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J DENT RES published online 6 May 2013

DOI: 10.1177/0022034513488787

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J Dent Res XX(X):1-7, 2013

ABSTRACT

In orthodontic tooth movement (OTM), we should be concerned about external root resorption (ERR) as an undesirable iatrogenic problem, but its mechanisms are not fully understood. Since our previous epidemiologic studies found that patients with allergic diseases showed higher rates of ERR during orthodontic treatment, we explored the possible effect of allergic sensitization on ERR. In ovalbumin (OVA)-sensitized Brown–Norway rats, the amounts of ERR and OTM were greater than those in animals subjected to orthodontic force alone. The expression levels of RANKL and pro-inflammatory cytokines were increased in the periodontal tissues of sensitized rats with OTM, compared with control rats. Furthermore, leukotriene B₄ (LTB₄), a potent lipid mediator of allergic inflammation, and enzymes of the 5-lipoxygenase pathway, the biosynthetic pathway of leukotrienes, were also up-regulated. We found that low doses of aspirin suppressed ERR in allergen-sensitized rats, as well as the expressions of RANKL, pro-inflammatory cytokines, and LTB₄. The present findings indicate that allergen sensitization has adverse effects on ERR under OTM, and that aspirin is a potential therapeutic agent for combating ERR.

KEY WORDS: allergy, tooth movement, cytokines, leukotrienes, aspirin, rats.

DOI: 10.1177/0022034513488787

Received November 14, 2012; Last revision March 13, 2013; Accepted April 10, 2013

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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INTRODUCTION

Many orthodontists consider that undesirable external root resorption (ERR) is an unavoidable and unpredictable pathologic consequence of orthodontic tooth movement (OTM). Serious cases may involve shortening of the tooth roots, which in turn shortens the life of the tooth. This problem remains a matter of grave concern for orthodontic treatments. A few clinical reviews concerning ERR have suggested the involvement of systemic conditions, including allergic conditions (McNab *et al.*, 1999; Davidovitch *et al.*, 2000). Indeed, we previously showed that Japanese patients with allergic diseases had higher rates of orthodontically induced root resorption (Nishioka *et al.*, 2006). However, the mechanisms of ERR under allergic conditions are still poorly understood.

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , and receptor activator of nuclear factor κ B ligand (RANKL) play well-known roles in bone resorption through the induction of osteoclast differentiation (Heath *et al.*, 1985; Bertolini *et al.*, 1986), and have been suggested to be involved in OTM (Lowney *et al.*, 1995; Kanzaki *et al.*, 2002). TNF- α and IL-1 β induce local expression of RANKL, which is critical for the terminal differentiation of osteoclast precursor cells (Suda *et al.*, 1999; Azuma *et al.*, 2000). Furthermore, leukotrienes, which are metabolites of arachidonic acid, are potent lipid mediators that play important roles in allergic inflammation and constitute several products of the 5-lipoxygenase (5-LOX) pathway (Murphy and Gijon, 2007). Increased levels of leukotrienes are found in inflammatory diseases such as asthma and periodontal diseases (Emingil *et al.*, 2001; Hallstrand and Henderson, 2010). Hikiji *et al.* (2009) reported that leukotriene B₄ (LTB₄) modulates bone metabolism by increasing bone resorption. These LTB₄ actions are mediated by specific receptors, BLT1 and BLT2 (Yokomizo *et al.*, 1997, 2000).

Brown–Norway (BN) rats, which spontaneously produce high levels of serum IgE and show further highly enhanced serum IgE levels after sensitization with ovalbumin (OVA), have been extensively used as animal models of asthma (Bellofiore and Martin, 1988) and atopic dermatitis (Imada *et al.*, 2002).

To explore the possibility that allergies increase OTM and ERR, we examined the effects of allergen sensitization on ERR and OTM using OVA-sensitized BN rats. Furthermore, we attempted to prevent ERR progression during OTM by aspirin administration, since aspirin has been reported to improve bone mineral density in human epidemiological studies (Bauer *et al.*, 1996; Carbone *et al.*, 2003).

