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ORIGINAL ARTICLE

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Effects of local osteoprotegerin gene transfection on orthodontic root resorption during retention: an *in vivo* micro-CT analysis

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Structured Abstract

Objectives – External root resorption (ERR) is a serious complication of orthodontic treatment. Aim of this study was to evaluate the effects of local osteoprotegerin (OPG) gene transfection on ERR during retention.

Material and Methods – Eighteen 6-week-old male Wistar rats were divided into three groups. All the rats were subjected to 2 weeks of orthodontic tooth movement followed by a 2-week retention period. During retention, the three groups of rats received local OPG gene transfection (OPG transfection group, n = 6), mock vector transfection (mock group, n = 6), or no injections (control group, n = 6). ERR of all three groups was evaluated with *in vivo* micro-CT analysis at three different time points: baseline, the last day of orthodontic tooth movement, and the last day of retention.

Results – In the OPG transfection group, there was no significant difference between ERR at the baseline and ERR on the last day of retention. By the last day of retention, the repair ratio of ERR in the OPG transfection group was statistically higher in relation to the repair ratio of the other groups ($p < 0.001$).

Conclusion – The results indicated that local OPG gene transfection significantly enhanced the repair of ERR during retention. Local OPG gene transfection might therefore be a useful tool for ERR repair during retention.

Key words: micro-CT; orthodontic tooth movement; osteoprotegerin; root resorption

Introduction

External root resorption (ERR) has been recognized as a frequent iatrogenic complication of orthodontic treatment. ERR is undesirable because it can provoke the loss of hard tissue of

teeth and affect the long-term viability of the dentition (1, 2). However, the exact mechanisms of ERR are not yet fully understood. ERR can occur during orthodontic treatment and continue after the orthodontic force is removed (3–8). Odontoclasts play an important role in ERR, in a similar manner to osteoclasts during bone resorption (3–5, 9).

The receptor activator of the nuclear factor-kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system is a key regulator of osteoclastogenesis and bone resorption (10). The differentiation and activation of osteoclasts can be inhibited by OPG (10). The RANK/RANKL/OPG axis might also contribute to ERR (11–14). Low et al. reported that RANK/RANKL regulates ERR (12). Yamaguchi et al. found that the levels of RANKL had increased while those of OPG had decreased in the compressed periodontal ligament cells from patients with severe ERR (13). Tyrovola et al. reported that the levels of OPG and soluble RANKL in blood serum and in gingival crevicular fluid were relative to the degree of ERR severity (14).

It has been confirmed that exogenous OPG could inhibit the activation of osteoclasts and reduce bone resorption in many disease processes (10). The effects of exogenous OPG on orthodontic tooth movement have been investigated (15, 16). Dunn et al. demonstrated that a fusion protein, OPG-Fc, effectively inhibited orthodontic tooth movement in rats (15). In our previous study, we reported that local *OPG* gene transfection significantly reduced orthodontic tooth movement (16) and *RANKL* gene transfection enhanced orthodontic tooth movement (17). As ERR is performed by odontoclasts, these data suggest the possibility that ERR could be reduced with the local transfection of the *OPG* gene.

Recently, several approaches to prevent ERR have been reported, including the administration of thyroid hormone (18), bisphosphonates (19–21), celecoxib (22), and an antibody against the macrophage colony-stimulating factor (M-CSF) receptor c-Fms (23), among others. In these studies, ERR inhibitors were administered during active orthodontic tooth movement (18–23). However, because of the similarities between odontoclasts and osteoclasts, some inhibitors used to reduce ERR can also inhibit orthodontic tooth movement (19–24), which constitutes a disadvantage for orthodontic treatment. Therefore, this study was designed to administer the ERR inhibitor during the retention phase.

In summary, the aim of this study was to evaluate the effects of local *OPG* gene transfection on ERR during the retention phase of orthodontic treatment.

Materials and methods

Animals

Eighteen 6-week-old male Wistar rats (approximate weight, 180–190 g) were utilized in this study. All the rats were housed under normal conditions with a 12-h circadian cycle and were fed with a standard rat-chow diet and water *ad libitum*. The experimental procedures were approved by the Ethics Committee of Peking University Health Science Center.

