Original Article

Occlusal hypofunction causes periodontal atrophy and VEGF/VEGFR inhibition in tooth movement

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ABSTRACT

Objective: To examine changes in microvasculature and the expression of vascular endothelial growth factor A (VEGF-A) and VEGF receptor 2 (VEGFR-2) in rat hypofunctional periodontal ligament (PDL) during experimental tooth movement.

Materials and Methods: Twelve-week-old male Sprague-Dawley rats were divided into normal occlusion and occlusal hypofunction groups. After a 2-week bite-raising period, rat first molar was moved mesially using a 10-gf titanium-nickel alloy closed coil spring in both groups. On days 0, 1, 2, 3, and 7 after tooth movement, histologic changes were examined by micro–computed tomography and immunohistochemistry using CD31, VEGF-A, VEGFR-2, and the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method.

Results: Hypofunctional molars inclined more than normal molars and did not move notably after day 1 of tooth movement. Blood vessels increased on the tension side of the PDL in normal teeth. Immunoreactivities for VEGF-A and VEGFR-2 in normal teeth were greater than those in hypofunctional teeth during tooth movement. Compressive force rapidly caused apoptosis of the PDL and vascular endothelial cells in hypofunctional teeth, but not in normal teeth.

Conclusions: Occlusal hypofunction induces vascular constriction through a decrease in the expression of VEGF-A and VEGFR-2, and apoptosis of the PDL and vascular cells occurs during tooth movement. (*Angle Orthod.* 2013;83:48–56.)

KEY WORDS: Occlusal hypofunction; Tooth movement; VEGF; VEGFR

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INTRODUCTION

Loss of occlusal function occurs in teeth with malocclusion such as with high-positioned canines. Orthodontic movement of teeth with occlusal hypofunction induced by long-term loss of occlusion is associated with undesirable tissue changes.[1,2] Root resorption during orthodontic treatment has been reported for such teeth, and occlusal hypofunction has been suggested to be a possible cause.[3,4] Histologically, occlusal hypofunction leads to atrophic changes in the periodontium, such as narrowing of the periodontal space, constriction of the periodontal fiber arrangement, and the collapse of blood vessel formation.[5–7]

The vascular system in the periodontal ligament (PDL) delivers cells and chemical mediators to periodontal tissue that is stimulated by mechanical

Accepted: April 2012. Submitted: January 2012.
Published Online: June 18, 2012

 DOI: 10.2319/011712-45.1

force during orthodontic tooth movement and plays a key role in tissue remodeling of the periodontium and alveolar bone under orthodontic treatment.[8] Under a loss of occlusion, PDL blood vessels are subjected to various influences, such as vascular constriction and changes in vasodilatory factors.[7] We hypothesized that nonoccluded teeth would have a different initial response to orthodontic compressive force due to changes in angiogenic activity in the PDL.

Vascular endothelial growth factor (VEGF) potently regulates the formation of new blood vessels through VEGF receptors (VEGFRs) and has been reported to be a key factor in periodontal angiogenesis during tooth movement.[9] VEGF-A is a member of the VEGF family that specifically acts on vascular endothelial cells. The two major receptors for VEGF signals are the structurally related tyrosine kinases VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). The angiogenic, mitogenic, and permeability-enhancing effects of VEGFR-1 are primarily mediated by VEGFR-2. Moreover, the VEGF family plays a role in bone resorption[10,11] and generation[12] due to its effect on the proliferation and differentiation of osteoblasts and osteoclasts in vitro.

Our objective was to clarify the differences in orthodontic tooth movement between the occlusal hypofunctional tooth and normal teeth, and the changes in the vascularization of hypofunctional periodontium through changes in expression of VEGF-A and VEGFR-2 during experimental tooth movement in rats.

MATERIALS AND METHODS

Animals

The experimental procedures described here were approved by the Animal Welfare Committee (0110-200A) and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University. Twelve-week-old male Sprague-Dawley rats were used because the molars have complete roots. Rats were divided into normal occlusion (n = 30) and occlusal hypofunction (n = 30) groups. Orthodontic appliances were placed for 1 week after a 2-week occlusal hypofunctional period. All rats were fed a powdered diet and given water ad libitum throughout the experimental period. All procedures were carried out under general anesthesia.

