Occlusal hypofunction mediates alveolar bone apposition via relative augmentation of TGF-β signaling by decreased Asporin production in rats

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Abstract
The periodontal ligament (PDL) maintains alveolar bone homeostasis against mastication force. Occlusal hypofunction, which lost mastication force, narrows the PDL by alveolar bone apposition, though the mechanisms remain unclear. Asporin is a secreted extracellular matrix protein and preferentially expressed in the PDL. Asporin binds directly with transforming growth factor-beta (TGF-β) and inhibits TGF-β/Smad signaling, resulting in the inhibition of bone formation. In the present study, we hypothesized that Asporin is downregulated by occlusal hypofunction, which results in the increased alveolar bone apposition via relative upregulation of TGF-β signaling. To clarify the hypothesis, we employed in vivo experiments using rats.

Crowns of lower right first molar in Wistar rats were removed to induce occlusal hypofunction in upper right first molar. Alveolar bone apposition was examined by histomorphometric analysis of Calcein/Xylenol-orange vital staining. Asporin, TGF-β, and its signaling molecule, Smad3 expression were examined in mRNA and protein levels. Osteoblastic differentiation of the cell in the PDL under the occlusal hypofunction were observed using Bone sialoprotein (BSP), Osteocalcin and Osteopontin expression as osteoblastic differentiation marker.

We discovered that occlusal hypofunction increased the alveolar bone apposition and downregulated Asporin expression in PDL fibroblasts. Furthermore, occlusal hypofunction relative augmented TGF-β signaling in PDL judged by phosphorylated (p)-Smad2/3 immunohistochemical staining, and upregulated osteoblastic differentiation in PDL.

In the present study, we firstly reported that occlusal hypofunction mediates alveolar bone apposition via relative augmentation of TGF-β signaling by decrease of Asporin production.

Introduction
The periodontal ligament (PDL) is a fibrous connective tissue that is interposed between the roots of the teeth and the alveolar bone. The PDL plays an important role in supporting and preserving the tooth against external force including mastication force [1]. Optimal mechanical stimuli are essential to maintain healthy alveolar bone by inducing the formation and remodeling of the alveolar bone [2,3]. However, in the situation of loss of the tooth, antagonizing tooth lose the occlusion, (ie occlusal hypofunction), clinically exhibits extrusion of the tooth due to disuse atrophy of PDL [4].

The effects of occlusal hypofunction on the periodontal tissue have been studied [2-8]. These studies indicated that occlusal hypofunction results in atrophic changes of the PDL, narrowing of periodontal width [5-7], and increased alveolar bone formation [2,3,8]. Taken together, occlusal hypofunction narrows the PDL by alveolar bone apposition [2,3]. At this point, the molecules involved in the alveolar bone apposition by occlusal hypofunction are unknown.

TGF-β superfamily is known to play an important role in osteogenic cell differentiation and consequently bone formation in PDL [9,10]. Regarding the antagonizing molecule for TGF-β, Asporin is one of the well-known inhibitor for TGF-β signaling. Asporin binds directly with TGF-β and inhibits TGF-β/Smad signaling, which is resulting in the inhibition of bone formation, and is thought to be main regulator of alveolar bone homeostasis [11,12]. Asporin is a secreted extracellular matrix protein that contains 380 amino acids. It is preferentially expressed in the PDL and encodes a novel small leucine-rich repeat proteoglycan protein [13].

In the present study, we hypothesized that Asporin is downregulated by occlusal hypofunction, which results in the increase in alveolar bone apposition via upregulation of TGF-β signaling. To clarify the hypothesis, we employed in vivo experiments.

Materials and methods
Animals
All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Tsurumi University School of Dental Medicine (approved numbers; 25A062, 26A105, 25A063, 26A104).

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Experimental design

Crowns of lower right first molar were removed to induce occlusal hypofunction [7]. Briefly, the crowns of the lower right molars were cut using small pliers under a combination anesthesia (Medetomidine/Midazolam/Butorphanol:0.15/2.0/2.5 mg/kg body weight, respectively) (Figure 1). Right upper jaws were used as experimental side, and the left were used as control side. Rats were sacrificed by overdose pentobarbital sodium anesthesia, and then they were fixed with perfusion of 10% Formalin Neutral Buffer Solution (Keyence BZ-9000 Osaka, Japan). Bone apposition, which is identified by two fluorescent dyes, were measured both in frontal and cross sections. The distance between two fluorescent dyes was measured at the depth of 7-800 µm from interradicular septum in frontal sections. The distance was also measured at buccal and palatal side of distobuccal and distopalatal roots in the cross sections. 4 regions of alveolar bone in each section, three sections in each group, total 12 regions were measured using Image J [16].