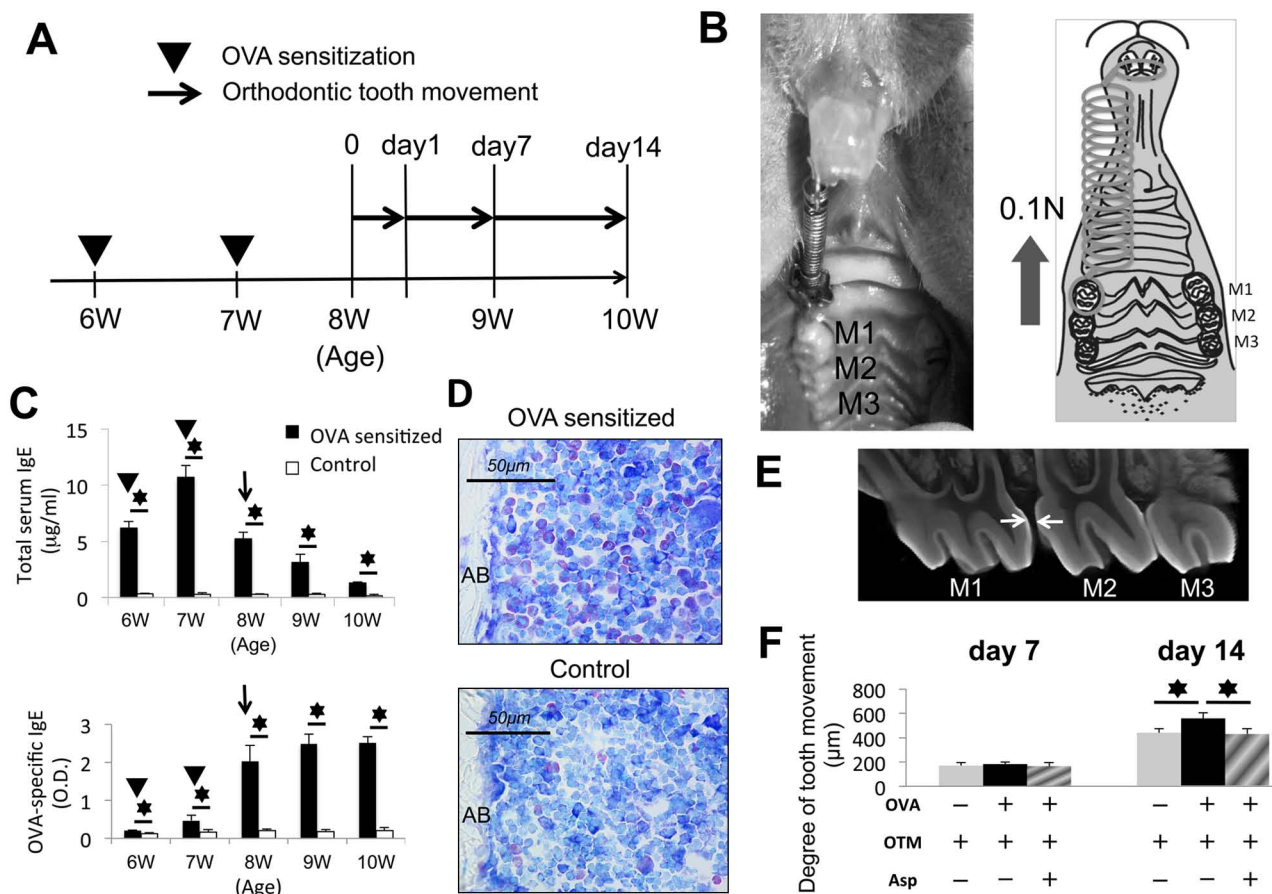


Figure 1. Experimental design, changes in serum IgE, histology of alveolar bone, and degrees of tooth movement. **(A)** Scheme showing the experimental design regarding OVA sensitization, orthodontic force application, and aspirin administration. **(B)** Photomicrograph and scheme of rat maxillae with the orthodontic appliance. The force level of the closed-coil spring after activation is approximately 0.1 N. M1, first molar; M2, second molar; M3, third molar. **(C)** The total and OVA-specific IgE levels in serum are greatly elevated after the first OVA sensitization, and further elevated after the second OVA sensitization. The arrowheads indicate the times of the 2 OVA sensitizations. The arrows indicate the start of the orthodontic force application. All data are expressed as means $\pm$ SD. * $p < .05$ by Student's t test ($n = 7$ animals per group). **(D)** Granulocytes including eosinophils are increased in OVA-sensitized alveolar bone marrow compared with control alveolar bone marrow. The sections are stained with Giemsa. AB, alveolar bone. **(E)** Micro-CT photograph showing the maxilla from a rat in the OVA with OTM group. The arrows indicate the dissociation of tooth movement. **(F)** The degree of tooth movement is significantly greater in OVA-sensitized animals than in non-sensitized animals after tooth movement. Aspirin treatment reverses the increase in OVA-sensitized animals. All data are expressed as means $\pm$ SD. * $p < .05$ by one-way ANOVA with Tukey's multiple-comparison test ($n = 7$ animals per group).

MATERIALS & METHODS

Rats

The experimental protocols were approved by the Animal Care and Use Committee of Kyushu University. Six-week-old male BN rats (Kyudo, Tosu, Japan) weighing 110 to 140 g were fed a powdered diet (CE-2; Clea Japan, Tokyo, Japan) and given water *ad libitum*.

Allergen Sensitization

The rats were sensitized by a subcutaneous injection of saline (1 mL) containing 1 mg of OVA (grade V; Sigma-Aldrich, St. Louis, MO, USA) and 200 mg of aluminum hydroxide. A *Bordetella pertussis* vaccine containing 1×10^{10} heat-killed bacilli (Wako, Osaka, Japan) was given intraperitoneally as an adjuvant, and OVA was injected after 7 days. ELISAs were used

to detect total serum IgE (Shibayagi, Gunma, Japan) and OVA-specific IgE (Cusabio, Newark, DE, USA) in the OVA-alone and control groups ($n = 7$ per group).

OTM and Aspirin Administration

At 7 days after the second sensitization, an Ni-Ti closed-coil spring at a force of 0.1 N (Sentallony®, Ultra Light; Tomy International Inc., Tokyo, Japan) was inserted between the upper right first molar (M1) and the incisors (Figs. 1A, 1B).

Aspirin dissolved in feed water (250 μg/kg/day) was administered to OVA-sensitized rats at the time of orthodontic force application. For ELISA and RT-PCR analyses, aspirin was administered from 24 hrs before OTM.

The animals were divided into 5 groups: control group (no OVA or OTM); OVA-alone group (no OTM); OTM-alone group (no OVA); 'OVA with OTM' group; and 'aspirin administration

to OVA with OTM' group. We used separate animals for the different analyses.

Histology

After 7 and 14 days of OTM, the animals ($n = 7$ per group) were perfused transcardially with 200 mL of heparinized phosphate-buffered saline, pH 7.4, followed by 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer. The right halves of maxillae decalcified in 10% EDTA were cut into 8- μ m-thick parasagittal sections, and every fifth section was collected. The sections showing the greatest length of the distopalatal root together with the 5 adjacent sections on either side (total of 11 sections per animal) were stained for tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich) or Giemsa (Merck, Darmstadt, Germany). All sections were observed under a digital microscope with MZ-II Analyzer software (BZ-9000; Keyence, Osaka, Japan).