Induction of Occlusal Hypofunctional Condition

The maxillary first molar (M1) was induced into hypofunctional occlusion before tooth movement by the bite-raising technique. In the hypofunction group, an anterior metal bite plate and metal cap constructed from stainless band material (0.180 × 0.005 inches; Rocky Mountain Morita, Tokyo, Japan) were attached to the maxillary and mandibular incisors using light-curing composite resin (Clearfil Liner Bond II; Kuraray, Okayama, Japan).[13]

Experimental Tooth Movement

After a 2-week period of bite-raising, a 10-gf titanium-nickel alloy (TiNi) closed coil spring (Furukawa Electric, Tokyo, Japan) was attached to M1 and the maxillary incisors with light-curing composite resin, and M1 was moved mesially for a week in both the normal and occlusal hypofunction groups. Rats were sacrificed at 0, 1, 2, 3, and 7 days after attachment of the coil spring (Figure 1).

Micro–Computed Tomography Analysis

The hemi-maxilla was scanned by micro–computed tomography (CT) (SMX-100CT, Shimadzu, Kyoto, Japan) at 0, 1, 2, 3, and 7 days after the start of tooth movement. Image reconstruction in the appropriate cross-section was carried out using software (VGStudio MAX, Nihon Visual Science, Tokyo, Japan). The amount of tooth movement was evaluated by measuring the molar distance, the distance from the mesial crown surface of the second molar (M2) to the distal crown surface of M1, and the angle of inclination of the moved molar (MA). The difference was in angle between the mesial root of M1 and the occlusal plane of the molars from that in the normal group at day 0 in micro–CT images (Figure 2A).

Preparation of Histologic Sections

Frozen nondecalcified sections were prepared with a cryofilm-transfer kit (Finetec, Tokyo, Japan).[14] The isolated hemi-maxilla was frozen by quenching in cold

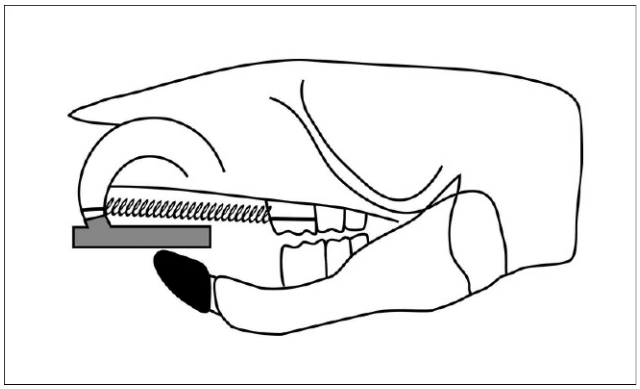

Figure 1. Experimental model. Occlusal hypofunctional condition in the molar region was achieved by the attachment of an anterior bite plate and metal cap to the maxillary and mandibular incisors. A 10-gf NiTi closed coil spring was attached to the maxillary M1 and the maxillary incisors to move M1 mesially.