Histomorphometry of alveolar bone apposition

The Calcein/Xylenol-orange fluorescent labels in the undecalcified frozen sections were examined under a fluorescent microscope (Keyence BZ-9000 Osaka, Japan). Bone apposition, which is identified by two fluorescent dyes, were measured both in frontal and cross sections. The distance between two fluorescent dyes was measured at the depth of 7-800 µm from interradicular septum in frontal sections. The distance was also measured at buccal and palatal side of distobuccal and distopalatal roots in the cross sections. 4 regions of alveolar bone in each section, three sections in each group, total 12 regions were measured using Image J [16].

RNA extraction

1/3 of the central portion of the PDL (360 µm²) in the distobuccal and distopalatal roots were microdissected from the sections by using laser capture microdissection (LCM) PALM MicroBeam system (PALM Microlaser Technologies AG, Bernried, Germany) (Figure 2) [7]. After LCM, total RNA was extracted using an RNeasy micro kit (Qiagen GmbH, Hilden, Germany) with on-column genomic DNA digestion.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Isolated RNA was reverse transcribed with iScript cDNA-Supernmix (Bio-Rad, Hercules, CA), and cDNA stock was diluted (2×) with Tris/ethylenediaminetetraacetic acid (EDTA) buffer. Real-time RT-PCR was performed with SsoFast EvaGreen-Supermix (Bio-Rad). The PCR primers used in the experiments were described in Table 1. Fold changes of gene of interests were calculated with ∆∆Ct method using β-actin as reference gene.

Histological examination

Perfusion fixed upper jaws, including the molars, were excised, further fixed with 10% Formalin Neutral Buffer Solution (Wako) overnight, and decalcified with 10% EDTA in PBS for 4 weeks at 4°C. Then the specimen were dehydrated in an ascending ethanol series and embedded in paraffin. Serial paraffin sections (5 µm-thick) of periodontal tissues of the distal root of the upper first molars were prepared, and they were used for hematoxylin and eosin staining or immunohistochemical staining.

Immunohistochemistry

The sections were deparaffinized, and inactivated endogenous peroxidase activity for 30 min (3% hydrogen peroxide). Then they were preincubated in 2.5% normal horse serum for 1 h (Vector Laboratories, Burlingame, CA), and were subsequently incubated with primary antibodies overnight. The primary antibodies we used were anti-Asporin antibody (Actis Antibodies, Inc. Herford Germany), anti-TGF-β antibody (R&D systems, Inc. Minneapolis, MN), anti-phosphorylated (p)-Smad2/3 antibody (Santa Cruz Biotechnology, Inc. Dallas, TX), anti-Osteocalcin antibody (R&D systems, Inc. Minneapolis, MN) and anti-Osteopontin antibody (Proteintech Group, Rosemont, IL). Antigen retrieval procedure were performed in the sections for p-Smad2/3 and Osteopontin staining with L.A.B Solution (Polysciences, Inc. Warrington, PA) prior to the blocking step.
After being rinsed, the sections were incubated with the peroxidase micropolymer-conjugated secondary antibody (Vector Laboratories). To visualize immunoreactivity, the sections were flooded with a diaminobenzidine solution (Vector Laboratories). Counterstaining was carried out with hematoxylin (Merck Japan, Tokyo, Japan). Sections were mounted with Entellan (Merck) and observed with a microscope. Intensity levels were measured using Image J (colour deconvolution) [16].

**Statistical analysis**

The results were presented as the mean ± standard deviation. The Student’s t-test was used for comparisons between the control and each experimental group, and the one-way analysis of variance (ANOVA) and Dunnett analysis was used for multiple comparisons using SPSS® 11.0 (SPSS Inc., Chicago, IL). p<0.05 and p<0.01 were considered to be statistically significant.

**Results**

**Occlusal hypofunction increased the alveolar bone apposition**

Histological observation demonstrated that the PDL width in the occlusal hypofunction side (experimental side) was narrowed as compared to that in control side. A considerable amount of new bone was formed on the old bone in the occlusal hypofunction side (Figure 3B and 3C), which resulted in the decrease in PDL width as compared to these in control sections (Figure 3B and 3E). This was observed not only in the frontal section but also in the cross section (Figure 3C and 3F). These histological results confirmed by the measurement of PDL width in the frontal section but also in the cross section (Figure 3C and 3F). The 14day/ control ratio of alveolar bone apposition were 1 to 6-folds in frontal sections, showing increased bone apposition at whole measured points (Figure 4G). These results indicate that the occlusal hypofunction increases the alveolar bone apposition.

**Occlusal hypofunction downregulated asporin expression**

It was reported that Asporin negatively regulates osteoblastic bone formation [11], therefore we examined the Asporin expression in the PDL under occlusal hypofunction. The expression of Asporin was significantly downregulated after 1, 3, and 7 day in the PDL in the experimental group as compared to that in the control group (Figure 5A-5C).

