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Phagocytic osteoclasts in the alveolar bone of diabetic rats with periodontitis

Eun-Jung Bak[1], Ae Ri Kim[1,2,3], Ji-Hye Kim[4], and Yun-Jung Yoo[1]*

[1]*Department of Oral Biology, Yonsei University College of Dentistry, Seoul 03722, Republic of Korea*
[2]*BK21 PLUS Project, Yonsei University College of Dentistry, Seoul 03722, Republic of Korea*
[3]*Department of Applied Life Science, The Graduate School, Yonsei University, Seoul 03722, Republic of Korea*
[4]*Department of Dental Hygiene, Baekseok University, Cheonan 31065, Republic of Korea*

Periodontitis is a bacteria-induced inflammatory disease associated with alveolar bone loss. Osteoclast is a macrophage-lineage cell that exhibits phagocytic activity; however, osteoclast phagocytic activity has not been demonstrated under pathological conditions. Diabetes is a pathological condition that exacerbates alveolar bone loss via periodontitis; therefore, we examined phagocytic osteoclasts in diabetic rats that had periodontitis. The rats were divided into the control (C), periodontitis (P), and diabetes with periodontitis (DP) groups. Diabetes and periodontitis were induced by streptozotocin injection and ligature of the mandibular first molars, respectively. On days 3 and 20 after the ligature, the rats were sacrificed, and osteoclasts containing inclusions were quantified by tartrate-resistant acid phosphatase staining. On day 3, there were more osteoclasts containing inclusions in the DP group than in the C group. Among inclusions, osteocyte-like cells and dense bodies were more frequently observed in the DP group than in the C group. Cytoplasm-like structures were elevated more in the DP group than in the C and P groups. However, no differences were observed on day 20. Interestingly, some osteoclasts were in contact with the osteocytes within the exposed lacunae and contained several inclusions within a large vacuole. Thus, the elevation of phagocytic osteoclasts in rats with diabetes and periodontitis provides insight into the role of osteoclast phagocytic activity under pathological conditions.

Keywords: Periodontitis, Diabetes mellitus, Osteoclasts, Phagocytosis

Introduction

Osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells that degrade bone by secreting protons and enzymes. However, the roles of osteoclasts are not limited to bone resorption. For example, osteoclasts are involved in coupling bone resorption and formation by stimulation of osteoblast differentiation [1]. Additionally, several studies suggest that osteoclasts have phagocytic activity under physiological conditions. Osteoclasts containing cells are found in various bones, such as tibia, femur, calvaria, and alveolar bone of young animals that show active bone modeling or remodeling [2-5]. Cells phagocytosed by osteoclasts have been characterized as macrophages, osteoblasts, or osteocytes based on morphological features or immunohistochemical analysis [3,4,6-9]. Apoptotic bone cells have also been identified within osteoclasts in the alveolar bone of normal young rats [5,10]. In another study, rats treated with parathyroid hormone showed

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***Correspondence to:** Yun-Jung Yoo, E-mail: yu618@yuhs.ac https://orcid.org/0000-0002-0045-9597

increased osteoclasts in tibiae, but did not exhibit an increase of osteoclasts with cellular inclusions [3]. These studies provide insight into the role of phagocytic osteoclasts under physiological conditions, but the frequency and significance of phagocytic osteoclasts under pathological conditions remains unknown.

Periodontitis is an inflammatory disease caused by bacteria that is characterized by alteration of the alveolar bone that surrounds the roots of teeth [11]. We have previously reported that osteoclast formation and alveolar bone loss is increased in diabetic rats with periodontitis as well as in rats with periodontitis compared to control rats [12,13]. Therefore, rats with periodontitis or coincident diabetes and periodontitis provide models for observing osteoclast formation in the alveolar bone under pathological bone resorption. We hypothesized that osteoclast phagocytic activity as well as osteoclast formation would be increased under bone resorption-stimulated conditions. In this study, in order to estimate increase of osteoclast phagocytic activity in diabetes with periodontitis, we evaluated osteoclasts with inclusions in rats with streptozotocin (STZ)-induced diabetes with periodontitis.