The rats were randomly divided into three groups: the control group, mock group, and the OPG transfection group (n = 6 rats in each group). All the rats were subjected to a 2-week period of orthodontic tooth movement followed by a 2-week retention phase. During retention, the rats in the control group received no injections, the rats in the mock group received localized mock vector transfection, and the rats of the OPG transfection group received the localized transfection of the *OPG* gene.

All the three groups of rats were scanned with a micro-CT (SkyScan 1076 X-ray microtomography system; SkyScan, Kontich, Belgium) at three different time points: at the baseline before orthodontic tooth movement, on the last day of orthodontic tooth movement, and on the last day of retention. Based on the results of micro-CT scanning, the degree of ERR at different time points was analyzed, while the amount of bone mineral density (BMD) and bone volume fraction (BVF) of the alveolar bone and tibiae was quantified. The BMD and BVF values were analyzed using the in-house software of the SkyScan 1076.

The density and volume fraction are important parameters in describing the status of bone metabolism (25, 26). BVF is the fraction of solid bone volume/total volume, which is measured to evaluate the trabecular microstructure. The accuracy of BVF and BMD measurements by micro-CT scanning was confirmed by a previous study (27).

Orthodontic appliance placement

The method used for the application of orthodontic force has been described previously in detail (22). Briefly, a closed coil nickel–titanium spring (Sentalloy®; Tomy Inc., Tokyo, Japan) providing a force of 60 gf at activation was connected between the maxillary right first molar and maxillary incisors with 0.20-mm steel ligatures (Fig. 1). During retention, the spring was removed and light-cured resin was placed in the interdental space between the right maxillary first and second molars. The diet of the rats did not change after the orthodontic device had been applied.

In vivo gene transfer

For *in vivo* gene transfer, we used an inactivated hemagglutinating virus of the Japan envelope vector (HVJ-E) (GenomONE®; Ishiara-Sangyo Kaisha Ltd., Osaka, Japan). The details of constructing the *OPG* expression plasmid [pcDNA3.1(+)-mOPG] have been described in our previous study (16). The vector map of pcDNA3.1(+)-mOPG is shown in Fig. 2. The administration of HVJ-E containing pcDNA3.1(+)-mOPG to the rats in the OPG transfection group was commenced on the initial day of retention. The rats received general anesthesia, and 5 μl of vector solution was injected into the palatal mucosa adjacent to the mesial surface of the upper right first molar using 26 s-gauge microneedles (Hamilton Company, Reno, NV, USA). The volumetrically equivalent mock vector solution (HVJ-E containing a mock plasmid of pcDNA3.1+) was injected into rats in the mock group. All the injections were administered twice weekly during the retention phase. The rats of the control group received no injections.

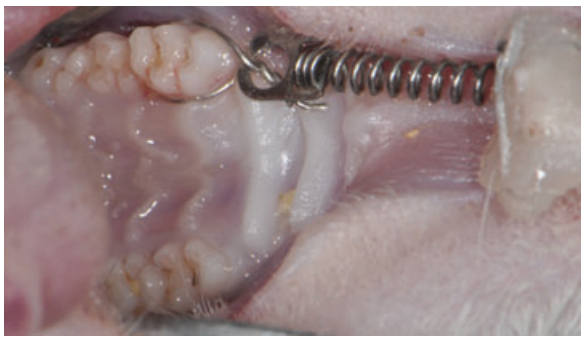

Fig. 1. Intraoral photograph of the orthodontic appliance.

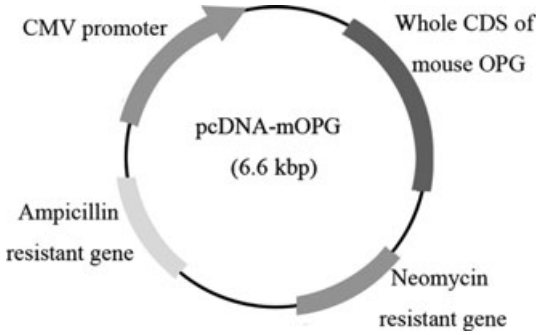

Fig. 2. The vector map of pcDNA3.1(+)-mOPG. The vector contained cytomegalovirus (CMV) promoter and antibiotics-resistant genes (ampicillin-resistant gene and neomycin-resistant gene). Whole coding sequence (CDS) of mouse OPG was inserted into the multiple cloning site of the vector. OPG, osteoprotegerin.

