ORIGINAL ARTICLE

Inhibition of orthodontically induced root resorption with echistatin, an RGD-containing peptide

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Introduction: Induced dental root resorption is a common side effect of orthodontic treatment. It is an unpredictable phenomenon, and its etiology is unknown. Odontoclasts responsible for the resorption of the dental tissues—ie, cementum and dentin—share many cytochemical and morphological characteristics with osteoclasts, which are responsible for bone resorption. The aim of this study was to explore cellular mechanisms that decrease induced root resorption in orthodontically treated teeth. **Methods:** The effects of targeting the αvβ3 integrin receptor, expressed by odontoclasts, on induced root resorption surface areas and the number of root resorption lacunae were investigated by using an RGD-containing peptide, echistatin. The effect of echistatin on the number of clast cells in the periodontium was also examined. Tooth movement was achieved in 14 Sprague-Dawley rats by placing elastic bands between the right maxillary first and second molars for 24 hours. The animals were equally divided into 2 groups; the experimental animals received echistatin intravenously for 8 hours (0.8 μg/kg/min), and the controls received sterile water. The specimens obtained were processed for light microscopy. The surface area and the number of root resorption lacunae were measured histomorphometrically by using digital photomicrographs. Echistatin labeled with a fluorescent marker was used to confirm its presence in clast cells with fluorescent microscopy. Cytochemically, tartrate-resistant acid phosphatase was used to quantify mature and committed clast cells. Echistatin was localized in targeted cells in the periodontium. **Results:** Echistatin significantly decreased root resorption surface areas ($P < .01$) and reduced the number of root resorption lacunae ($P < .01$). There was no statistically significant difference in clast cell numbers. **Conclusions:** Targeting αvβ3 integrin receptor expressed by odontoclasts can be effective in reducing root resorption during tooth movement. Further studies are needed to elucidate the mechanism of this inhibition. (Am J Orthod Dentofacial Orthop 2006; 129:252-60)

Root resorption is a common, but unpredictable, adverse reaction to orthodontic tooth movement (TM). It also occurs under physiologic conditions.[1] The exact etiology of root resorption is unknown, but most likely it is a multifactorial problem involving genetic predisposition, environmental factors related to the morphology of the roots, and orthodontic treatment-related issues.[2-4]

The cells responsible for dental root resorption are called odontoclasts. They share many functional and morphological similarities with osteoclasts.[5-8] Therapeutic modulation of these cells in high-risk patients is warranted. A few attempts to prevent root resorption at the molecular and cellular levels have been made. The use of bisphosphonates, which putatively interfere with the function of clast cells and, hence, inhibit both bone and root resorption, was reported.[9] It has been shown that thyroid hormone administered in high-risk orthodontic patients reduces induced root resorption.[10]

The process of mineralized tissue resorption involves several tightly regulated steps.[11] Among these steps is the adhesion of clast cells to the mineralized surface with a cell surface integrin receptor, which is essential in forming the cytoplasmic tight seal, in demineralization of the inorganic matrix of the mineralized tissue, and in the microenvironment at the resorptive front. The microenvironment has a low pH, which is necessary for the optimal functioning of lysosomal enzymes.[11]

Clast cells express αvβ3, α2β1, αvβ5, and αvβ1 integrin heterodimers.[12] Immunocytochemical studies of the distribution of integrin αvβ3 in rat tissues

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showed strong labeling on the osteoclast domains facing the bone surface, corresponding to the areas of the clear zone around the ruffled borders. Other integrins showed low-intensity labeling throughout all parts of the osteoclasts.[13] Recently, it has been shown, by using immunohistochemical localization, that the αvβ3 integrin is expressed by odontoclasts in root resorption lacunae and by osteoclasts active in different forms of alveolar bone resorption during experimental TM.[14]

A potential molecular-based treatment modality for excessive bone resorption seen in osteoporosis involves blocking the activity of the αvβ3 integrin to inhibit bone resorption.[11] Monoclonal antibodies were developed to block the αvβ3 integrin by immunization of mice with purified recombinant human αvβ3 receptor proteins.[15] Exposing isolated clast cells to a monoclonal anti-αvβ3 integrin antibody inhibits bone resorption.[16] Inhibition of clast cell adhesion isolated from chicks and rats, and cultured on both glass and dentin slices was achieved by using an arginine-glycine-aspartic acid (RGD) containing peptide (GRGDSP). These effects were not observed when a non-RGD containing peptide (GRGESP) was used [16].