Materials and Methods

1. Induction of diabetes and periodontitis

Six-week-old male inbred F344 rats were divided into control (C; n = 6 per time point), periodontitis (P; n = 8 on day 3 and n = 12 on day 20), and diabetes with periodontitis (DP; n = 8 on day 3 and n = 11 on day 20) groups. Diabetes was induced by intravenous administration of STZ (50 mg/kg dissolved in 0.1 M citrate buffer; Sigma-Aldrich, St. Louis, MO, USA) after fasting for 16 h. The C group was injected with 0.1 M citrate buffer alone. To confirm induction of diabetes, blood glucose levels were measured using the Accu-Check active system (Roche Diagnostics, Mannheim, Germany) after overnight fasting in accordance with the manufacturer's instructions. Induction of diabetes was considered successful when fasting glucose levels were higher than 300 mg/dL. One week after administration of STZ or citrate buffer, periodontitis was induced by ligature of the mandibular first molars using dental floss. Rats were sacrificed on days 3 and 20 after ligature. All animal protocols were approved by the Institutional Animal Care and Use Committee of Yonsei University (2014-0393).

2. TRAP staining and quantification

After fixation with 10% neutral buffered formalin for 2 days, mandibles were decalcified with 10% ethylenediaminetetraacetic acid for 2 months, embedded in paraffin, and cut into serial 4-μm thick sagittal sections. To identify osteoclasts, sections were stained using a TRAP kit (Sigma-Aldrich) according to the manufacturer's protocol. Measurement of TRAP-positive multinucleated osteoclasts with cellular inclusions was performed in a region of interest (ROI) from the alveolar bone crest to the root apex in the furcation (Fig. 1A). The number of phagocytic TRAP-positive multinucleated osteoclasts was counted along the bone surface in the ROI using Viewpoint software (Precipoint, Silver Spring, Freising, Germany). The number of phagocytic osteoclasts is presented relative to the total number of osteoclasts and was calculated per mm of bone surface. Based on the criteria of previous studies [5,9], inclusions within osteoclasts were classified into three types: osteocyte-like cells with distinct nuclei and faint basophilic cytoplasm similar to surrounding osteocytes located in lacuna, dense round or ovoid bodies (dense bodies) with nuclei strongly stained by hematoxylin and a few cytoplasm, and cytoplasm-like structures with lightly stained cytoplasm and no nucleus. The number of osteocyte-like cells, dense bodies, and cytoplasm-like structures phagocytosed by TRAP-positive multinucleated osteoclasts in the ROI was counted. Images were taken using an Olympus microscope system (BX53; Tokyo, Japan). The individual performing the analyses was blinded to the experimental conditions.

3. Statistical analyses

All statistical analyses were performed using a statistical analysis program (SPSS ver. 25; IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Scheffé's method was used to determine significant differences. $p < 0.05$ was considered statistically significant. Data are expressed as the means ± standard error of the mean. In the Figs. 1B and 2B, homogeneous subsets are not marked with asterisks.