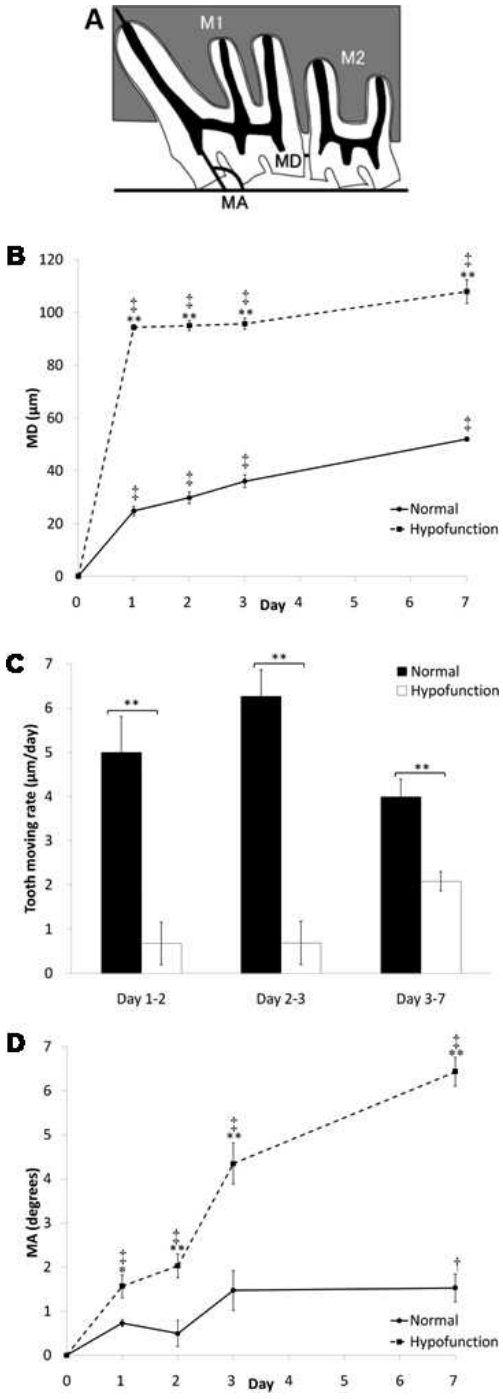

Figure 2. Micro–CT analysis of tooth movement. (A) Measurement design for micro–CT analysis. (B) Molar distance. (C) Angle of inclination of the moved molar. (D) Tooth moving rate per day. Values of the normal group at day 0 are the baseline level in micro–CT analysis. *, $^{\dagger}P = .05$, **, $^{\ddagger}P = .01$. * Significant difference vs the normal group at each day. † Significant difference vs the normal group at day 0.

hexane, then embedded in 5% SCEM gel, and further frozen in cold hexane. The frozen SCEM blocks of samples were cut horizontally with a disposable carbide tungsten steel blade (Leica Microsystems, Nussloch, Germany). The trimmed surface was covered with an adhesive film (Fintec), and the sample was sectioned horizontally along with the film at a thickness of 20 μm.

Immunohistochemistry

Film-supported undecalcified frozen sections were stained with antibodies to CD31, VEGF-A, and VEGFR-2. The sections were incubated with the CD31 antibody (diluted 1:100; Millipore, Mass) and visualized using Alexa Fluor 546 (diluted 1:300; Molecular Probes, Eugene, Ore). For VEGF-A and VEGFR-2, sections were incubated with antibodies against VEGF-A and VEGFR-2 (each diluted 1:50; Abcam, Cambridge, Mass) overnight at 4°C and visualized using Alexa Fluor 488 and Alexa Fluor 546 (each diluted 1:300; Molecular Probes). The apoptotic change was checked by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) using an in situ apoptosis detection kit (Takara Biomedicals, Shiga, Japan). Fluorescence images of immunostained sections were obtained by confocal laser scanning microscopy (FV10i, Olympus, Tokyo, Japan).

Histologic and Quantitative Analysis

Cross-sections at the level of 500 to 600 μm from the furcation of the distobuccal roots of M1 were subjected to a histologic investigation. The area of immunopositive cells for CD31, VEGF-A, and VEGFR-2 was measured in a square area of interest (200 × 200 μm^2) (Figure 2A).[7] Five consecutive sections per animal were counted to correct for differences in observation.

Statistical Analysis

The quantitative results of micro–CT and immunohistochemistry in each group were represented as the mean ± standard error of the mean. Data of the normal group at day 0 were considered as the baseline for all evaluations. Comparisons between each group were analyzed by analysis of variance (ANOVA) followed by Scheffe post hoc test using statistical analysis software (Stat View 5.0 software, SAS Institute, Cary, NC).