After investigation of all 5 roots of M1 with surrounding structures, we found that ERR was conspicuous in the distopalatal root. We measured the area of ERR as well as the numbers of odontoclasts and osteoclasts within a defined area (Appendix Fig. 1) according to the method of Mavragani *et al.* (2005). The resorption area (eroded surface per bone surface; ES/BS) and adjacent osteoclasts (osteoclast number per bone surface; N.Oc/BS) were also measured in the adjacent alveolar bone. We counted TRAP-positive cells in resorption lacunae facing the tooth roots as odontoclasts, and other TRAP-positive cells (containing more than 2 nuclei) as osteoclasts.

Cytokines and Leukotriene

The periodontal tissues, including the bone, surrounding M1 of the right maxillae after 24 hrs of OTM ($n = 7$ per group) were immediately frozen in liquid nitrogen and homogenized in Tris-HCl buffer, pH 7.4, containing protease inhibitors. After centrifugation, the supernatants were collected and stored at -80°C . TNF- α , IL-1 β , and IL-6 were evaluated with a TNF- α ELISA Kit (Shibayagi), an IL-1 β ELISA Kit, and an IL-6 ELISA Kit (BioSource, Camarillo, CA, USA), respectively, according to the manufacturers' protocols.

Lipid was extracted in 100% methanol at -30°C overnight, and the level was evaluated with an EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).

RT-PCR

The periodontal tissues around M1 after 24 hrs of OTM were rapidly frozen in liquid nitrogen and homogenized ($n = 4$ per group). After mRNA isolation, quantitative real-time PCR or conventional RT-PCR was performed with gene-specific primer sets (Appendix Table), as described previously (Wang *et al.*, 2011). Amplification for real-time PCR was performed with a Rotor Gene 3000 (QIAGEN K.K., Tokyo, Japan). All samples were normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase or 18S ribosomal RNA.

Micro-CT

Micro-CT images were acquired at 9- μ m resolution with a SkyScan 1076 (SkyScan, Antwerp, Belgium) used to assess the degree of OTM. At day 14 (10 wks of age), the hemi-maxillae were excised ($n = 6$ per group). The hemi-maxilla samples were directed parallel to the occlusal plane and scanned for 2.70 mm. The distance from the distal surface of M1 to the mesial surface of the second molar (M2) was measured with DataViewer software (ver. 1.4.3; SkyScan).

Statistical Analysis

The serum IgE levels were compared by Student's *t* test. The means of multiple groups were compared by one-way analysis of variance (ANOVA). Other values were compared by ANOVA followed by Tukey's multiple-comparison test. Values of $p < .05$ were considered to indicate statistical significance.

RESULTS

Systemic and Local Changes with OVA Sensitization

The total serum IgE and OVA-specific IgE levels were greatly elevated after the first OVA sensitization, and further elevated after the second sensitization (Fig. 1C). Interestingly, the numbers of granulocytes including eosinophils were increased in the alveolar bone marrow at 7 days after the second OVA sensitization compared with those in non-sensitized control alveolar bone marrow (Fig. 1D). Acute lung inflammation was confirmed by intranasal OVA challenge (Appendix Fig. 2).

To explore whether allergen sensitization had specific effects on only the alveolar bone with OTM or systemic effects on the long bones as well, we examined the proximal metaphyseal region of the tibia using micro-CT. There were no significant differences in the bone structural parameters between the groups (Appendix Fig. 3).

The body weights showed no significant differences among the groups (Appendix Fig. 4).

OTM and Root and Alveolar Bone Resorption

At day 14, the degree of OTM was significantly larger in the 'OVA with OTM' group than in the OTM-alone group (Fig. 1E). Without orthodontic force, no osteoclasts and/or odontoclasts were observed on the medial surface of the alveolar bone in any of the groups (Figs. 2A–2E). Marked ERR was observed on the pressured side in the OTM-alone and 'OVA with OTM' groups (Figs. 2C, 2D). The 'OVA with OTM' group exhibited broad and deep TRAP-stained areas, with resorption lacunae on the root and surrounding bone on days 7 and 14 (Figs. 2D, 2F). In addition, the numbers of odontoclasts and osteoclasts were significantly greater in the 'OVA with OTM' group than in the OTM-alone group (Figs. 2G, 2H). The number of osteoclasts peaked on day 7 in the 'OVA with OTM' group, and then decreased by day 14. While the area of ERR increased from day 7 to day 14, the number of odontoclasts was maintained during this period (Figs. 2F, 2H). The alveolar bone resorption and osteoclast numbers were significantly greater in the