Using RGD containing peptides—ie, echistatin—inhibits the resorptive activity of isolated clast cells in vitro.[17] It blocks the attachment of clast cells to the substrate via interaction with the functional part of the integrin structure and prevents tight seal formation.[17,18] Echistatin binds with high affinity (Kd = 0.5 nM) to and colocalizes with αvβ3 receptor.[17] The effect of echistatin is specific for αvβ3, as shown by experiments on a number of integrins that are expressed by osteoclasts and osteoblasts.[19,20] In addition, the in-vivo effect of echistatin on bone resorption was examined in models of excessive bone resorption such as ovariectomized rodents and in the case of secondary hyperparathyroidism.[21-23] It was concluded that echistatin significantly reduces bone resorption. Recently, the effect of echistatin on TM was examined by using radiographs of rat skulls.[21] The amount of TM was measured at 5 different times: at activation of the closed spring and at 1, 3, 7, and 10 days. The TM at day 10 in the control group that did not receive echistatin was significantly different from that of the other groups.[24]

The overall aim of this study was to explore cellular mechanisms that could be used in developing therapeutic approaches to inhibit induced root resorption in orthodontically treated teeth. The specific objectives were to examine the effects of systemic delivery of echistatin on surface areas and the number of induced root resorption lacunae affecting maxillary molars during TM. Additional objectives were to examine the effects of echistatin on the number of odontoclasts and osteoclasts associated with molar roots and alveolar bone, respectively.

MATERIAL AND METHODS

Experimental model

We used 14 male Sprague-Dawley rats. They were equally divided into 2 groups, an experimental group (n = 7) that received echistatin (Sigma, St Louis, Mo) in sterile water for intravenous injections (Baxter, Toronto, Ontario, Canada) and a control group (n = 7) that received sterile water only. TM was achieved by placing elastic bands between the right maxillary first and second molars.[25] The right maxillary side represented TM. The left side did not receive an elastic band and represented no tooth movement (NTM) and physiological root resorption. All procedures were carried out in accordance with the Animal Care Committee recommendations at the University of Illinois at Chicago. A specially designed cage was used for the echistatin intravenous infusion (Coulbourn, Allentown, Pa). A catheter placed in the internal jugular vein was attached to fine tubing that passed subcutaneously to the shoulder region (Charles River Laboratories, Willmington, Mass). The tubing passed through an incision that allowed a second needle to be placed in the extended tubing, and infusion was carried out with an automatically adjustable syringe infusion pump (Coulbourn). Echistatin was infused at a rate of 0.9 mL/hour by using a dose of 0.8 μg/kg/min for approximately 8 hours 30 minutes immediately after the placement of the elastic band. After 24 hours of TM, the animals were killed by placing them in a carbon-dioxide chamber. The maxillae were dissected and divided into halves. The tooth-bearing segments were obtained. These specimens were fixed overnight in 4% paraformaldehyde, pH 7.2, at 4°C. Specimens from 7 animals were demineralized overnight with a rapid decalcifier (RDO, Apex Engineering, Plainfield, Ill). Specimens from the rest of the animals were demineralized with 4.13% EDTA, pH 7.4, for 4 to 6 weeks. The specimens demineralized in EDTA were used for quantification of odontoclasts and osteoclasts by a specific tartrate-resistant acid phosphatase (TRAP) histochemical procedure. After demineralization, the specimens were processed for light microscopy. Alternate sections were stained with hematoxylin and eosin. The unstained sections were used for histochemical assay.

Localization of fluorescein isothiocyanate-labeled echistatin

Fluorescein isothiocyanate (FITC)-labeled echistatin is used in various applications involving fluorescence detection of proteins. The fluorescent properties

of FITC include an absorbance maximum of about 495 nm and an emission wavelength of 520 nm.