Micro-CT scanning and analysis

Micro-CT was used to analyze the volume of ERR, measure the BMD and BVF of the alveolar bone and tibiae. Each rat was scanned with the SkyScan 1076 X-ray microtomography system. The SkyScan 1076 is a low-dose *in vivo* X-ray micro-CT scanner for slice imaging and the 3D image reconstruction of small animals. The head and the left tibiae of every rat were scanned individually. The micro-CT settings for the maxilla images were 70 kV and 141 μA, with a rotation step of 0.8° and an image voxel size of 9.488 μm; the scan duration was approximately 20 min. Micro-CT settings for the tibiae images were 70 kV and 139 μA, with a rotation step of 0.6° and the image was voxel size 18.97 μm; the scan duration was approximately 8 min.

The Mimics 13.1 software (Materialise, Leuven, Belgium) was used to carry out the 3D image

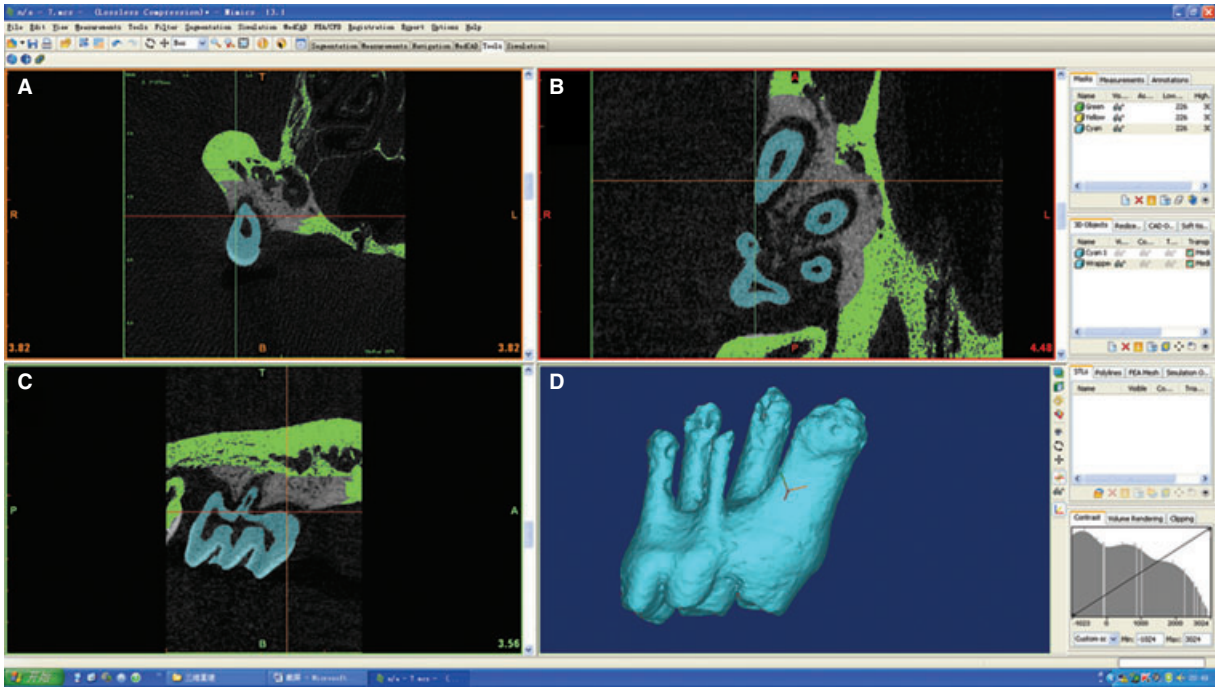

Fig. 3. 3D-reconstruction of the maxillary right first molar using Mimics 13.1 software: (A, B, C) three different directional slices of the maxillary first molar; (D) 3D image of the maxillary first molar.

reconstruction at the appropriate cross-section and calculate the ERR volume quantification. The 3D reconstruction of teeth is shown in Fig. 3.