Results

1. Phagocytic osteoclasts are elevated in diabetic rats with periodontitis during early bone loss

Three days after ligature, the number of TRAP-positive mul-

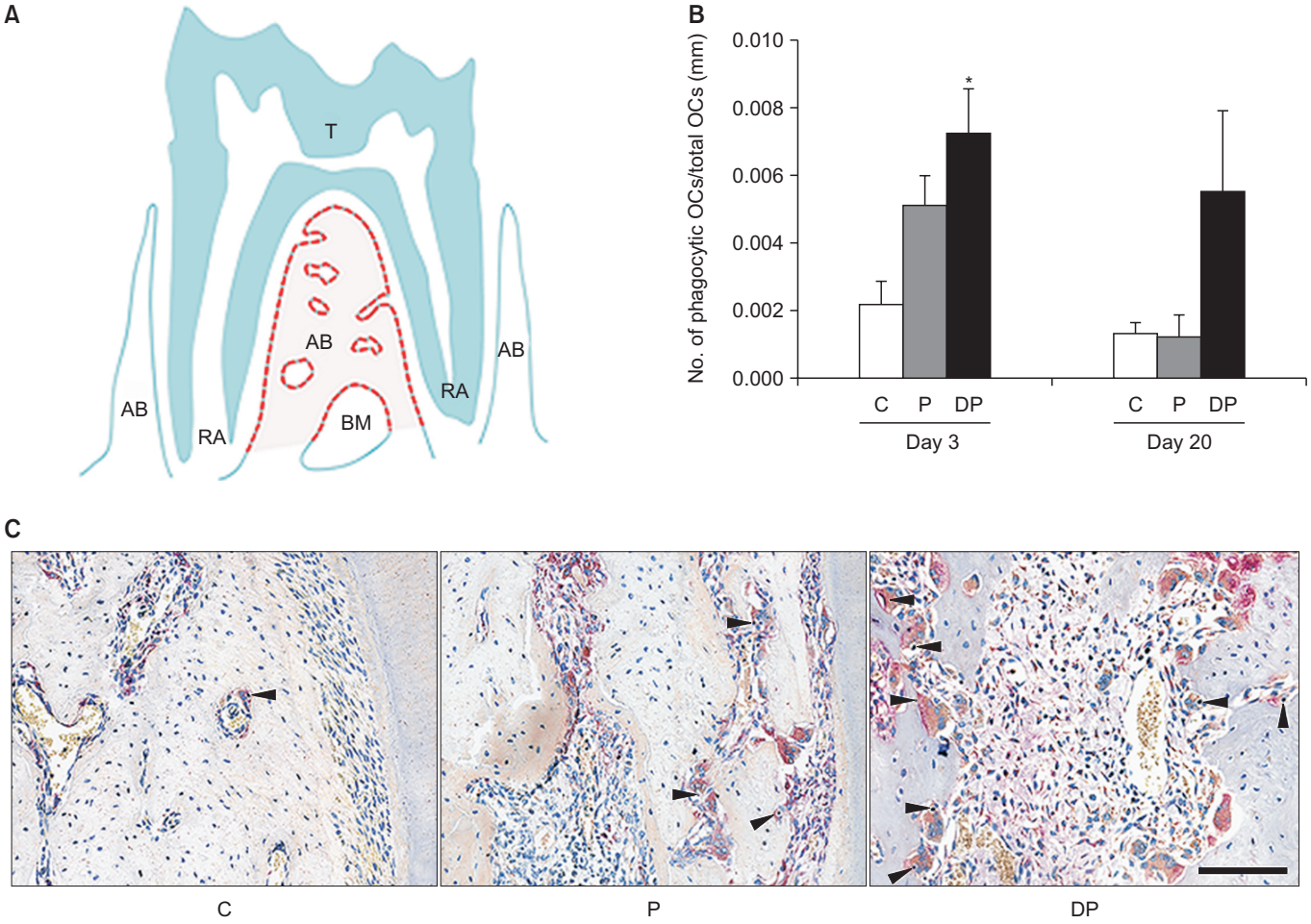

Fig. 1. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts containing inclusions. (A) Region of interest for counting TRAP-positive osteoclasts with inclusions in the furcation area of mandibular first molar (red dotted lines in pale pink color). (B) The number of TRAP-positive osteoclasts with inclusions in the C, P, and DP groups on days 3 and 20 after ligature. The number of phagocytic osteoclasts is presented relative to the total number of osteoclasts and was calculated per mm of bone surface. (C) Representative images of TRAP-positive osteoclasts within in the C, P, and DP groups on day 3. Black arrowheads indicate TRAP-positive osteoclasts with inclusions. Data are represented as the means ± standard error of the mean. Data were evaluated by ANOVA. Scale bar = 100 μm.
AB, alveolar bone; BM, bone marrow; T, tooth; RA, root apex; No, number; OCs, osteoclasts; C, control; P, periodontitis; DP, diabetes with periodontitis.
*p < 0.05 compared with the C group.

tinucleated osteoclasts with inclusions in the DP group was higher than the C group (Fig. 1B, F = 5.816, p = 0.012 and 1C). The P group showed a similarly increased pattern compared to the C group, but there was no statistical difference. Twenty days after ligature, there were no differences among C, P, and DP groups.

2. Cellular inclusions are elevated in phagocytic osteoclasts of diabetic rats with periodontitis

Three types of phagocytosed inclusions were quantified within TRAP-positive multinucleated osteoclasts: osteocyte-like cells, dense bodies, and cytoplasm-like structures (Fig. 2A). On day 3, osteocyte-like cells (F = 5.870, p = 0.012) and dense bodies (F = 11.510, p = 0.001) were higher in the DP group than the C group (Fig. 2B). Cytoplasm-like structures (F = 10.097, p = 0.001) were also higher in the DP group than both the C and P groups. On day 20, there were no significant differences among the three groups.