Before FITC labeling of echistatin, the molecular weight of echistatin was determined to be 5425 kDa with mass spectrometry. Then echistatin was dissolved in 0.1 M sodium carbonate buffer solution, pH 9, at a concentration of 2 mg/mL. In a darkened laboratory, 50 to 100 μL of FITC solution was added slowly to each milliliter of protein solution. The labeled echistatin was purified by using high performance liquid chromatography. The isolated FITC-labeled echistatin molecular weight was remeasured with mass spectrometry. It was determined to be 5813 kDa, and the increase corresponded to the molecular weight of FITC, which is 389 kDa. The labeled echistatin was used for intravenous infusion in 2 animals with a concentration similar to that used for the other experimental animals. The specimens were processed as previously described. The unstained sections were used for detection and localization of FITC-labeled echistatin by using fluorescence microscopy with fluorescein/green fluorescent protein filter. Unstained sections from unlabeled echistatin infusion were used as negative controls.

Histomorphometry

To standardize the measurements of root resorption in the experimental and control groups, photomicrographs were taken digitally with a 5X objective lens. All measurements were made with Image Pro computer software (MediaCybernetics, Des Moines, Iowa). The measurements included percentages of root resorption and numbers of root resorption lacunae. To calculate the percentages of root resorption, 2 reference points were selected that could be reliably identified in all sections: the cementoenamel junction and the root furcation point. For the mesiobuccal roots of the first molars, the 2 reference points were the furcation points. A line was drawn between the reference points. By tracing the pulp area, the computer software calculated the area (μm^2) of the pulp. The whole root was traced, and the area (μm^2) of the whole root was also calculated by the software. Then the root resorption lacunae were traced, and the combined surface areas were measured by the software (Fig 1). To determine the percentages of root resorption for each root, the following formula was used: combined surface area of root resorption lacunae divided by surface area of the whole root minus surface area of the radicular pulp multiplied by 100. Measurements were taken from 4 sections at 30-μm intervals and averaged for each root of the first, second, and third molars. Values of root resorption for all roots were pooled. To document the effect of blocking αvβ3 on root resorption and to evaluate for statistical significance,

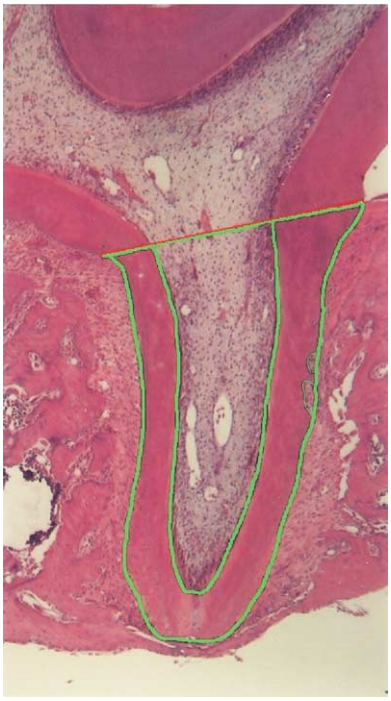

Fig 1. Photomicrograph showing tracing of root, pulp, and root resorption lacunae for calculating percentage of root resorption and number of lacunae.

the independent *t* test was used to compare the means of percentages of root resorption areas and numbers of resorption lacunae in both groups. Also, the independent *t* test was used to compare the means of resorption percentages and numbers of lacunae in the TM side that represented the induced root resorption and the NTM side that represented physiologic root resorption.

To examine the root resorption measurement error, 10 photomicrographs were randomly selected. The roots in these images were traced, and the surface area of each root was measured and documented with the Image Pro software. Ten days later, a second measurement of the surface area of the roots was made. The paired *t* test showed no statistically significant differences between the 2 measurements ($P = .73$).

Enzyme histochemical quantification of clast cell number with tartrate-resistant acid phosphatase staining

The identification of odontoclasts was based on their morphology and by using tartrate-resistant acid phosphatase (TRAP) marker for clast cells. The rationale for using TRAP in addition to light microscope examination is to help in identifing committed monocytes that do not exhibit the multinucleated characteristic morphology.