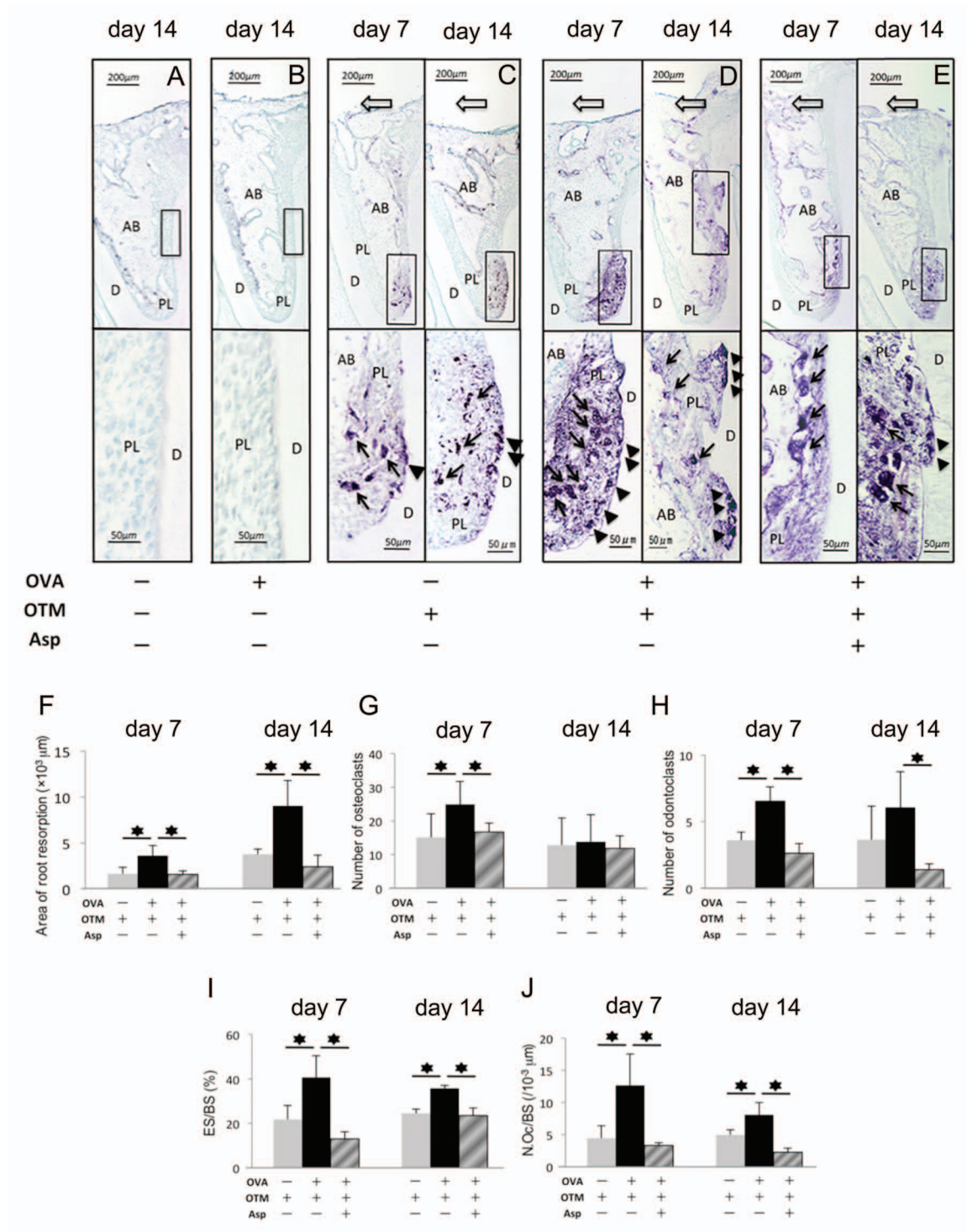


Figure 2. Increased numbers of osteoclasts/odontoclasts and amounts of root/bone resorption on the pressured side after tooth movement in OVA-sensitized animals, and inhibition of these increases by aspirin administration. Histologic sections of periodontal tissues around the distopalatal root of the first molar stained for TRAP after 7 and 14 days of tooth movement (**A–E**) and quantitative analyses (**F–J**). The upper panels show low-magnification images and the lower panels show high-magnification images of the boxed areas in the upper panels. (**A**) Control group (without treatment). (**B**) OVA-alone group. (**C**) OTM-alone group. (**D**) 'OVA with OTM' group. (**E**) 'Aspirin administration to OVA with OTM' group. (**F**) Area of root resorption ($\times 10^3 \mu m^2$). (**G**) Number of osteoclasts. (**H**) Number of odontoclasts. (**I**) Eroded surface per alveolar bone surface (ES/BS). (**J**) Number of osteoclasts per alveolar bone surface (N.Oc/BS). The white arrows indicate the orthodontic force direction. The black arrows indicate TRAP-positive osteoclasts. The arrowheads indicate TRAP-positive odontoclasts. The sections are counterstained with toluidine blue. AB, alveolar bone; D, dentin; PL, periodontal ligament. All data are expressed as means $\pm$ SD. * $p < .05$ by one-way ANOVA with Tukey's multiple-comparison test ($n = 7$ animals per group).