3. Inclusion morphology in diabetic rats with periodontitis

A large number of osteoclasts in the DP group contained inclusions within clear vacuoles (Fig. 3A-3C). Many inclusions engulfed by osteoclasts were oval or round and stained dark

blue, and some inclusions were dense nuclei. Some osteoclasts were in contact with osteocytes within exposed lacunae (Fig. 3D–3F). This contact was observed under several conditions. Cytoplasmic extension of osteoclast was found to be inserted into exposed lacunae and in contact with osteocyte (Fig. 3E). Additionally, some osteocytes were localized partially within osteoclasts and partially within lacunae (Fig. 3F).

Interestingly, some osteoclasts contained several vacuoles with an individual type of inclusion, such as dense bodies or cytoplasm-like structures (black and red arrowheads in Fig. 4A and 4B, respectively). And, one osteoclast had a large vacuole containing several inclusions with segmented condensed nu-

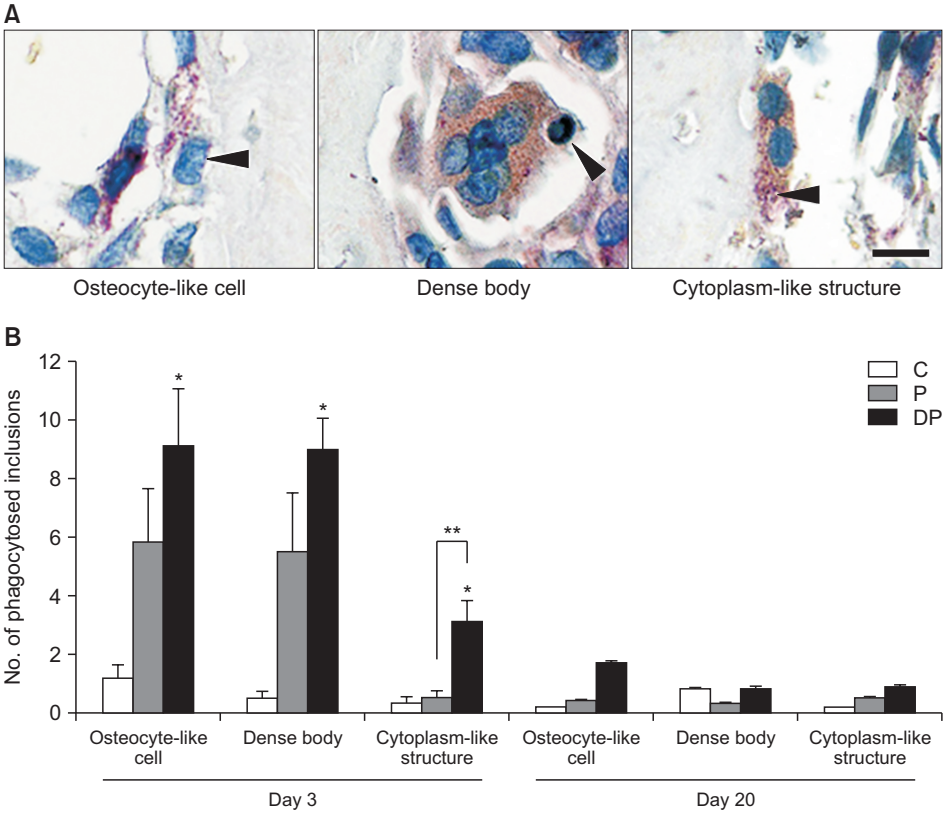

Fig. 2. Inclusions engulfed by tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts. (A) Representative images of inclusions within a vacuole on day 3. From left to right, the black arrowheads indicate an osteocyte-like cell in the C group, a dense body in the P group, and a cytoplasm-like structure in the C group, respectively. (B) The number of inclusions engulfed by TRAP-positive osteoclasts in the region of interest. Data are represented as the means ± standard error of the mean. Data were evaluated by ANOVA. Scale bar = 10 μm.
C, control; P, periodontitis; DP, diabetes with periodontitis.
*$p < 0.05$ compared with the C group. **$p < 0.05$.