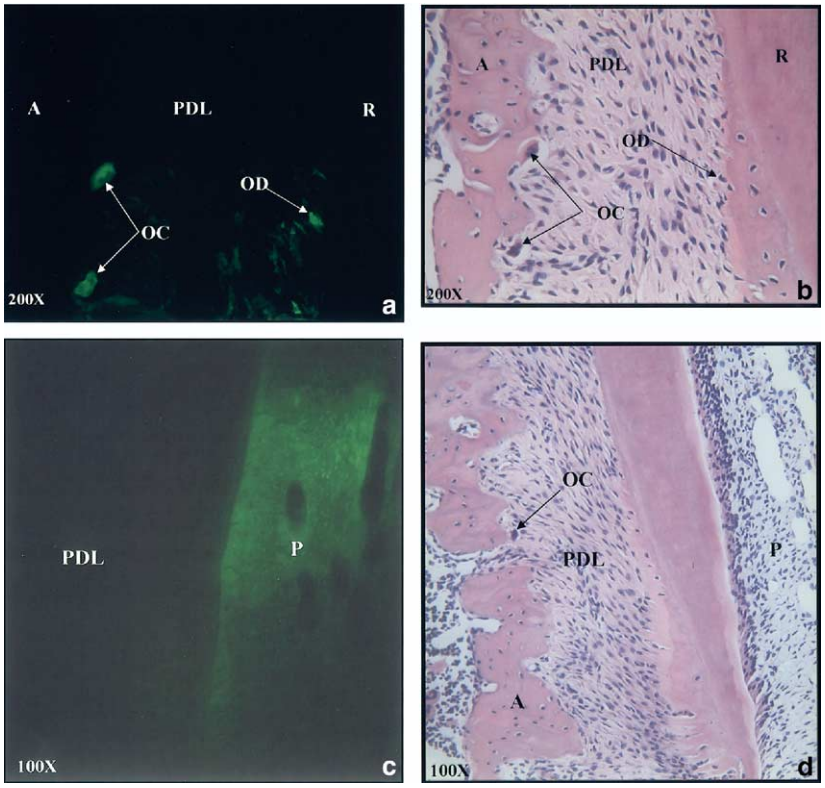

Fig 2. a, Photomicrograph showing FITC-labeled echistatin with fluorescence microscopy in osteoclasts (*OC*) and odontoclasts (*OD*). Alveolar bone (*A*), periodontal ligament (*PDL*), root (*R*). **b,** Same area as *a*, stained with hematoxylin and eosin. **c,** Negative control photomicrograph showing no FITC-labeled echistatin; **d,** Same area as *c*, stained with hematoxylin and eosin.

The unstained paraffin-embedded sections were deparaffinized, rehydrated, and incubated for 50 minutes at 37°C in a solution consisting of 1.0 mL fast garnet solution, 0.5 mL napthol AS-BI phosphate solution (12.5 mg/ml), 2.0 mL acetate solution (2.5 mmol/L, pH 5.2 ± 0.1), 1.0 mL tartrate solution (0.335 mol/L, pH 4.9 ± 0.1), and 45 mL deionized water. For the negative controls, additional sections were processed simultaneously, omitting the tartrate solution.

Photomicrographs were taken of the periodontal ligament (PDL) mesial and distal to the roots of the first and second molars with a digital camera at a magnification of 200 times and transferred into the Image Pro analysis software. A grid was superimposed on the photomicrographs to standardize the area to be included in cell counting, and a line was drawn, bisecting the PDL. The labeled cells on the half closer to the root surface were considered odontoclasts, and the labeled cells closer to the alveolar bone were considered osteoclasts. The average number of cells per square millimeter and the total number of cells in the PDL on the pressure and tension areas next to the roots of the maxillary first and second molars were quantified on the TM side. The cell numbers in the pressure and tension regions in the first and second molars were compared statistically for any significant difference between the experimental and control groups. The areas of marrow cavities and blood vessels were excluded from quantification of cell numbers.

RESULTS

The load delivered to the clinical crowns of the maxillary first and second molars with an elastic band caused uncontrolled tipping movement and created areas of compression and tension in the PDL. In the sections stained with hematoxylin and eosin, root resorption lacunae were seen on the root surface, and a few multinucleated odontoclasts were seen adhering to resorption lacunae along the root surface.