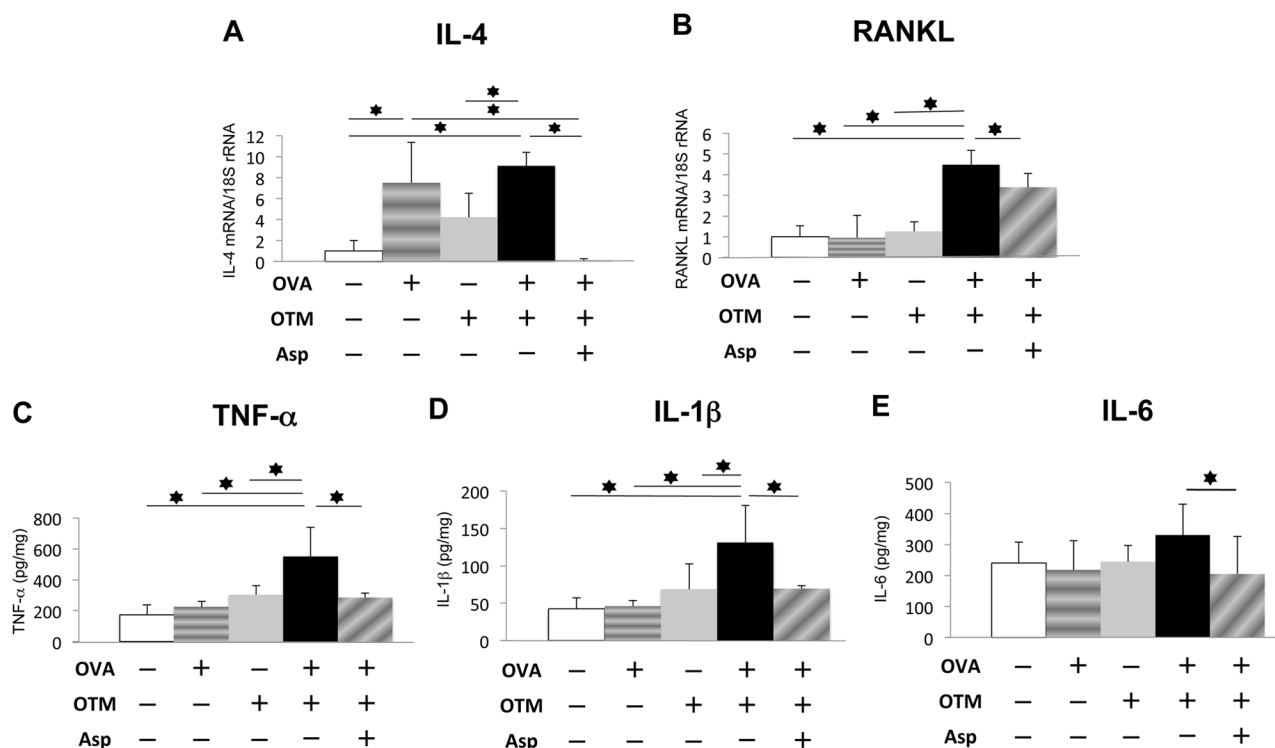


Figure 3. Elevated expressions of cytokines in periodontal tissues after 24 hrs of tooth movement in OVA-sensitized animals, and suppression of these elevations by aspirin administration. (A) IL-4. (B) RANKL. (C) TNF- α . (D) IL-1 β . (E) IL-6. All data are expressed as means $\pm$ SD. * $p < .05$ by one-way ANOVA with Tukey's multiple-comparison test ($n = 7$ animals per group).

'OVA with OTM' group than in the OTM-alone group on days 7 and 14 (Figs. 2I, 2J).

Cytokines

To identify the possible mechanism for the elevated ERR, we examined the levels of cytokines in the periodontal tissues surrounding M1 after 24 hrs of OTM. The IL-4 level was significantly higher in the OVA-alone and 'OVA with OTM' groups than in the control group (Fig. 3A). The levels of RANKL, TNF- α , and IL-1 β were significantly higher in the 'OVA with OTM' group than in the other groups (Figs. 3B–3D), while the difference in IL-6 was not significant (Fig. 3E). Although there were no significant differences between the OVA-alone and control groups, the OTM-alone group showed tendencies toward higher levels of TNF- α and IL-1 β (Figs. 3C, 3D).

LTB₄ and Related Factors

The LTB₄ level and mRNA levels of the synthases 5-LOX and LTA₄ hydrolase (LTA₄h) were significantly higher in the 'OVA with OTM' group than in the other groups after 24 hrs of OTM (Figs. 4A–4C, 4F). The mRNA levels of the LTB₄ receptors BLT1 and BLT2 were also higher in the 'OVA with OTM' group (Figs. 4D–4F).

Effects of Aspirin Administration

Aspirin treatment reversed the degree of OTM in the 'OVA with OTM' group to a level similar to that in the OTM-alone group

on day 14 (Fig. 1E). Histologic examinations revealed that the area of ERR and numbers of odontoclasts/osteoclasts were decreased to the levels observed in the OTM-alone group (Figs. 2E–2J). The levels of IL-4 and RANKL mRNAs, and TNF- α , IL-1 β , and IL-6 proteins were also suppressed to the levels detected in the OTM-alone group (Fig. 3). Furthermore, the amounts of LTB₄, BLT1, BLT2, 5-LOX, and LTA₄h were also suppressed by aspirin treatment (Fig. 4).

DISCUSSION

The main findings of the present study are that the amounts of OTM, alveolar bone resorption, and ERR appear to be associated with systemic allergen sensitization. The increased numbers of odontoclasts/osteoclasts on the pressured side during OTM in OVA-sensitized rats, suggesting odontoclastogenesis/osteoclastogenesis under the condition of orthodontic force, were stimulated by systemic allergen sensitization. Since we observed these changes in maxillae after OVA sensitization (under high serum IgE conditions) without further OVA challenge to the skin or nose, the allergic background may be associated with susceptibility to ERR. These findings may support the idea that allergic diseases affect OTM, which was raised by our previous epidemiologic observations showing increased rates of ERR in patients with allergic diseases (Nishioka *et al.*, 2006).

We found markedly elevated serum IgE and OVA-specific IgE levels and, at the same time, observed increased infiltration of inflammatory granulocytes and IL-4 mRNA levels in the periodontal tissue. These findings suggest that OVA sensitization stimulates