The ERR volume measurement was based on the application of a 3D convex hull. The convex hull algorithm assumes that the surface of the root is convex; therefore, when a crater is present, this assumption is not met and a break in the convexity is detected, which enables the volume to be calculated by using the Mimics 13.1 software. Therefore, the ERR of all five roots of the upper right first molar were calculated, and the whole surface of each root was included.

During BMD and BVF measurements, the cortical and trabecular bones of tibiae were separated manually using the in-house SkyScan 1076 software. After the images were captured, 100 slices beginning approximately 1.9 mm beneath the growth plate of the tibiae were established as the 'volume of interest'. BMD and BVF of alveolar bone were measured in the first molar furcation area. The furcation area was chosen because it provides reproducible morphological landmarks (15).

Histological and immunohistochemical analysis of OPG expression

The animals were killed with an overdose of anesthetic, and the tissues were fixed in 10% neutral-buffered formalin solution for 24 h at 4°C. The upper jaw, including all molars, was dissected and decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 4 weeks at 4°C before they were embedded in paraffin. Horizontal specimens were obtained from the root of each maxillary first molar. The sections were stained with hematoxylin and eosin (H&E) for descriptive histology. Immunohistochemical staining of OPG was performed to identify the site of *OPG* gene transfection. A minimum of six slides per animal were deparaffinized and then incubated with anti-OPG antibody (1:200 dilution; sc-8468; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then, the slides were stained with the 2-step plus poly-horseradish peroxidase (HRP) anti-Goat IgG detection system (ZSGB-Bio, Beijing, China), followed by color development with diaminobenzidine (DAB). Integrated optical density (IOD) was measured to describe the intensity of immunohistochemistry with the Image-Pro Plus

6.0 software (Media Cybernetics Inc., Bethesda, MD, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significance of differences among the three groups. Two-way ANOVA was used to calculate the significance of differences among the three different time points. Pairwise comparisons between groups and between different time points were performed with the least-significant difference test. All data are expressed as mean ± standard deviation. Differences where p-values were <0.05 were considered significant.

Results

Animal status

The orthodontic appliance and force only slightly affected the weight of animals during the first 3 days of the experiment. At the baseline, the average weight of all rats was 184.1 ± 2.6 g; 3 days after the initiation of orthodontic tooth movement, the average weight of all the rats was 176.1 ± 2.2 g. The weight of the rats then increased, and the average was 212.9 ± 2.8 g on the last day of orthodontic tooth movement. On the last day of retention, the average weight of rats in the control, mock, and OPG transfection groups were 268.5 ± 6.9, 283.2 ± 5.7, and 271.2 ± 8.3 g, respectively, and there were no significant differences among the groups. These data suggest that localized *OPG* gene transfection did not affect the growth of rats.

Twice-weekly localized OPG gene transfection appeared to cause no appreciable macroscopic changes, including edema, erythema, or erosion at the site of injection. The results of histological examination showed that there was no severe inflammation, such as lymphocytic infiltration, in the periodontal tissue (Fig. 4).

Three-dimensional (3D) analysis of ERR at different time points in different groups

The volume of ERR in the three groups was measured at three different time points: baseline, the last day of orthodontic tooth movement, and the last day of retention (Table 1). At baseline, the amount of ERR in the three groups was minimal and there were no significant differences among the three groups at the first two time points. By the last day of orthodontic tooth movement, the volume of ERR in all three groups was significantly increased ($p < 0.001$). After 2 weeks of retention, the volume of ERR in all three groups was significantly decreased ($p < 0.001$), especially in the OPG transfection group. In the control and mock groups, there was significantly more ERR by the last day of retention than that at baseline (Fig. 5). However, in the OPG transfection group, there was no significant difference between the volume of ERR at the baseline and ERR on the last day of retention (Fig. 6).