Fluorescent microscopic analysis of tissue sections from animals intravenously infused by FITC-labeled echistatin showed that labeled echistatin was localized in osteoclasts and odontoclasts in the periodontium of the maxillary rat molars (Fig 2, *a* and *b*). Negative

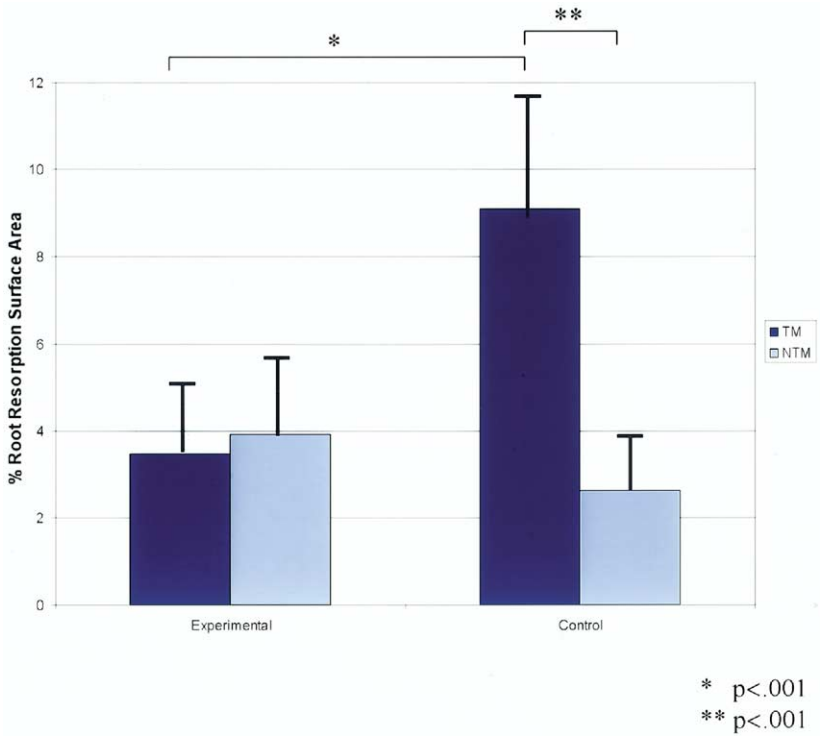

Fig 3. Relative surface area of root resorption expressed as percentage of total surface area of root on TM and NTM sides. Difference between experimental and control groups statistically significant on TM side ($P < .01$). Difference between TM and NTM statistically significant in control group ($P < .001$).

control tissue sections from animals that received unlabeled echistatin showed no echistatin in the periodontium of their molars (Fig 2, *c* and *d*).

Histomorphometric analysis of the relative root resorption expressed in percentages showed that the average relative root resorption affecting maxillary molars on the TM side was 3.46 ± 1.62 in the experimental group and 9.09 ± 2.85 in the control group (Fig 3). The average relative root resorption on the NTM side was 3.91 ± 2.02 in the experimental group and 2.62 ± 1.16 in the control group (Fig 3). There was a statistically significant difference in relative root resorption between the TM and the NTM sides in the control group ($P < .001$). There was a statistically significant inhibition of root resorption in the experimental group ($P < .01$) on the TM side. There was no statistically significant difference of relative root resorption between the TM and the NTM sides in the experimental group. Furthermore, there was no statistically significant difference of relative root resorption on the NTM side in the experimental and control groups.

The average numbers of root resorption lacunae on the TM side were 13.57 ± 4.04 in the experimental group and 21.86 ± 3.67 in the control group (Fig 4). The average numbers of root resorption lacunae on the NTM side were 11.86 ± 4.1 in the experimental group and 10.86 ± 3.57 in the control group (Fig 4). There was a statistically significant difference in the number of root resorption lacunae between the TM and the NTM sides in the control group ($P < .001$). There was a significant decrease in the number of root resorption lacunae in the experimental group ($P < .01$). There was no statistical difference in the number of root resorption lacunae between the TM and the NTM sides in the experimental group. Furthermore, there was no statistical difference in the number of root resorption lacunae on the NTM sides in the experimental and control groups.