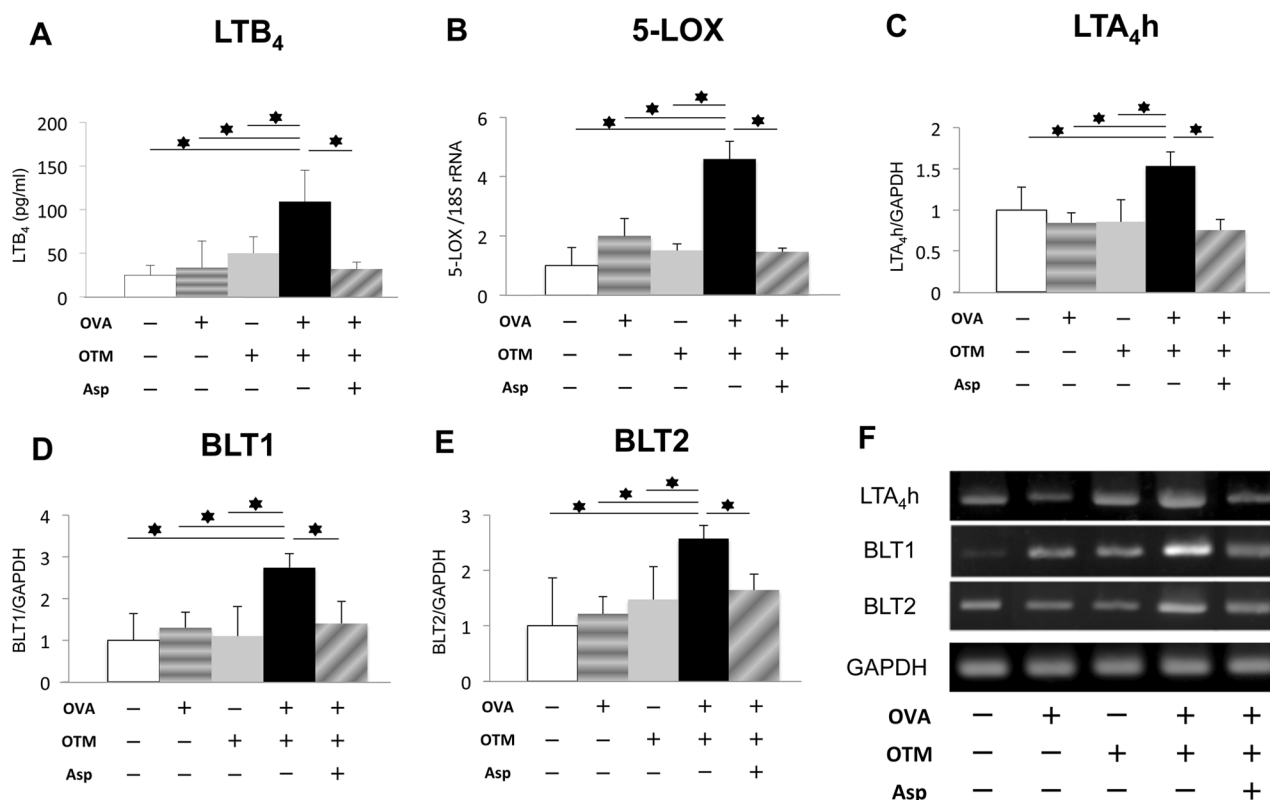


Figure 4. Augmentation of LTB₄ levels and mRNA levels of LTB₄ synthases and receptors in periodontal tissues after 24 hrs of tooth movement in OVA-sensitized animals, and inhibition of these elevations by aspirin administration. (A) Concentration of LTB₄ in periodontal tissues. (B–F) mRNA levels of leukotriene synthases and receptors determined by real-time (B) and conventional (C–F) RT-PCR. The mRNA levels of the leukotriene synthases 5-LOX (B) and LTA₄h (C), and BLT1 (D) and BLT2 (E) and the electrophoretograms (F) are shown. All data are expressed as means ± SD. **p* < .05 by one-way ANOVA with Tukey's multiple-comparison test (*n* = 4 animals per group).

IL-4 production, consistent with a previous report (Renzi *et al.*, 1996). Furthermore, higher expressions of TNF- α and IL-1 β , well-known pro-inflammatory cytokines, were detected in the periodontal tissues of the 'OTM with OVA' group. Recently, Polzer *et al.* (2011) reported that TNF- α -transgenic IL-1 β -deleted mice were protected against bone loss, suggesting that IL-1 β contributes to TNF- α -mediated inflammatory osteopenia. The elevated levels of TNF- α and IL-1 β , together with RANKL as an essential cytokine for osteoclastogenesis, suggest increased rates and magnitudes of ERR and OTM for the differentiation of odontoclasts/osteoclasts. IL-4 is known to suppress TNF- α and RANKL-mediated osteoclast formation *in vitro* (Kong *et al.*, 1999; Kitaura *et al.*, 2003) and *in vivo* (Fujii *et al.*, 2012), while mice overexpressing IL-4 develop systemic bone loss (Lewis *et al.*, 1993). In the periodontal tissues of allergen-sensitized animals, clarification of the cytokines that cause the exacerbation of tooth or bone resorption requires further studies.

Leukotrienes have been implicated in bone remodeling in several reports. Garcia *et al.* (1996) showed that LTB₄ stimulated osteoclastic bone resorption, and Hikiji *et al.* (2009) demonstrated that bone resorption was attenuated in ovariectomized BLT1-knockout mice. We observed higher levels of LTB₄ together with increased expressions of the receptors BLT1 and BLT2, as well as 5-LOX and LTA₄h, biosynthetic enzymes for leukotrienes, suggesting enhanced production of LTB₄ in the

tissues during OTM with allergic inflammation. Based on these findings, the elevated expressions of LTB₄ and its receptors together with the pro-inflammatory cytokines TNF- α and IL-1 β may augment the ERR and OTM through RANKL-mediated osteoclastogenesis. It can be considered that the reciprocal induction of the leukotriene pathway and pro-inflammatory cytokines is involved in the mechanisms of ERR and OTM.

Elderly men and women taking aspirin were reported to show higher bone mineral density than non-takers (Carbone *et al.*, 2003). Yamaza *et al.* (2008) showed that low-dose aspirin inhibited ovariectomy-induced osteoporosis by inhibiting T-cell activation in mice. Administration of aspirin at much lower doses than for general use as a painkiller was able to reverse the increases in ERR, degrees of OTM, and the levels of leukotriene signaling pathway components in our allergen-sensitized model. These findings raise the possibility that low-dose aspirin administration inhibits osteoclastogenesis under inflammatory conditions through the production of anti-inflammatory lipid mediator resolvins (Bento *et al.*, 2011). In our limited experimental period, we did not find that allergy affected the bone structural parameters of the tibia. However, repeated sensitization with allergens over a longer period suggested effects on bone metabolism (unpublished observations). Thus, the increased degree of OTM could be caused by alterations in the structural properties of alveolar bone as well as the enhanced osteoclastogenesis. For

further understanding of the complexity and diversity of molecules associated with the regulation of root and alveolar bone resorption with allergic inflammation, both systemic and loco-regional experiments are needed.