The repair ratios of ERR during retention were 37.1 ± 4.3% (control group), 35.9 ± 6.7% (mock group), and 75.7 ± 6.9% (OPG transfection group), respectively (Fig. 7). At this time point, the degree of repair of ERR in the OPG transfection group

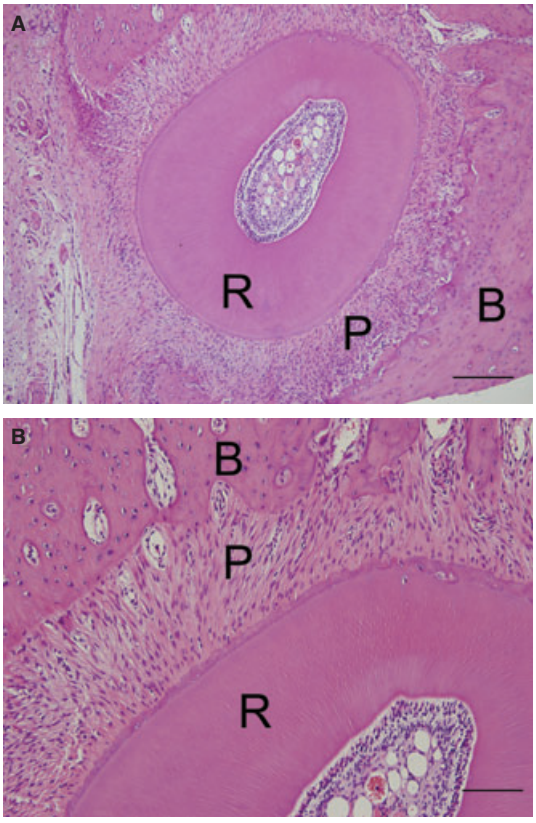

Fig. 4. H&E staining of the mesial root of the maxillary right first molar: (A) scale bar = 200 μm; (B) scale bar = 100 μm. No severe inflammation of the periodontal tissue was observed. R, root; P, periodontal ligament; B, bone.

Table 1. **ERR at three different time points in the three groups**

	Control group	Mock group	OPG transfection group
ERR at the baseline (mm^3)	0.06 ± 0.03[a]	0.06 ± 0.02[a]	0.06 ± 0.04[a]
ERR on the last day of orthodontic tooth movement (mm^3)	0.58 ± 0.11[b]	0.57 ± 0.12[b]	0.56 ± 0.15[b]
ERR on the last day of retention (mm^3)	0.36 ± 0.08[c]	0.36 ± 0.08[c]	0.13 ± 0.02[a]

Mean values with the same superscript letters are not significantly different ($p > 0.05$). Mean values with the different superscript letters indicate statistically significant differences ($p < 0.001$). Localized *OPG* gene transfection significantly reduced ERR during retention. ERR, external root resorption; OPG, osteoprotegerin.

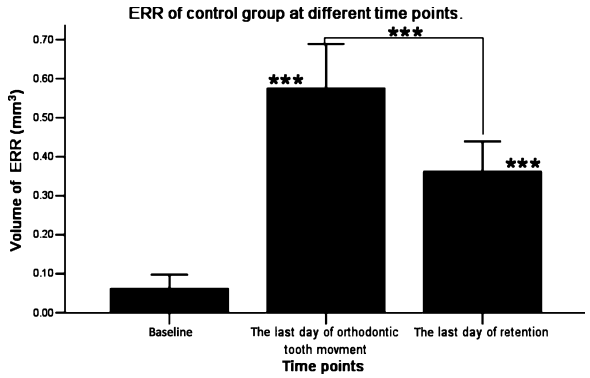

Fig. 5. External root resorption (ERR) of the control group at different time points. ERR on the last day of retention was significantly less than ERR on the last day of orthodontic tooth movement. The repair ratio of ERR was 37.0 ± 1.2%. ***$p < 0.001$.

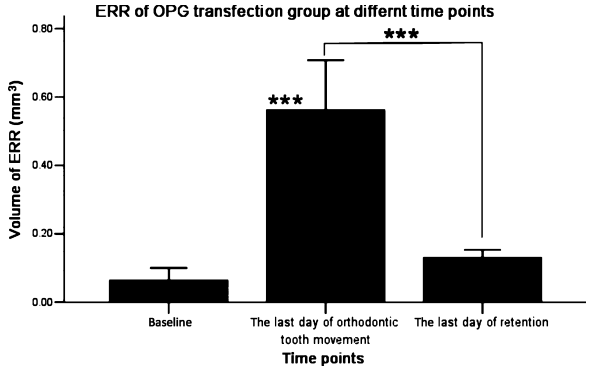

Fig. 6. External root resorption (ERR) of the osteoprotegerin transfection group at different time points. The average amount of ERR at different time points is shown. ERR on the last day of retention was significantly less than ERR on the last day of orthodontic tooth movement. There was no significant difference between ERR at the baseline and ERR on the last day of retention. The repair ratio of ERR was 76.9 ± 3.3%. ***$p < 0.001$.

was significantly higher relative to control and mock groups, respectively ($p < 0.001$ for both) (Table 1, Fig. 7).