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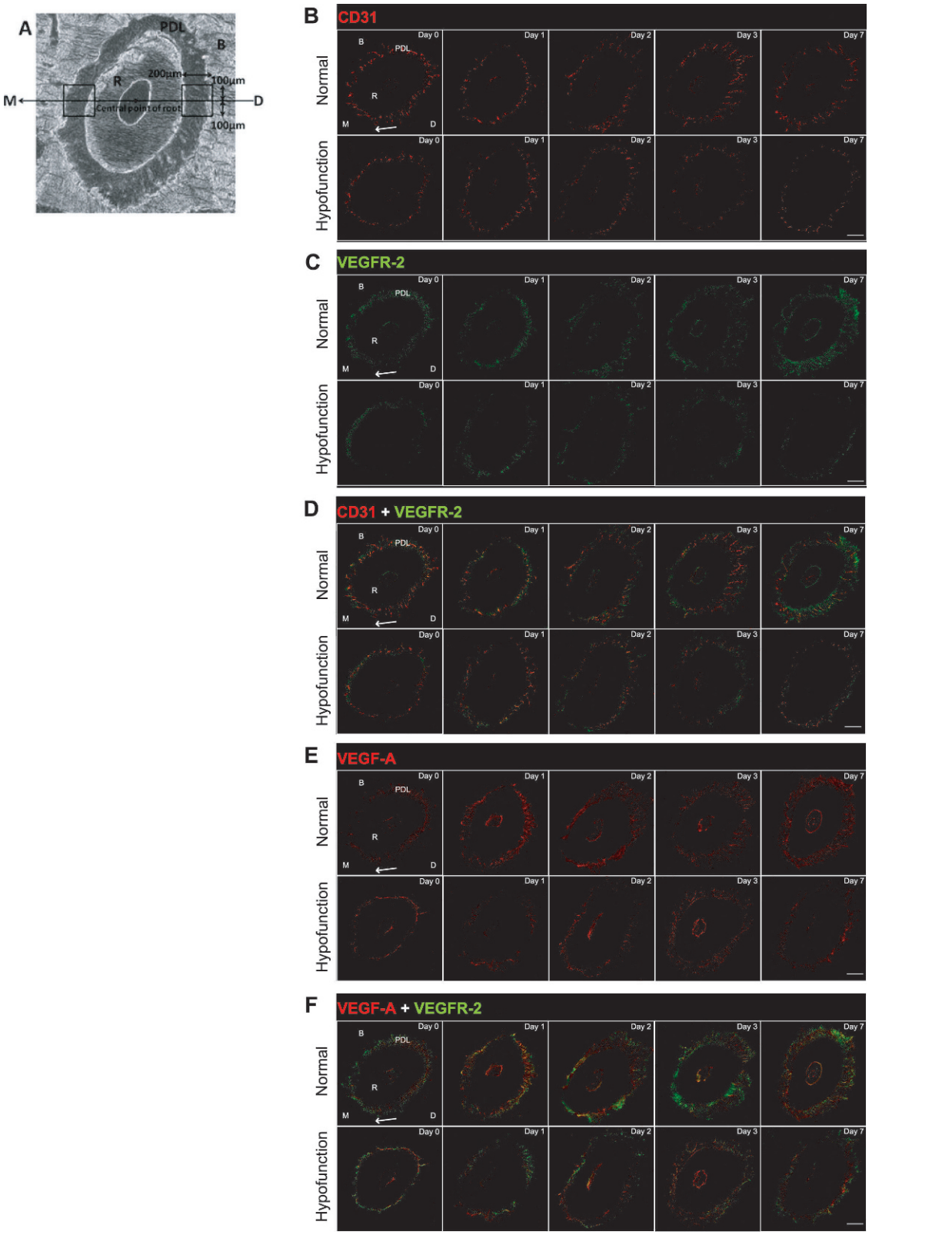
Figure 3. Immunohistochemical staining for CD31, VEGFR-2, and VEGF-A in periodontal tissues of the distobuccal roots of maxillary first molars. (A) Observation area. Immunoreactivity was recorded at 200 × 200 μm^2 s in the center of mesial and distal periodontal areas.



(B) CD31. (C) VEGFR-2. (E) VEGF-A. (D) Superimposed images of CD31 and VEGFR-2, VEGF-A (F) VEGFR-2. The upper panels in B though F show immunostained sections in the normal group from days 0 to 7, and the lower panels show those in the hypofunction group. CD31 and VEGF-A were stained by Alexa Fluor 546, VEGFR-2 was stained by Alexa Fluor 488. The arrows show the direction of tooth movement. B indicates bone; PDL, periodontal ligament; R, root; and bar, 200 μm.

RESULTS

Experimental Movement of Molars After Occlusal Hypofunction

At day 0 the maxillary M1 and M2 were in contact, and mesial movement of M1 was initiated by the attachment of a 10-gf coil spring. While the occlusal hypofunction group showed large movement from days 0 to 1, there was no movement from days 1 to 7 (Figure 2B). On the other hand, in the normal group molar distance significantly increased with time from days 1 to 7. Based on the increase in the amount of tooth movement per day from days 1 to 7, tooth movement in the normal group was significantly greater than that in the hypofunction group (Figure 2C).