In conclusion, we have proposed a model for studying the effects of allergen-induced inflammation and/or mechanical stress on ERR during OTM. This process was affected by pro-inflammatory cytokines, together with lipid mediators. Furthermore, our findings suggest that pro-inflammatory cytokines and leukotrienes, together with low-dose aspirin, may represent pharmacological targets for ERR during orthodontic treatment with allergic conditions.

ACKNOWLEDGMENTS

We thank Dr. I. Takahashi for valuable advice. We appreciate the technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University. This study was supported by a Grant-in-Aid for Scientific Research (C) (to H.I.) and by a Grant-in-Aid for Young Scientists (B) (to N.M.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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J Dent Res DOI: 10.1177/0022034513488787

Effect of Allergen Sensitization on External Root Resorption

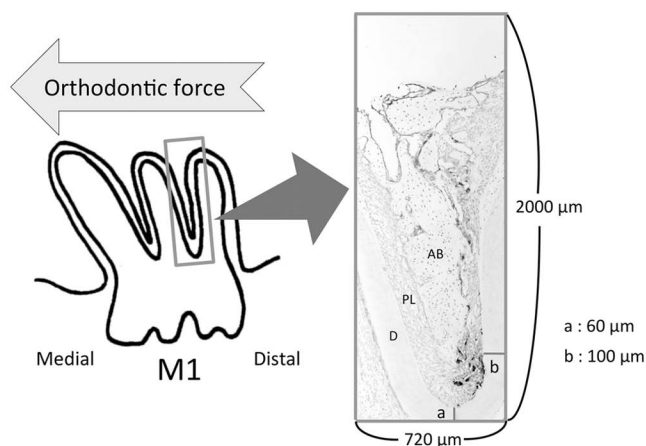
APPENDIX

Appendix Table. Primers Used for Conventional and Real-time RT-PCR

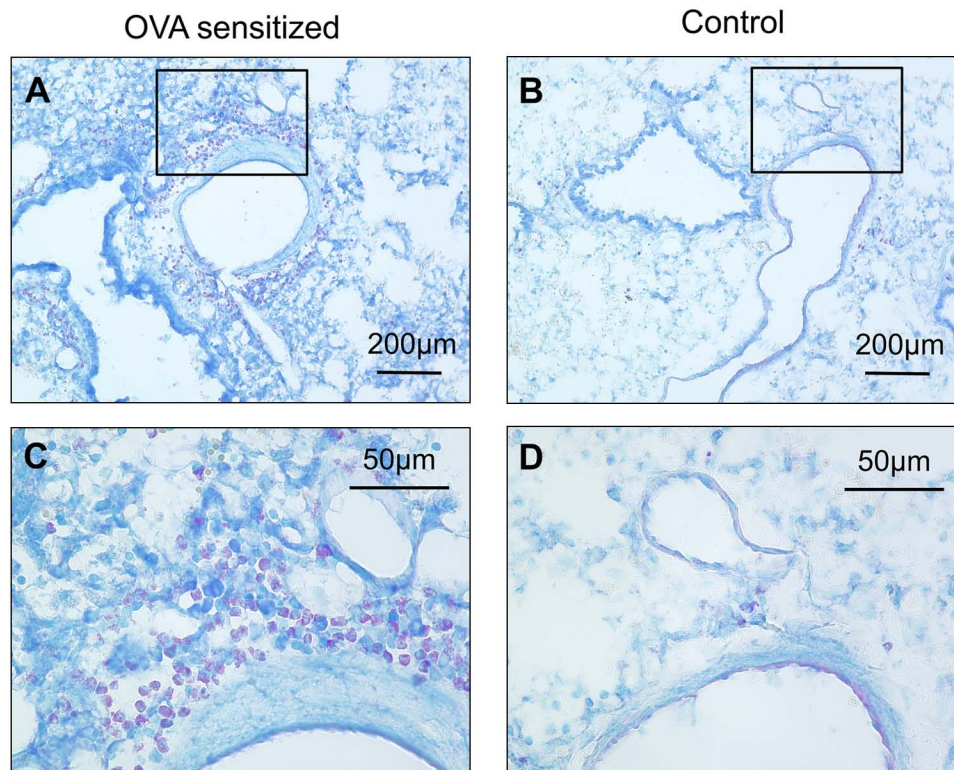
Target Gene	Primer Sequences (forward/reverse)	PCR Product (bp)	Accession Number
IL-4*	5'-TGCACCGAGATGTTGTACCAGA-3' / 5'-TTGCGAAGCACCTGGGAAG-3'	92	NM_201270
RANKL*	5'-CATCGGGTCCCATAAAG-3' / 5'-GAAGCAAATGTTGGCGTA-3'	139	NM_057149
5-LOX*	5'-AGCCAACAAGATTGTTCCCATCGG-3' / 5'-TGGCAATACCGAACACCTCAGACA-3'	196	NM_012822
LTA4h	5'-GGGGACCCTATGTTTGGGGA-3' / 5'-CAGGAGCATAGAGCCAGGCA-3'	612	S_87522
BLT1	5'-GGTGGCATGTCCCTGTCTC-3' / 5'-GACCGGTATGGCCAGCAGAA-3'	420	AB_025230
BLT2	5'-GGGGTGATCGCGTGTGTCAA-3' / 5'-CTCCCCAGAGCCTTCGAAGA-3'	552	AB_052660
GAPDH**	5'-AAACCCATCACCATCTTCCA-3' / 5'-GTGGTTCACACCCATCACAA-3'	178	NM_017008
18S rRNA*	5'-AAGTTTCAGCACATCCTGCGAGTA-3' / 5'-TTGGTGAGGTCAATGTCTGCTTTC	140	NM_213557

*Primers used for real-time PCR.