Effects of OPG gene transfer on bone remodeling: alveolar bone and tibiae

The values for the BMD and BVF of alveolar bone were analyzed to evaluate the effects of *OPG* gene transfection on local bone remodeling. On the last day of retention, BMD and BVF were significantly increased in the OPG transfection group compared to the control and mock groups, respectively ($p < 0.05$) (Table 2). BMD and BVF were analyzed in the tibiae to evaluate the effects of *OPG* gene transfection on systemic bone metabolism. The average BMD values and other parameters of the tibiae are shown in Table 3. There were no significant differences among the three groups.

Confirmation of transfection *in vitro* and *in vivo*

In vitro confirmation of *OPG* transfection has been shown in our previous research (10). OPG

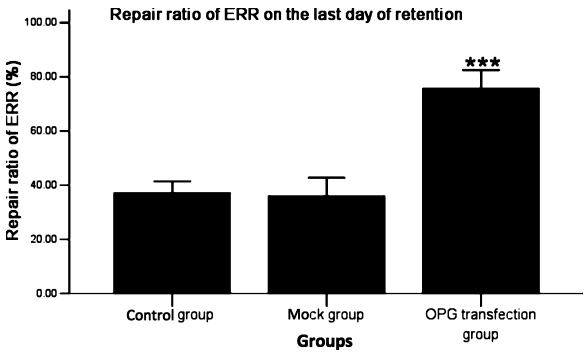

Fig. 7. The repair ratio of external root resorption (ERR) on the last day of retention. ERR repair ratio in the osteoprotegerin transfection group was significantly higher than that in the other groups. ***$p < 0.001$.

Table 2. **BMD and BVF of alveolar bone on the last day of retention**

	Control group	Mock group	OPG transfection group
BMD (g/cm^3)	0.51 ± 0.03	0.52 ± 0.03	0.59 ± 0.07*
BVF (%)	64.33 ± 1.92	67.56 ± 4.36	71.69 ± 1.30*

The mean values from six rats in each group are shown.
Local *OPG* gene transfection significantly increased the BMD and BVF values of alveolar bone in the OPG transfection group.
BMD, bone mineral density; BVF, bone volume fraction; OPG, osteoprotegerin.
*$p < 0.05$.

Table 3. **BMDs and BVF of tibiae on the last day of retention**

	Control group	Mock group	OPG transfection group
BMD of cortical bone (g/cm^3)	0.96 ± 0.06	0.90 ± 0.03	0.96 ± 0.07
BMD of cancellous bone (g/cm^3)	0.26 ± 0.07	0.22 ± 0.02	0.24 ± 0.04
BVF (%)	21.47 ± 7.92	20.37 ± 1.77	19.35 ± 3.99

The mean values from six rats in each group are shown. BVF: Percent bone volume, bone volume/total volume. There were no significant differences among the three groups.
BMD, bone mineral density; BVF, bone volume fraction; OPG, osteoprotegerin.

protein production was confirmed by Western blot analysis, and the functional activity of OPG was tested with a bone resorption analysis.

The immunohistochemical analysis of OPG showed that OPG protein expression was facilitated locally in the periodontium when the HVJ envelope vector containing pcDNA-mOPG was injected in the OPG transfection group, especially on the root surface (Fig. 8). Very few areas of ERR were found in the OPG transfection group (Fig. 8C, D). In contrast, OPG expression levels were very low in the control and mock groups, and areas of ERR were obvious (Fig. 8A, B).

Discussion

Experimental procedure

The HVJ envelope vector is a non-viral gene transfer system; its advantages compared to the commonly used gene transfer system have been reported before, including high safety, low immunogenicity, and long-term gene expression, among others (28). In our previous research, it has been demonstrated that 5 μl of OPG transfection solution and twice-weekly transfection could maintain a locally effective concentration and prolonged protein expression (16). In this study, the injection of vector solution with or without the *OPG* gene did not affect body weight of animals or systemic bone metabolism.