The angle of inclination of the moved molar (MA) gradually increased in the hypofunction group throughout the overall movement period (Figure 2D). However, MA did not change significantly from days 0 to 3 in the normal group compared to the baseline, and remained virtually constant from days 3 to 7. According to the value of MA, the movement of M1 in the hypofunction group depended on the greater inclination of the crown compared to that in the normal group from days 0 to 1 (Figure 2B,D). It was observed that M1 root apex drifted distally to M2 in the hypofunction group during day 1 to 7, and the inclination of M1 in the hypofunction group increased more than that in the normal group despite small tooth movement rate per day from days 1 to 7.

Localization of Blood Vessels in the Periodontal Tissues During Tooth Movement

Immunoreactivity for CD31, which is a marker protein of blood vessels, was observed in the whole periodontal tissue area of M1 in both the normal and occlusal hypofunctional groups before tooth movement (Figure 3B). The expression level of CD31 in the hypofunction group was lower than that in the normal group at day 0 (Figure 4A,B). After the mesial movement of M1 was begun, CD31-positive cells increased on the distal side of the PDL in the normal group from days 3 to 7. On the other hand, CD31-positive cells decreased in both the mesial and distal areas of M1 in the hypofunction group from days 1 to 7.

Immunolocalization of VEGFR-2 and VEGF-A in the Periodontal Tissues

At day 0, immunoreactivity for VEGFR-2 in the hypofunction group was lower than in the normal group. VEGFR-2 expression was sustained on the mesial and distal sides in the normal group from days 1 to 3 and increased at day 7 (Figure 4C,D). On the other hand, the expression level of VEGFR-2 significantly decreased in the hypofunction group from days 1 to 3, and did not recover on the distal side at day 7. Before tooth movement the expression level of VEGF-A in the hypofunction group was higher than that in the normal group (Figure 4E,F). After the start of tooth movement, VEGF-A positive cells dramatically increased in the whole PDL of the normal group, and VEGF-A expression in this group was significantly higher than both the baseline level and hypofunction group from days 1 to 7.

Apoptosis of Periodontal Cells in Occlusal Hypofunctional Tooth During Tooth Movement

TUNEL-positive cells were observed in the PDL of the hypofunction group and dramatically increased during the early phase of tooth movement. This positivity was localized in various PDL cells including CD31-positive cells on days 1 and 3 (Figure 5). On the other hand, little immunoreactivity for TUNEL was observed in the normal group during the experimental period (Figure 6). The data showed that apoptosis of PDL and vascular endothelial cells were induced in the PDL of occlusal hypofunctional teeth during the early phase of tooth movement.

DISCUSSION

Occlusal hypofunction induces a decrease of PDL cells and extracellular matrix in the PDL and the change in the viscoelasticity of the PDL. The normal molar was moved continuously with a 10-gf force, which has been shown to be comparable to the force used in human tooth movement.[15] Displacement of the hypofunctional tooth was significantly unchanged after day 1, though M1 root drifted distally during day 1 to 7. Heavy orthodontic force applied to molar generates a classic three-part tooth movement curve: an initial mesial movement period, followed by a lag period and a late mesial movement period. It was reported that the molar with an applied force of 40 gf drifted distally during a lag period despite continuous mesial force. On the other hand, a moderate force of 1.2–10 gf induces continuous molar movement without a lag phase.[16] In rat hypofunctional molar, a force of 15 gf decreased the production of extracellular matrix and induced the irregular distribution of periodontal fiber during tooth movement, whereas a force of 2 gf had no atrophic effect.[1] A force of 10 gf exerts the effect of strong force to the hypofunctional tooth, though the same force moderately affects on the normal tooth, which results in the drift movement of the hypofunctional molar.

Atrophic changes in vascular and PDL cells were induced in the hypofunctional teeth during tooth