Tissue sections stained with TRAP marker showed positive identification of mature multinucleated osteoclasts and odontoclasts in the periodontium of the maxillary molars. TRAP marker was also identified in mononuclear clast cells in the periodontium. The labeled cells exhibited maroon staining in these tissue sections (Fig 5). Histomorphometric analysis and statistical comparison of the number of odontoclasts and osteoclasts per square millimeter of the periodontium on the pressure and tension regions of the maxillary first and second molars on the TM side showed no

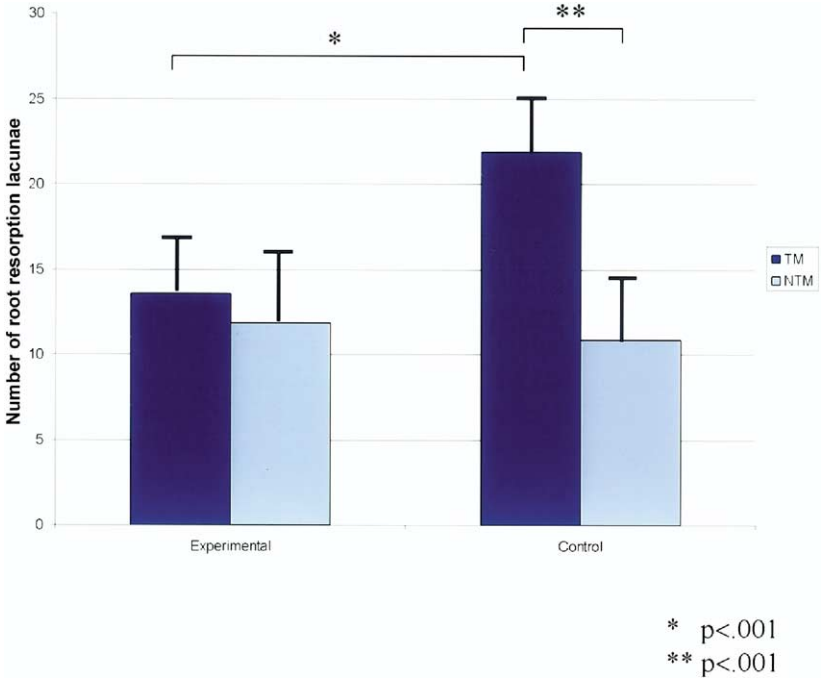

Fig 4. Number of root resorption lacunae in experimental and control groups on TM and NTM sides. Difference between experimental and control groups statistically significant on TM side ($P < .01$). Difference between TM and NTM statistically significant in control group ($P < .001$).

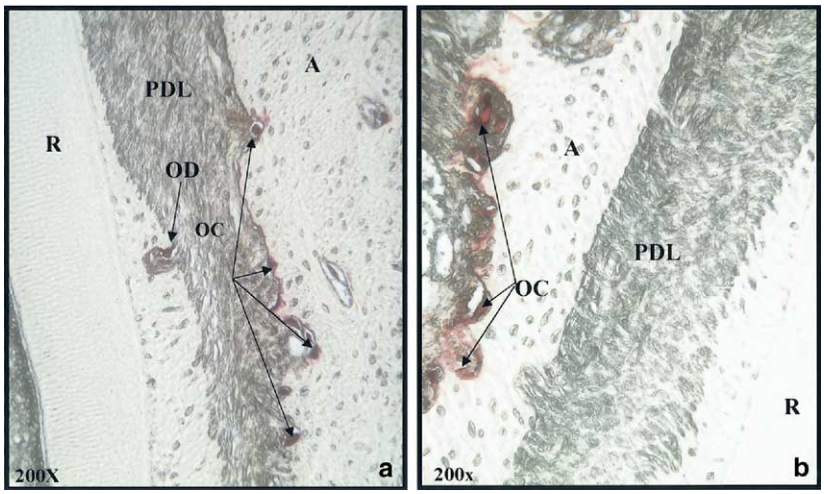

Fig 5. a, Photomicrograph showing number of osteoclasts (*OC*) lining alveolar bone (*A*), positively labeled for TRAP, and similarly labeled odontoclasts (*OD*) at edge of root (*R*) resorption lacuna. **b,** Photomicrograph showing positive TRAP-labeled osteoclasts lining alveolar bone.

statistically significant difference between the 2 groups (Tables I and II).

DISCUSSION

Induced root resorption is considered a risk of orthodontic TM. Identifying high-risk patients for developing root resorption during orthodontic TM is a prerequisite for developing clinical or cellular treatment modalities to prevent or reduce the incidence of root resorption.

