, which ultimately results in tooth movement (Melsen, 2001). To maintain the optimal periodontal ligament (PDL) distance, PDL cells on the compressed side release osteoclast inductive molecules such as interleukin-1 (IL-1) or receptor activator of nuclear factor kappa-B ligand (RANKL) (Nakao et al., 2007; Pavasant & Yongchaitrakul, 2011; Sokos, Everts, & de Vries, 2015; Yamaguchi, 2009), resulting in osteoclast resorption of alveolar bone. If excessive force is applied, the root of the tooth and alveolar bone become attached and form ankylosis (Krishnan & Davidovitch, 2006; Lindskog, Pierce, Blomlof, & Hammarstrom, 1985). To avoid ankylosis, compressed PDL cells secrete inhibitory factors for osteoblasts and activating factor for osteoclasts.

Mechanical stress produces a compressive force (CF) that affects osteoblastic bone formation (Kawashima, 2000; Naito, Matsuzaka, Ishigami, & Inoue, 2009; Robling et al., 2008). However, during orthodontic tooth movement, no activation of bone formation is observed on the compressed side. These contradictory findings may be explained by active inhibition of bone formation on the compressed side, which is likely inhibited by factors from the compressed PDL cells. The candidate inhibitory molecules for osteoblastic bone formation by PDL cells are sclerostin (encoded by *SOST*) (Jager, Gotz, Lossdorfer, & Rath-Deschner, 2010; Tu et al.,

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2012) and periodontal ligament-associated protein 1/asporin (PLAP-1/ASPN) (Henry et al., 2001; Li et al., 2012; Lorenzo et al., 2001; Yamada et al., 2001; Yamada et al., 2007). *SOST* inhibits bone formation by inhibiting Wnt signaling (Li et al., 2005; Piters, Boudin, & Van Hul, 2008; van Bezooijen et al., 2007). ASPN binds directly with bone morphogenetic protein (BMP)-2, and inhibits transforming growth factor (TGF)-β/Smad signaling, resulting in the inhibition of bone formation (Henry et al., 2001; Lorenzo et al., 2001; Yamada et al., 2001). Recently, upregulated expression of ASPN in PDL cells was demonstrated *in vitro* during CF (Li, Yang, Yue, & Bai, 2014); however, the types of molecule involved in the inhibition of bone formation on the compressed side during orthodontic tooth movement are unknown.

We investigated the inhibitory effects of bone formation by PDL cells subjected to mechanical CF, by measuring expression changes in *ASPN* and *SOST* in PDL cells subjected to CF.

2. Materials and methods

2.1. Cell culture

Human periodontal ligament (hPDL) cells were isolated from healthy PDLs of premolar teeth that were extracted for orthodontic reasons. All the patients gave informed consent before providing the samples. All cells were cultured in alpha-minimum essential medium (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Cellgro; Mediatech Inc., Herndon, VA, USA), 50 U/mL penicillin G (Gibco-BRL), 50 μg/mL fungizone (Gibco-BRL), and 50 μg/mL gentamicin (Gibco-BRL), and incubated at 37 °C in a 5% CO_2 incubator. Cells underwent 3–8 passages prior to use in the experiments. All procedures were approved by the Research Ethics Committee of Kyushu Dental University (permission number: 14-8).

2.2. Application of CF in vitro

We applied mechanical force *via* centrifugation according to a previous report with some modifications (Redlich et al., 1998). When the cells reached subconfluence in culture flasks (Becton Dickinson, San Jose, CA, USA), the medium was changed to HEPES-buffered Dulbecco's modified eagle medium without bicarbonate (Gibco, Invitrogen Co., Carlsbad, CA, USA), and 90 μL HCl (Nacalai Tesque, Inc., Kyoto, Japan) was added to stabilize the pH; then, cells were incubated in the incubator (Yamato Scientific Co., Ltd., Tokyo, Japan) at 37 °C for 24 h. Next, cell culture flasks were centrifuged at 40, 90, 135, and 160 × *g* (Kubota Co., Tokyo, Japan) in the incubator at 37 °C for 24 h.