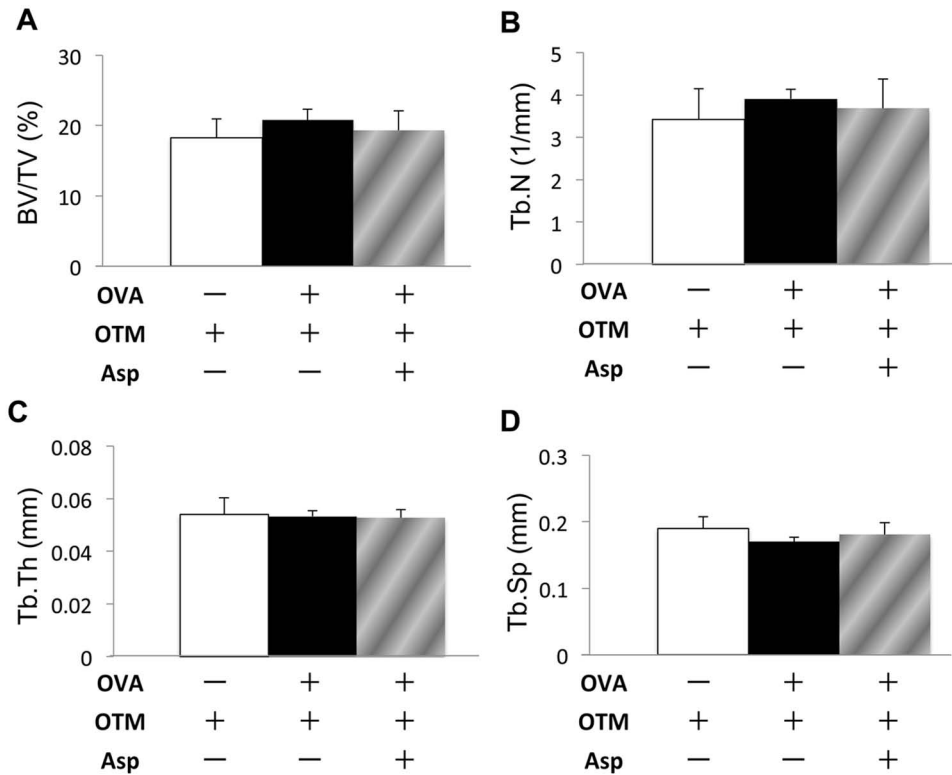
**Primers used for both conventional RT-PCR and real-time PCR.



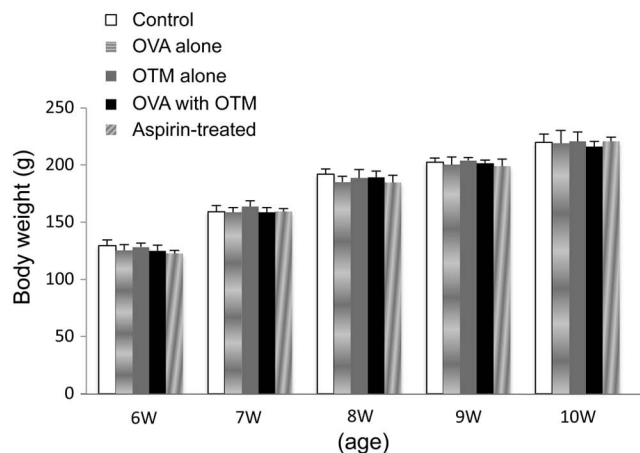
Appendix Figure 1. Area for morphometric analysis. An area of 2,000×720 μm² around the mesial aspect of the distopalatal root of M1 was investigated at ×200 magnification. The observation area was determined at 60 μm distant from the furcation and 100 μm distant from the root. AB, alveolar bone; D, dentin; PL, periodontal ligament.



Appendix Figure 2. Photographs showing lung inflammation. **(A, C)** Infiltration of eosinophils and macrophages around the blood vessels and bronchi was observed after intranasal OVA challenge (100 µg in 40 µg PBS) at 7 days after the second OVA sensitization. **(B, D)** No apparent histological changes are observed in the lungs of control animals (PBS alone). The upper panels show low-magnification images and the lower panels show high-magnification images of the boxed areas in the upper panels. The sections are stained with Giemsa.



Appendix Figure 3. Computerized morphometries of tibiae. No significant changes are observed in the bone structural parameters in the proximal tibiae of the animals after orthodontic force application and aspirin administration. Scanning of the proximal region of the tibia was initiated at the level of the growth plate and extended distally for 2.70 mm. Thereafter, 0.90-mm reconstructed images at 1.00 mm distal to the growth plate were evaluated. The bone structural parameters were analyzed with CTAn software (ver. 1.8.1.5; SkyScan). **(A)** Bone volume fraction (BV/TV). **(B)** Trabecular number (Tb.N). **(C)** Trabecular thickness (Tb.Th). **(D)** Trabecular separation (Tb.Sp). All data were $p > .05$ by one-way ANOVA with Tukey's multiple-comparison test ($n = 6$ animals per group).



Appendix Figure 4. No significant changes in the body weights of the animals after OVA sensitization, orthodontic force application, and aspirin administration during the experimental period. All data are expressed as means $\pm$ SD. All data were $p < .05$ by one-way ANOVA with Tukey's multiple-comparison test ($n = 7$ animals per group).