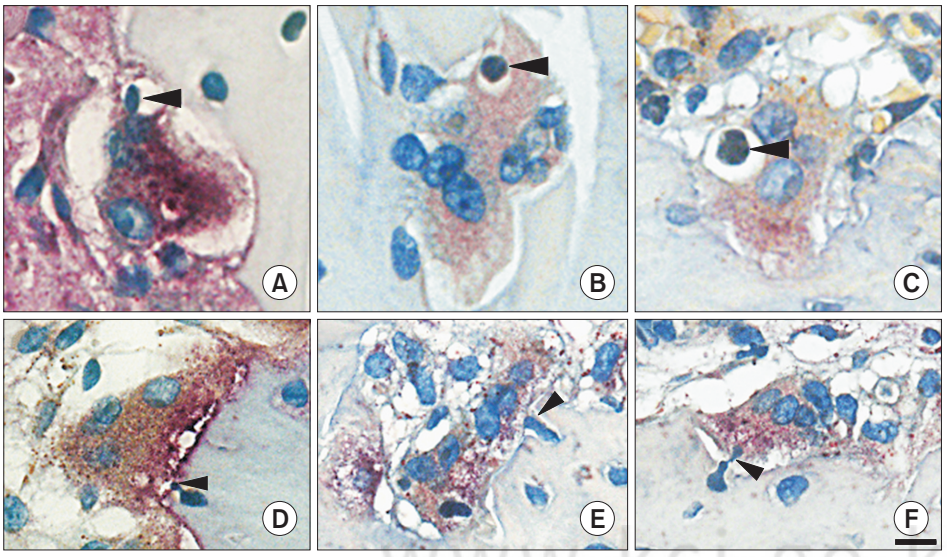

Fig. 3. Representative images of tartrate-resistant acid phosphatase-positive multinucleated osteoclasts contained inclusions and contacted with osteocytes in the diabetes with periodontitis group. (A-C) Osteoclasts with oval or round dense body within a vacuole on day 3 (black arrowheads). (D) An osteoclast in contact with an osteocyte within the exposed lacuna on day 3 (black arrowhead). (E) A cytoplasmic extension of an osteoclast inserted into the exposed lacuna and in contact with an osteocyte on day 3 (black arrowhead). (F) An osteoclast with an osteocyte partially placed within lacuna on day 3 (black arrowhead). Scale bar = 10 μm.

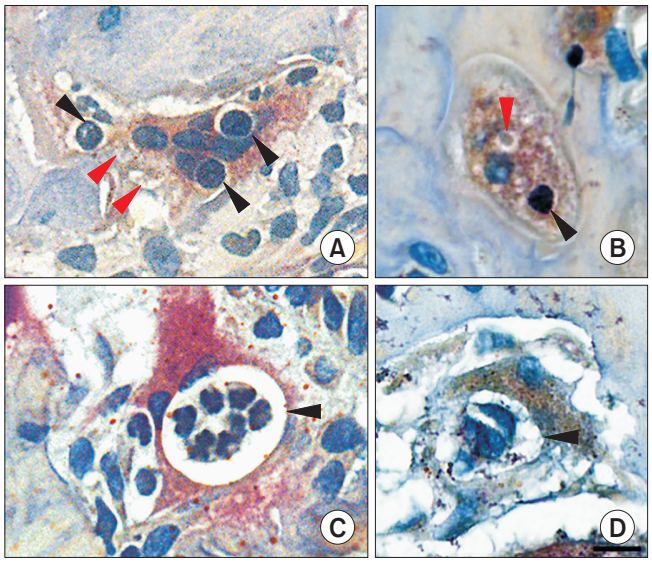

Fig. 4. Representative images of tartrate-resistant acid phosphatase-positive multinucleated osteoclasts containing several vacuoles with individual inclusions or several inclusions within one vacuole in the diabetes with periodontitis group. (A, B) Osteoclasts containing several vacuoles with individual inclusions on day 20 (black arrowheads indicate dense bodies and red arrowheads indicate cytoplasm-like structures). (C) An osteoclast containing several segmented condensed nuclei in one large vacuole on day 20 (black arrowhead). (D) An osteoclast containing several inclusions within a large vacuole on day 3 (black arrowhead). Scale bar = 10 μm.

clei (Fig. 4C). Moreover, another osteoclast engulfed several osteocyte-like inclusions within a large vacuole (Fig. 4D).