In this study, the SkyScan 1076 system was used to analyze ERR. Micro-CT scanning is a non-destructive examination, and the rats can be scanned while still alive. The accuracy and repeatability of measuring ERR with micro-CT scanning have been confirmed previously (7, 29, 30). Darendeliler et al. analyzed ERR of rats and human with micro-CT scanning in several studies (7, 29), and the SkyScan 1076 system has been used to analyze ERR in the study by Wierzbicki et al. (30).

Local *OPG* gene transfection reduced ERR during retention

The results showed that the volume of ERR in the three groups was not zero (Table 1), even at baseline when the rats had not yet received any orthodontic force. Similar results have been shown in the study by Matthew et al. (29). A possible explanation for this is that cementum, in a similar manner to alveolar bone, can remold under physiological conditions without the application of an orthodontic force (29).

In the control and mock groups, ERR significantly increased after 2 weeks of orthodontic tooth movement and significantly decreased after 2 weeks of retention (Table 1, Fig. 5). Approximately 35–37% of ERR reduced during the retention stage (Fig. 7). These data suggest that ERR might be partly repaired during retention.

In the OPG transfection group, approximately 75% of ERR reduced during the retention phase (Fig. 7), and there was no significant difference between ERR on the last day of retention and at baseline (Table 1, Fig. 6). On the last day of retention, the volume of ERR in OPG transfection group was significantly less than that in either the

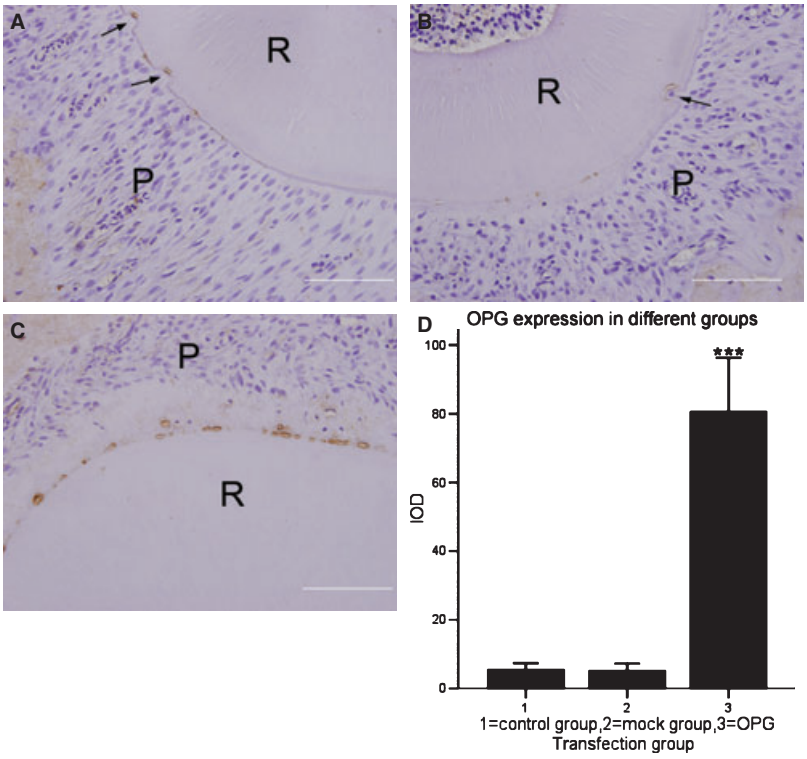

Fig. 8. Immunohistochemical analysis of osteoprotegerin (OPG) expression: (A) control group, (B) mock group, and (C) OPG transfection group. Brown staining shows the immunoreaction for OPG detected by the DAB reaction. Scale = 100 μm. Black arrow, crater of external root resorption; R, root; P, periodontal ligament. (D) The intensity of immunohistochemistry measured at samples from six photographs. IOD, integrated optical density. ***$p < 0.001$.

control or the mock groups (Table 1). These data suggest that *OPG* gene transfection can enhance ERR repair during retention.