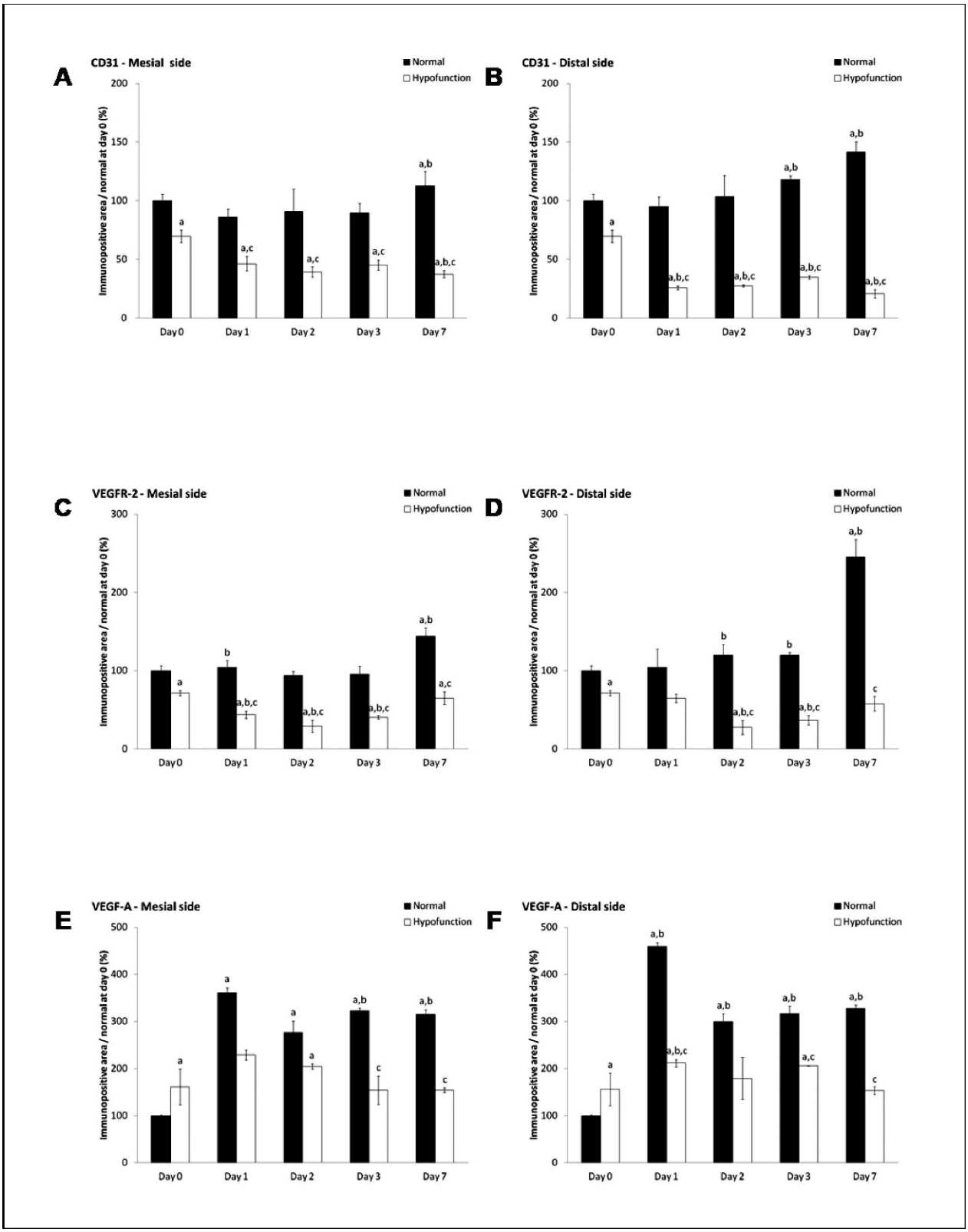


Figure 4. Quantitative analysis of the immunopositive area in the normal and hypofunction group. (A, B) CD31. (C,D) VEGFR-2. (E, F) VEGF-A. Data show the total immunopositive area per unit area (200 × 200 μm²) on the mesial side (A, C, E) and distal side (B, D, F) relative to those in the normal group at day 0. Statistically significant differences ($P = .05$) are represented as follows: a = relative to the normal group at day 0; b = relative to the hypofunction group at day 0; and c = relative to the normal group at each day.