Then we immunohistochemically examined the Asporin expression in the PDL. In the control group, Asporin was located in the most of fibroblasts in the PDL. However, consistent with the results of real-time RT-PCR, the number of Asporin positive fibroblasts was gradually decreased with experimental periods (Figure 5D-5I). Significantly small number of Asporin positive fibroblasts was observed at experimental group (Figure 5C). These results suggest that the occlusal hypofunction downregulates Asporin expression in the PDL.

**Occlusal hypofunction upregulated TGF-β signaling**

Since Asporin binds directly to TGF-β and inhibits TGF-β signaling [11,12,17], expression of TGF-β in the PDL was immunohistochemically examined in the PDL. TGF-β was located in the most of fibroblasts in the PDL irrespective of control and experiment (Figure 6A). Considering the reduction of Asporin in the PDL, TGF-β has chance to escape from Asporin and works as stimulating factor for osteoblastic differentiation. The localization in the osteoblast in experimental group was somewhat different from that in control group. TGF-β was located in the osteoblasts in the PDL in control group. In the experimental group it was also located in the osteoblasts at day-1 but its localization in osteoblast was gradually reduced in the PDL on day-7 and 14.
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Then we examined the major signaling molecule for TGF-β superfamily signaling, Smad3 [9,18]. Real-time RT-PCR revealed that Smad3 was significantly upregulated after day-7 of the occlusal hypofunction (Figure 6B). Immunohistochemistry demonstrated that p-Smad2/3 was found in several fibroblasts in the control group. p-Smad2/3 was found in considerable number of fibroblast in the PDL in experimental group at day-7 (Figure 6C). These results suggest that the occlusal hypofunction relatively augments TGF-β signaling in PDL.

**Occlusal hypofunction upregulated osteoblastic differentiation**

It is well known that TGF-β superfamily signaling promotes osteoblastic differentiation [9,19], therefore we examined osteoblastic differentiation marker expression in the PDL under occlusal hypofunction. Osteopontin mRNA was significantly upregulated after occlusal hypofunction (Figure 7C), though bone sialoprotein (BSP) and Osteocalcin exhibited tendency toward upregulation (Figure 7A and 7B). Then localization of the Osteocalcin and Osteopontin in the PDL was immunohistochemically examined. Osteocalcin and Osteopontin were detected in the osteoblast on the bone surface and fibroblast in the PDL even in control. Osteocalcin and Osteopontin were observed

![Figure 3. Hematoxylin and eosin staining of PDL.](image_url)

(A) The frontal section of the upper first molar in control. Higher magnified image of the area indicated by black box is shown in Fig.3B.

(B) Higher magnified image of frontal section of control.

(C) Higher magnified image of frontal section of day-21 after occlusal hypofunction. Corresponding area indicated by the black box in Fig.3A is shown. Black double arrow indicates the osteoid which can be recognized by the staining properties.

(D) The cross section of the upper first molar in control. Higher magnified image of the area indicated by black box is shown in Fig.3E.

(E) Higher magnified image of cross section of control.

(F) Higher magnified image of cross section of day-21 after occlusal hypofunction. Corresponding area indicated by the black box in Fig.3D is shown. Black double arrow indicates osteoid.

B: buccal side, P: palatal side, M: mesial side, D: distal side. ab: alveolar bone, p: PDL, r: tooth root. Scale bar: 300 µm (A, D), 100µm (B, C, E, and F).

![Figure 4. Occlusal hypofunction increased the alveolar bone apposition.](image_url)

(A) The measured points of distobuccal and distopalatal roots in frontal section are illustrated. 4 regions marked as red, a: buccal side of buccal root, b: palatal side of buccal root, c: buccal side of palatal root, and d: palatal side of palatal root, were measured.

(B) Representative photographs of the fluorescent labeling in frontal section of control. Higher magnified image of the area indicated by white box is shown in Figure 4C. Green indicates Calcein label, and red indicates Xylenol-orange label.

(C) Higher magnified image of frontal section of 14 day after occlusal hypofunction. The distance of two fluorescent dye indicated by white arrow were measured.

(D) Representative photographs of the fluorescent labeling in frontal section of 14 day after occlusal hypofunction. Higher magnified image of the area indicated by white box is shown in Figure 4E. Green indicates Calcein label, and red indicates Xylenol-orange label.

(E) Higher magnified image of frontal section of 14day after occlusal hypofunction. The distance of two fluorescent dye indicated by white arrow were measured.

(F) The distance of two fluorescent dye in frontal sections.

(G) The 14day/control ratio of alveolar bone apposition in frontal sections.