The biological mechanism behind these findings might be that OPG inhibits the resorptive activity of odontoclasts and/or promotes the differentiation of cementoblasts during retention and so can enhance the repair of ERR. Odontoclasts and cementoblasts play an important role in root resorption and repair (3–5, 9). It is reported that odontoclasts still have resorptive activity and ERR continues during retention (5–8). Orthodontic force application stimulates the differentiation and activation of odontoclasts. However, even when the active orthodontic force is terminated, odontoclasts are still present in the resorptive lesions and the ERR process continues in the area of hyalinized tissue (5). When all necrotic tissue in the periodontal ligament is resorbed, the odontoclasts lose their resorption activity and detach from the resorbed surface. Then, the cementum is repaired by fibroblast-like and cementoblastic cells (3, 5, 9). Odontoclasts and cementoblasts are considered to be similar to osteoclasts and osteoblasts, respectively (3–5, 9). In many studies, it has been confirmed that OPG can inhibit the differentiation and activation of osteoclasts (10, 15–17). Yu et al. reported that the overexpression of OPG promoted the differentiation of osteoblasts (31). Therefore, *OPG* gene transfection might have inhibited the resorptive activity of odontoclasts and/or promoted the differentiation of osteoblasts during retention in this study.

Local OPG transfection affected bone remodeling in alveolar bone but not in tibial bone

As OPG is a key regulator of bone remodeling, we tested whether localized *OPG* gene transfer affected local and systemic bone metabolism. In the studies conducted on bone metabolism, the values of BMD and BVF are usually used to evaluate the status of bone metabolism; BMD is a strong predictor of bone strength and BVF is an important parameter to describe the trabecular microstructure (25, 26). Micro-CT analysis, as a non-destructive examination, has demonstrated the ability to measure BMD and BVF with high accuracy and precision (27).

In this experiment, the results of the immunohistochemistry analyses showed that local *OPG*

gene transfection induced OPG expression locally in periodontal tissue (Fig. 8). This exogenous OPG expression might affect the local bone remodeling, as the BMD and BVF values of alveolar bone in the OPG transfection group significantly increased compared to the other groups. A similar result was shown by Duun et al. (15). However, there were no significant differences in the BMD values and other parameters of the tibiae. These data suggest that the overexpressed OPG inhibited osteoclastogenesis only in alveolar bone, but not in tibial bone, which was distant from the injection site.

Other ERR inhibitors used in previous studies

In the past decade, the modifying effect of several pharmacological agents on orthodontic ERR has been examined. Bisphosphonates inhibit the function of odontoclasts and result in the inhibition of ERR (19, 20). However, osteonecrosis of the jaw has recently become a concern with bisphosphonate therapy (21, 32). Bisphosphonates incorporate directly into bone where they can persist for many years, and their effects are not readily reversible (21, 32). The effects of celecoxib on ERR are still controversial (22, 33). It has been shown that celecoxib caused a significant dose-dependent inhibition of ERR in rats (33). However, the results of the study by Gameiro et al. showed that short- and long-term celecoxib administration did not reduce ERR but inhibited orthodontic tooth movement (22, 34). Kitaura et al. focused on M-CSF, which is another factor related to the formation of osteoclasts; they demonstrated that an antibody against M-CSF receptor inhibited ERR and orthodontic tooth movement (23, 24).

In previous studies associated with ERR, inhibitors were administered to animals during active orthodontic tooth movement (18–23, 33). However, because of the similarities between odontoclastogenesis and osteoclastogenesis, orthodontic tooth movement was inhibited by the same inhibitors of ERR (19–24, 33, 34). In this study, local *OPG* gene transfection was performed during the retention stage, so it did not interfere with active orthodontic tooth movement.

Conclusions

The results of this study indicated that local *OPG* gene transfection significantly enhanced ERR repair during the retention stage of orthodontic treatment without affecting systemic bone metabolism. However, the precise biological mechanism behind this finding has not yet been fully elucidated. Further studies are required to evaluate the role of the RANK/RANKL/OPG axis in ERR repair.

Clinical relevance

ERR is a serious complication of orthodontic treatment. This study focused on a new method to enhance ERR repair during retention. OPG is a key regulator of osteoclastogenesis. In this study, the *OPG* gene was transfected into the periodontal tissue of rats after orthodontic tooth movement. ERR was analyzed with *in vivo* micro-CT scanning. The results showed that local OPG gene transfection significantly enhanced the repair of ERR during retention.

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