Force was calculated using the following equation: $P = (m \times r \times \text{rpm}^2 \times \pi^2)/(A \times 9.8 \times 900)$, where P = kg compression/cm^2 of cells; m = mass of medium (0.005 kg); r = radius (0.1 m); rpm = revolution/min (600, 900, 1100, and 1200); and A = area of contact between medium and cells (12.5 cm^2). The CFs of 40, 90, 135, and 160 × *g* corresponded to 16.0, 36.0, 53.9, and 63.9 g/cm^2, respectively.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was isolated from CF-treated hPDL cells and purified using RNAqueous (Ambion; Life Technologies, Austin, Texas, USA) according to the manufacturer's instructions followed by DNase digestion. cDNA was synthesized from 2 μL total RNA in 30 μL reaction buffer containing 500 mM dNTPs, 20 U ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U Superscript II reverse transcriptase (Invitrogen; Life Technologies, Carlsbad, CA, USA). The following primers were used for amplification: *GAPDH*, 5′-TGA AGG TCG GTG TCA ACG GA-3′ and 5′-TAC TGG TGT CAG GTA CGG TAG-3′; *ASPN*, 5′-TCC TAG ACT GGT CTT CTA CAC T-3′ and 5′-GTG CTC AAC ATG TAA CGA GTC T-3′; *SOST*, 5′-GGA CTC CAG TGC CTT TTG AA-3′ and 5′-GTT CCA GTG AAG GTC TTA AGT C-3′. The PCR program consisted of an initial denaturation step (*GAPDH* and *SOST*, 94 °C for 2 min; *ASPN*, 95 °C for 9 min) followed by 40 cycles of denaturation (*GAPDH*, 94 °C for 30 s; *ASPN*, 95 °C for 60 s; *SOST*, 94 °C for 60 s), annealing (*GAPDH* and *ASPN*, 55 °C for 1 min; *SOST*, 62 °C for 1 min), and extension at 72 °C for 1 min. The PCR products were subjected to electrophoresis in 2% (*ASPN*) and 5% (*SOST*) agarose gels, and visualized with ethidium bromide. *GAPDH* expression was used as an internal control.

2.4. Real-time PCR analysis

Total RNA samples from CF-treated hPDL cells were treated with DNase and reverse transcribed with random primers using a Superscript First-Strand Kit (Invitrogen; Life Technologies). Real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and Eco Real Time PCR System (Illumina, San Diego, CA, USA). mRNA detection was performed using pre-developed proprietary TaqMan primers (*β-actin* [ACTB: Hs99999903_m1] and *ASPN* [ASPN: Hs01550903_m1]; Applied Biosystems). These analyses were conducted to determine the levels of *β-actin* for data normalization. Cycling conditions were 95 °C for 15 s and 60 °C for 60 s, for 40 cycles. The expression levels of target genes were normalized to *β-actin* expression and presented relative to the control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The protein release of ASPN was detected by ELISA using the culture medium of hPDL cells treated with or without CF. HPDL cells were cultured as mentioned above; 100 μL medium from each sample was pipetted into 96-well plastic plates and incubated at 4 °C overnight. After incubation, the plates were washed with PBS-Tween (0.5%, Tween 20) and blocked with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 30 min at room temperature. Then the plates were incubated with anti-human asporin (1:500 dilution; Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. After washing, donkey anti-goat IgG-HRP (1:5000 dilution; Santa Cruz Biotechnology) was added and incubated for 1 h at room temperature. After further washes, the alkaline phosphatase activity was detected by ELISA in the buffer. The ELISA buffer was obtained by adding OPD tablets (Wako, Osaka, Japan) and 4 μL H_2O_2 in 12 mL 0.1 M citrate phosphate buffer. The optical density was measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Orthodontic tooth movement model and immunocytochemistry

All procedures were approved by the Animal Research Committee of Kyushu Dental University (permission number: 15-004). Using male Sprague-Dawley (SD) rats weighing 200–250 g, we inserted an elastic band between their first and second upper molars. Untreated animals were used as a control group. Then the rats were perfused through the left ventricle with 4% paraformaldehyde (PFA). The maxillas were dissected, decalcified in 10% EDTA solution, and embedded in paraffin; then 5 μm horizontal sections of the molars in the maxilla were prepared. Immunohistochemical analysis was performed using rabbit polyclonal anti-asporin (1:100 dilution; Funakoshi Co., Tokyo, Japan) and anti-sclerostin (1:100 dilution; Santa Cruz Biotechnology) as the primary antibodies and goat anti-rabbit IgG (1:400 dilution; Invitrogen) as the secondary antibody. Positive reactions were visualized with 0.02% 3,3′-diaminobenzidine (DAB; Dojindo,

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