B: buccal side, P: palatal side. ab: alveolar bone, p: PDL, r: tooth root. Scale bar: 300 µm (B, D), 50µm (C, E). *: p<0.05, **: p<0.01 between the samples. n=3.
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in the most of fibroblast in the PDL and osteoblast on the bone surface expression at day-7 (Figure 7D and 7E). These results suggest that occlusal hypofunction upregulates osteoblastic differentiation in PDL.

Discussion

In the present study, we discovered that the occlusal hypofunction induce alveolar bone apposition through relative augmentation of TGF-β signaling by decrease of Asporin production. It has been well-known clinically and in basic science that occlusal hypofunction caused the narrowing the width of PDL by alveolar bone apposition [2,3], though the mechanism has remained unclear.

Consistent with previous reports, the middle of the distobuccal and distopalatal roots were examined in this study. Since physiological distal movement of the molars usually takes place in the 12-13 week old rat [20], bone apposition was observed on the alveolar bone at mesial side of the roots. Therefore, we mainly observed the PDL on the buccal and palatal side of roots to eliminate the effect of physiological distal movement of molars.

Figure 5. Occlusal hypofunction downregulated Asporin expression. Real-time RT-PCR analysis for of the Asporin in day-1 (A), day-3 (B), and day-7(C) mRNA expression in PDL tissue. **: p<0.01 between the samples. n=5. (D-I) Immunohistochemical staining of Asporin in PDL tissue. n=3. Whole image of upper molar in lower magnification is shown in each left side (Scale bar : 300 µm). The right side images are higher magnified images of the boxed area in the left side images. (1/3 of the central portion of the PDL tissue) (Scale bar : 25 µm). Expression of the Asporin protein in PDL of control (D), at day-1 (E), at day-3 (F), at day-7 (G), at day-14 (H), no primary antibody (I). B: buccal side, P: palatal side.

(J) The Intensity of the immuno-reactivity for Asporin. Mean value of 3 sections are shown. *: p<0.05 versus control.

Figure 6. Occlusal hypofunction upregulated TGF-β signaling. (A) Immunohistochemical staining for TGF-β in PDL tissue. Representative photographs are shown. Scale bar: 50 µm, n=3. (B) Real-time RT-PCR analysis for Smad3 mRNA expression in PDL tissue. *: p<0.05 between the samples. n=3. (C) Immunohistochemical staining for p-Smad2/3 in PDL tissue. Arrowhead indicates p-Smad2/3 positive cells. Scale bar: 50 µm. n=3. ab: alveolar bone, p: PDL, r: tooth root.
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The expression of Smad3 was significantly upregulated in the PDL and p-smad2/3 positive cell was evidently increased in the PDL under occlusal hypofunction. Smad3 is known as an intracellular signaling molecule for TGF-β signaling [9,18,19]. Positive relation between Smad3 and osteoblastic differentiation was also reported [28]. Consisted with the reports, occlusal hypofunction induced osteoblastic differentiation in our experiments.

This is supported by the results that the expressions of BSP, Osteocalcin, and Osteopontin were upregulated in the PDL under occlusal hypofunction. BSP, Osteocalcin, and Osteopontin are osteoblast-specific proteins and have been used for osteoblastic differentiation marker [29-37]. Occlusal hypofunction evidently stimulate the differentiation of PDL cells to osteoblasts in the PDL. It is interesting in this study that BSP Osteocalcin and Osteopontin were located not only in osteoblast at alveolar bone surface, but also in the most of fibroblast, as compared to that in the control. These might indicate that the fibroblast in the PDL also contribute to the bone formation in the PDL under hypofunction.

As to the source of osteoblastic differentiated cells in PDL, we presumed that one of them are mesenchymal stem cel, because mesenchymal stem cells are present in the PDL and are able to differentiate into multiple types of cells by responding to the mechanical forces [38]. Another possibility is direct osteoblastic differentiation of PDL cells because many researchers reported that PDL cells can differentiate into mature osteoblasts by suitable stimulation [39-42].

In the present study, we demonstrated that occlusal hypofunction induced rapid alveolar bone apposition through advantage relative augmentation of TGF-β signaling by decrease of Asporin production. Attenuation of Asporin expression would be therapeutic target for alveolar bone augmentation in periodontal disease on pharmacological retention after orthodontic treatment.

**Disclosure**

The authors have no conflicts of interest.

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Contributions
Kanako Itohiya: study design, data collection, analysis, and writing. Hiroyuki Kanazaki: study design instruction, editing assistance. Misao Ishikawa: animal experiments design instruction. Satoshi Wada: immunohistochemical staining technique instruction. Yutaka Miyamoto: LCM technique instruction. Tsuyoshi Narimiya: data collection. Yoshiki Nakamura: editing assistance.

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