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Original

VEGF Expression in Diabetic Rats Promotes Alveolar Bone Resorption by *Porphyromonas gingivalis* LPS

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Abstract: Diabetes mellitus is an important risk factor for periodontitis. Although numerous complications are associated with the disease, all of these are attributed to vascular disorders and are closely related to the potent angiogeneic factor vascular endothelial growth factor (VEGF). However, it remains unknown how diabetes mellitus/hyperglycemia-associated VEGF expression affects alveolar bone resorption in the periodontium. The aim of this study was to determine the level of adverse effect on bone resorption of diabetes mellitus-associated VEGF. Therefore, we induced experimental periodontitis with injections of the endotoxin lipopolysaccharide (LPS) from *Porphyromonas gingivalis* in diabetic rats, measured the level of bone resorption, and observed VEGF expression and localization of osteoclasts in the periodontium. Eight-week-old male Goto-Kakizaki (GK) rats were in the experimental group, and male Wistar rats were in the control group. Experimental periodontitis was induced by injecting *P. gingivalis* LPS and inserting ligatures. All rats were euthanized and underwent micro X-ray computed tomography (CT) to acquire bone resorption image, in which the distance between the cement–enamel junction and the alveolar bone crest was measured to determine the amount of bone resorption. Samples were prepared and underwent immunohistochemical staining with an anti-VEGF monoclonal antibody and tartrate-resistant acid phosphatase (TRAP) staining. The amount of bone resorption measured by micro X-ray CT images was significantly greater in the experimental group than in the control group. Immunohistochemical staining showed that VEGF expression levels on the alveolar bone surface and around microvessels in the gingival connective tissue were higher in the experimental group than in the control group. On the alveolar bone surface, localization of TRAP-positive cells and bone resorption lacunae from the same sites were observed in both groups. These results suggest that VEGF expression in the periodontium caused by hyperglycemia in rats with diabetes mellitus affects *P. gingivalis* LPS-induced alveolar bone resorption.

Key word: Bone resorption, Diabete mellitus, Osteoclast, *P.g* LPS, VEGF

Introduction

Diabetes mellitus is an important risk factor for periodontitis[1-4)]. Patients with diabetes mellitus frequently develop periodontitis and the disease tends to be severe. Therefore, the Japanese Diabetes Society has recognized periodontitis as the sixth common complication of diabetes mellitus[5)], following cerebrovascular disorders, cardiovascular disorders, retinopathy, nephropathy, and neurological disorders. Thus far, numerous mechanisms have been reported to underlie exacerbation of periodontitis caused by diabetes mellitus. Among them, the main mechanisms involve immune dysfunction[6,7)], cellular stress[8,9)], and cytokine imbalance[10,11)] caused by hyperglycemia, which enhances tissue destruction of periodontitis and impairs tissue repair. The mechanism of exacerbation of alveolar bone resorption due to cytokine imbalance is initiated when hyperglycemia increases the level of inflammatory cytokines, such as TNF-α[10)], IL-1[11)], and IL-6[11)]. These cytokines affect the interaction between RANKL and OPG[12)], and as a result, osteoclast differentiation is promoted. These inflammatory cytokines also act directly on osteoclasts, thereby promoting differentiation, activation, and alveolar bone resorption[13)].

Vascular endothelial growth factor (VEGF) is a glycoprotein with various effects including proliferation and differentiation of vascular endothelial cells, enhancement

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of vascular permeability, and vasodilation; it serves as an important angiogeneic factor[14]. VEGF is involved in normal angiogenesis, such as vasculogenesis and structural development in the embryonic period[15] and also plays an important role in angiogenesis and enhancement of vascular permeability in pathological conditions[14], such as solid tumors[16], chronic rheumatoid arthritis[17], diabetic retinopathy[18], inflammation[19], and wound healing[20]. VEGF is intralesionally overexpressed in chronic rheumatoid arthritis[17] and diabetic retinopathy[18] and involved in disease progression. Serum VEGF levels in patients with diabetes mellitus are significantly higher than those in healthy individuals[21]. We have previously reported that blood VEGF levels in model rats with type 2 diabetes mellitus are significantly higher compared with those in normal rats[22]. We have also reported that VEGF expression around microvessels in the wound healing sites in rats with diabetes mellitus that underwent periodontal surgery is enhanced compared with that in healthy rats and may be involved in impaired wound healing[23]. As summarized above, hyperglycemia due to diabetes mellitus has been demonstrated to induce VEGF expression in vivo and also have some effects on the periodontium. Furthermore, hyperglycemia-induced cytokine imbalance is a factor causing progression of periodontitis, such as enhanced resorption of the alveolar bone. However, how diabetes mellitus-associated VEGF expression affects alveolar bone resorption due to periodontitis-causing bacteria it remains unknown.