The use of elastic bands for TM is a well-characterized model. Latex elastic bands 0.5 mm thick deliver

Table I. Means and standard deviations of average total numbers of odontoclasts (*OD*) and per mm^2 of PDL on pressure and tension regions of first and second maxillary molars

	Experimental group	*Control group*	*Significance*
OD per mm^2, first molar, pressure side	0.71 ± 0.91	0.59 ± 0.62	NS
OD per mm^2, first molar, tension side	0.08 ± 0.29	0	NS
OD per mm^2, second molar, pressure side	0.5 ± 0.97	0.7 ± 0.82	NS
OD per mm^2, second molar, tension side	0.14 ± 0.38	0	NS
Total OD, first molar, pressure side	2.5 ± 3	1.5 ± 1	NS
Total OD, first molar, tension side	0.25 ± 0.5	0	NS
Total OD, second molar, pressure side	1.25 ± 1.89	2 ± 1.41	NS
Total OD, second molar, tension side	0.25 ± 0.5	0	NS

NS, Not significant on TM side.

Table II. Means and standard deviations of average total numbers of osteoclasts (*OC*) and per mm^2 of PDL on pressure and tension regions of first and second maxillary molars

	Experimental group	*Control group*	*Significance*
OC per mm^2, first molar, pressure side	1.39 ± 0.91	1.53 ± 1.12	NS
OC per mm^2, first molar, tension side	1.16 ± 1.26	0.75 ± 0.86	NS
OC per mm^2, second molar, pressure side	1.96 ± 1.6	1.57 ± 0.81	NS
OC per mm^2, second molar, tension side	0.14 ± 0.3	0.22 ± 0.44	NS
Total OC, first molar, pressure side	14.33 ± 13.65	17.25 ± 3.1	NS
Total OC, first molar, tension side	7.33 ± 9.29	2.75 ± 2.06	NS
Total OC, second molar, pressure side	11.75 ± 13.25	15.25 ± 8.66	NS
Total OC, second molar, tension side	0.25 ± 0.5	0.5 ± 0.57	NS

NS, Not significant on TM side.

forces of approximately 0.14 N.[26] In our study, a 1-mm thick latex elastic band was used; this was thicker than the previously characterized elastic band. Hence, the latex elastic bands produced forces presumably greater than 0.14 N. These elastic bands elicit tissue reactions that include the recruitment of clast cells and their progenitors; this results in bone and root resorption. This is evident in our study by the statistical difference in the extent of root resorption between the TM and the NTM sides of the control group.

Based on previous findings of vascular changes during tooth movement[27,28] and high vascularity of the maxillary complex, systemic delivery of therapeutic agents is likely to reach and be present in the PDL during experimental TM; this was confirmed in our study. The systemic delivery of FITC-labeled echistatin was localized in osteoclasts and odontoclasts in the PDL when observed with fluorescent microscopy, whereas the controls infused with unlabeled echistatin did not show fluorescence signals.

Targeting osteoclast adhesion both in vitro and in vivo to prevent or inhibit bone resorption has been explored by using peptides containing RGD sequence—ie, echistatin that was either naturally present in snake venom or synthetically produced.[17,18] Others used monoclonal antibodies containing the RGD sequence, and inhibition was shown to be RGD sensitive.[15,16,19-23] In our study, systemic delivery of naturally occurring echistatin significantly reduced induced root resorption surface areas and the number of root resorption lacunae. Echistatin reduced root resorption to physiologic root resorption levels. There was no evidence that echistatin affects physiologic root resorption because this resorption could have been present before the experiment, or longer echistatin exposure might be needed to affect it. The effect of echistatin on induced root resorption suggests that the mechanisms of root and bone resorption are similar. In addition, targeting αvβ3 integrin receptor can be effective in reducing induced root resorption associated with forces used to move teeth in the alveolar bone. Our findings that RGD-containing peptides inhibit root resorption agree with previous findings about reducing bone resorption in vivo.[21-23] However, the exact mechanism by which echistatin inhibits bone resorption is still not clear. Several modes of action are suggested, including the steps involved in mineralized tissue resorption. Echistatin might inhibit the differentiation, proliferation, and migration of clast cells to the site of resorption, and that is expressed as a decrease in the number of clast cells involved in resorption. It also might disrupt the tight seal formation and the actin microfilament ring that depend on the