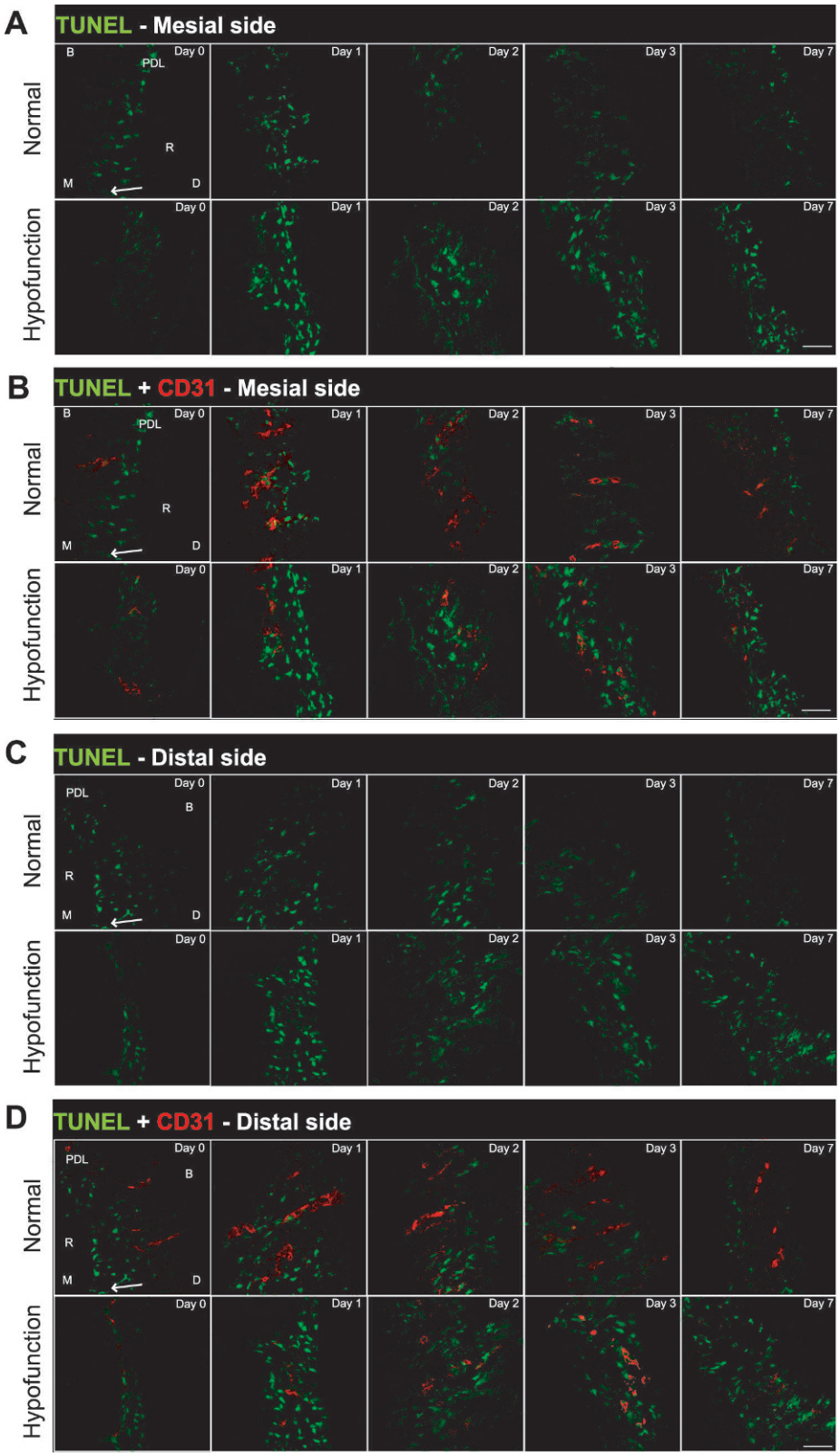


Figure 5. Immunohistochemical staining for TUNEL (A, C) and superimposition of CD31 and TUNEL (B, D). The upper panels in A through D show immunostained sections in the normal group from days 0 to 7, and the lower panels show those in the hypofunction group. They are each an enlarged image of the mesial and distal side in Figure 3A. The arrows show the direction of tooth movement. B indicates bone; PDL, periodontal ligament; R, root; and bar, 40 μm.