Discussion

Alveolar bone loss is characteristic of periodontitis, and diabetes aggravated alveolar bone loss induced by periodontitis [13,14]. In previous report, we have demonstrated that the distance from the cemento-enamel junction to the alveolar bone crest and the number of osteoclasts is markedly increased on days 3 and 20 in rats with periodontitis and diabetic rats with periodontitis compared with control rats [13]. Therefore, in this study, we examined phagocytic osteoclasts in alveolar bone at two time-points that have previously exhibited an elevated presence of osteoclasts. Osteoclasts with inclusions were significantly elevated on day 3 in the DP group compared to the C group. The differences in the number of phagocytic osteoclasts and inclusions between the DP group and the C group appeared to be consistent on days 3 and 20, but there was no statistical significance on day 20. The number of phagocytic osteoclasts and inclusions in the DP group tended to decrease on day 20 compared to day 3. In previous report, the number of osteoclasts in the DP group was increased on day 3 and day 20 than in the C group, but the increase on day 20 was less on day 3 [13]. The results of the number of phagocytic osteoclasts and inclusions in this study also showed similar trends. These results suggest elevation of osteoclast phagocytosis in diabetes and periodontitis condition.

Phagocytosis of osteoclasts has been suggested as a way removing exposed osteocytes or apoptotic cells during bone resorption. Suzuki et al. [9] reported that the osteocytes engulfed by osteoclasts had features similar to those of osteocytes embedded in the lacuna of the bone. Pebp2αA (isoform of Cbfa1, osteoblast marker)-positive osteocytes are found within osteoclasts in hamster jaw bones around developing tooth germs [8]. Other group has also reported ultrastructural contacts between osteoclasts and osteocytes in calvaria of young rats [4]. In addition, the femoral ultrastructure of young rabbits shows that the ruffled border of osteoclasts extends into lacuna and contacts osteocytes [2]. In this study, there were osteoclasts to be in contact with osteocytes within the exposed lacunae through ruffled borders or partially enclosed osteocytes similar to previous studies. Moreover, DP group showed the increase in inclusion of osteocyte-like cells. These suggest that engulfment of osteocytes by osteoclasts can be increased in diabetes with periodontitis conditions.

It has been suggested that osteoclasts engulf apoptotic cells based on the presence of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells within osteoclasts, gene expression profiles related to the clearance of apoptotic cells, and *in vitro* evidence of osteoclast phagocytosis of apoptotic thymocytes [5,10,15]. Other study suggests that apoptosis is enhanced in diabetic condition [16]. Consistent with previous report, our unpublished data showed that the number of TUNEL-positive osteocytes in diabetic rats with periodontitis was significantly higher than in control rats and rats with periodontitis on day 3, but not on day 20 (data not shown). Further, in this study, the DP group exhibited more dense bodies and cytoplasm-like structures as well as osteocyte-like cells in osteoclasts than the C group. These results suggest the possibility that elevated osteocyte apoptosis in combination of diabetes and periodontitis may affect osteoclast phagocytosis. For accurate determination of inclusions within phagocytic osteoclasts, immunohistochemistry using osteocyte or apoptosis markers is required in the future.

Macrophage is a representative phagocyte. We can observe some osteoclasts containing several vacuoles with an individual inclusion or several inclusions within one large vacuole

similar to phagocytic characteristic of macrophage [17]. After phagocytosis, professional antigen presenting cells such as macrophages and dendritic cells present fragmented peptides from phagocytosed antigen to T cells [18]. Processes after osteoclast phagocytosis have not been clearly defined. Some studies have shown induction of regulatory T cells to suppress the activation of T cells and the differentiation/activation of osteoclasts through antigen presentation by osteoclasts [19,20]. These groups suggested that induction of regulatory T cells through antigen presentation by osteoclasts protect bone from autoimmunity and bone loss. It will be interesting to clarify the progresses after osteoclast phagocytosis in diabetes with periodontitis.

This is the first report to demonstrate increased phagocytic activity of osteoclasts in diabetes with periodontitis. Our results are meaningful to indicate that further study is warranted to define the role of osteoclast phagocytic activity in the pathogenesis of diabetes with periodontitis.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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