adhesion of the αvβ3 integrin to the substrate. Therefore, to examine the effects of echistatin on the number of odontoclasts and osteoclasts, a specific clast cell marker, TRAP, was used. The determination of the number of odontoclasts is relevant to clarify the mechanism of inhibition by echistatin—ie, whether it interferes with clast cell function or interferes with differentiation and migration of these cells to the areas of resorption. In the latter case, clast cell numbers would be decreased compared with the controls. In this study, echistatin did not have an effect on the numbers of mature and committed osteoclasts and odontoclasts as shown by the quantification of osteoclasts and odontoclasts in the total area of the PDL or per square millimeter of the PDL, suggesting that echistatin at the concentration used in this study did not interfere with differentiation, proliferation, or migration of clast cells to the sites of mineralized tissue resorption. These findings agree with previous studies that showed that echistatin had no effect on clast cell proliferation and migration.[21,23]

TM in the experimental and control groups was quantified by using digital images of casts of maxillary impressions taken before infusion and 24 hours later when the rats were killed (data not shown). No statistically significant differences were found between the 2 groups in amount of TM or number of osteoclasts associated with alveolar bone. Because both osteoclasts and odontoclasts share numerous morphological and functional features, it is reasonable to assume that they would be similarly affected by echistatin. Yet in this study, a 24-hour infusion by echistatin significantly reduced orthodontically induced root resorption, but TM, which is linked to alveolar bone resorption, was not affected. Reitan[29] described 3 phases of orthodontic TM: an initial tipping phase, a lag phase, and a postlag phase, when TM occurs. The time parameter of our study was during the tipping phase; this might explain the observed lack of echistatin inhibition of TM. Echistatin inhibition of bone resorption is probably due to interference with the functional activity of osteoclasts because it was shown to disrupt integrin-mediated events[17]; however, the precise mechanism of echistatin action is yet to be elucidated. Because the number and surface area of resorption lacunae in the alveolar bone were not quantified in our study, whether echistatin infusion for 24 hours inhibits the functional efficiency of osteoclasts cannot be verified. If proven to be true, it is possible that the time frame of events involved in inhibition of bone resorption is different from that in root resorption, although the mechanisms might be similar. Such speculation is consistent with the findings of Dolce et al,[24] who showed no effect of echistatin on TM after 24 hours but significant inhibition after 10 days. In addition, it was reported that a minimum serum concentration of 20 nM of echistatin must be maintained for 14 days to produce significant inhibition of bone resorption in ovariectomized rats.[23] Additional investigation is needed to clarify the short-term effect of echistatin on alveolar bone resorption.

Clast cells are known not only to migrate toward the mineralized tissues but also to cycle after their adhesion between attachment and detachment from the mineralized tissue. Previous studies showed that the tight seal is a dynamic structure allowing molecules less than 10,000 kDa to pass across it.[30] Because echistatin has a molecular weight of less than 10,000 kDa, a possible mechanism of inhibition might be the diffusion of echistatin into the microenvironment and association with free αvβ3 integrin receptors, preventing their clustering and inhibiting the migration of clast cells on the mineralized tissue. Thus, these findings suggest that echistatin might affect the function of αvβ3 integrin receptor. This and the effect on local migration and spreading of clast cells on the mineralized tissue are the probable causes of the observed inhibition of induced root resorption during experimental TM. Further studies exploring the mechanism of action of RGD-containing peptides such as echistatin are needed.

This study provides a rationale for future development of drugs targeting the αvβ3 integrin that could be used to inhibit induced root resorption that is commonly associated with orthodontic TM. This study also shows that some treatment modalities for treating osteoporosis or bone metastasis by targeting osteoclast receptors or their intracellular proteins might be useful in orthodontics. Future drugs that are safe for clinical use and have the same properties of targeting the αvβ3 integrin might enhance anchorage during orthodontic treatment or inhibit induced root resorption.

CONCLUSIONS

A systemically delivered RGD containing peptide—echistatin—significantly reduced root resorption induced by TM. Treatment modalities based on targeting osteoclast receptors could have uses in clinical orthodontics.

Systemically delivered FITC-labeled echistatin localizes in targeted cells in the PDL. Echistatin does not affect the differentiation, proliferation, and migration of clast cells.

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