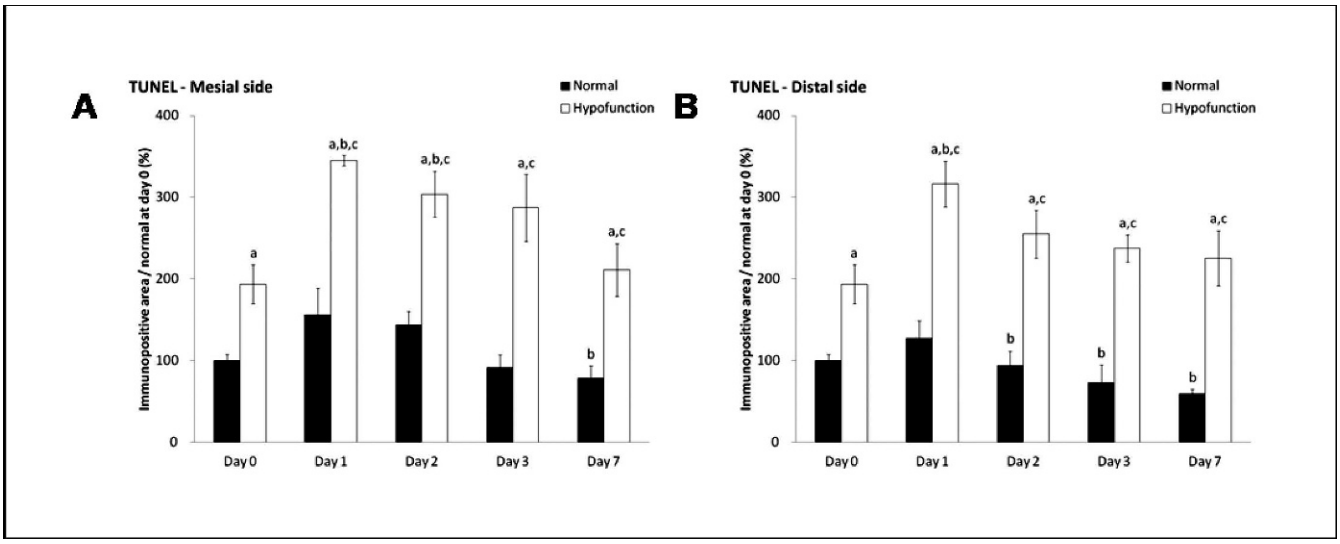


Figure 6. Quantitative analysis of the total TUNEL-immunopositive area in the normal and hypofunction group. Data show the total immunopositive area per unit area (200 × 200 μm²) on the mesial side (A) and distal side (B) relative to those in the normal group at day 0. Statistically significant differences ($P = .05$) are represented as follows: a = relative to the normal group at day 0; b = relative to the hypofunction group at day 0; and c = relative to the normal group at each day.

movement, while vessel formation occurred in normal teeth under the same amount of force. During the early phase of tooth movement, immunopositivities for VEGF-A and VEGFR-2 greatly increased on the tension side of M1 in normal teeth. The immunolocalization of VEGF-A has been observed in some cell types such as PDL cells, osteoblasts, and osteoclasts on the tension side of teeth during experimental tooth movement.[9,17] Cyclic tensile force promotes the expression of VEGF-A in PDL cells.[18] Colocalization of VEGFR-2 and CD31 was observed in both the normal and hypofunction groups. The localization of VEGF-A was not necessarily matched with that of VEGFR-2. VEGF exerts its angiogenic activity in the endocrine and paracrine manners.[19,20] The difference of localization of VEGF-A and its receptor occurred due to the paracrine system.

Apoptosis of the PDL and vascular endothelial cells rapidly occurred in hypofunctional teeth after the initiation of tooth movement. Long-term occlusal hypofunction induces the blockade of blood flow in the PDL, which results in a decrease in the oxygen level.[7] Abrupt reperfusion in low oxygen-maintained tissue induces the death of endothelial cells through an increase in active oxygen. After ischemic hypoxia, VEGF provides functional protection of endothelial cells after hypoxia in vessels.[21] The inhibition of VEGFR-2 signaling also worsens injury, affects cell death, and reduces endothelial cell proliferation after hypoxia.[22] VEGF-A is reported to have effects of proliferation and differentiation on PDL stem cells.[23] VEGFR-2 signaling induced by VEGF-A can suppress cell apoptosis and promote angiogenesis in recovery and regeneration after ischemia-reperfusion.

CONCLUSIONS

- The tooth movement rate in hypofunctional teeth was smaller than in normal teeth from days 1 to 7, and the inclination of hypofunctional teeth increased more than that in the normal teeth during tooth movement.
- Expression of VEGF-A and VEGFR-2 was decreased in the hypofunctional periodontium during tooth movement, which resulted in vascular constriction.
- Apoptosis of vascular and periodontal cells was observed in the hypofunctional periodontium during tooth movement.

ACKNOWLEDGMENTS

We wish to thank Dr Ikuo Morita, Dr Naoto Suda, and Dr Keiko Tachikawa for their valuable advice. This research was supported by Grants-in-Aid for Scientific Research to JH (20592393, 23792420) and NO (20451908) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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