In this study, we induced experimental periodontitis by injecting lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, a major periodontitis-causing bacterium, to the periodontium in Goto-Kakizaki (GK) rats and then inserting silk ligatures into the gingival sulcus. To determine the effects of VEGF expression on bone resorption caused by periodontitis in diabetes mellitus rats, we measured the level of bone resorption visualized on computed tomography (CT) scans and observed VEGF expression and localization of osteoclasts.

Materials and Methods

Experimental materials

For experiments, eight 8-week-old male GK rats (Shimizu Laboratory Supplies, Kyoto, Japan) as a model of type 2 diabetes mellitus were used as the experimental group[24], and eight 8-week-old male Wistar rats (Shimizu Laboratory Supplies, Kyoto, Japan) were used in the control group. In both groups, blood samples were collected from the caudal veins and blood glucose levels were measured with NITRO Stat Strip XP2 (NIPRO, Osaka, Japan). Only animals with a fasting blood glucose level of e”200 mg/dl were included in the experimental group. Body weights and blood glucose levels of both groups are shown in Figs. 1 and 2.

Experimental methods

Rats under inhalation anesthesia with isoflurane (Forane®, Abbott, North Chicago, IL, USA) received an intraperitoneal injection of 0.3 mg/kg of pentobarbital sodium (Somuno pentil injection, Kyoritsu Seiyaku Corporation, Tokyo, Japan) to induce general anesthesia and were fixed in a supine position with the mouth open. The periodontium including proximal and distal roots in the bilateral palatal side of maxillary second molars was observed.

To induce experimental periodontitis, 4-0 silk ligatures (PERMA-HAND™ Silk Suture, Johnson & Johnson, USA) were inserted into the gingival sulcus of left and right maxillary second molars, and 10 ml of *P. gingivalis* LPS (1.0 mg/ml; LPS-PG Ultrapure, InvivoGen, USA) was injected to the palatal side interdental papilla. Two additional injections of *P. gingivalis* LPS were given at intervals of 48 hours (Figs. 3 and 4)[25]. This study was approved by the Osaka Dental University Animal Experiment Committee and conducted in accordance with guidelines for animal experiments.

Micro X-ray CT

Twenty-four days after the final injection, rats in both groups were euthanized with an overdose of pentobarbital sodium (Somuno pentil injection®, Kyoritsu Seiyaku Corporation, Tokyo, Japan). The chest was opened immediately after euthanization, and a catheter was inserted into the ascending aorta from the left atrium to perform perfusion fixation with 10 % neutral buffered formaldehyde solution (Nacalai Tesque, Kyoto, Japan). The periodontium, including the roots of interest, was collected as a single mass, and micro X-ray CT (CT-Solver, SHIMADZU, Kyoto, Japan) was performed to acquire alveolar bone images. On CT images, the distance between the cement–enamel junction (CEJ) and alveolar bone crest (ABC) was measured (in mm)[26]. Measurement sites were the center of the first molar palatal distal root (M1D), the center of the second molar palatal mesial root (M2M), the center of the second molar palatal distal root (M2D), and the center of the third molar palatal mesial root (M3M; Fig. 5).

Methods for preparation of histopathological and immunohistochemical specimens

After immersion fixation in 10 % neutral buffered formaldehyde solution at 4 °C, the secondary fixation was performed in 80 % alcohol at 4 °C. Thereafter, decalcification was performed in hydrochloric acid buffer 5 % EDTA-Na solution

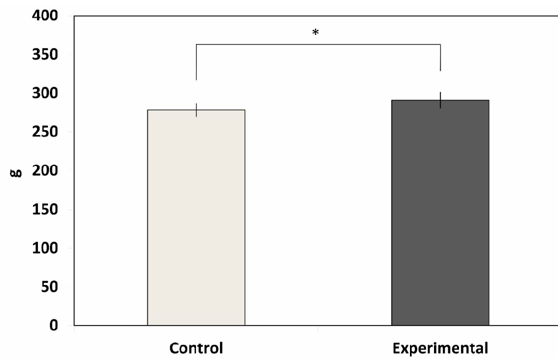

Figure 1. Body weight

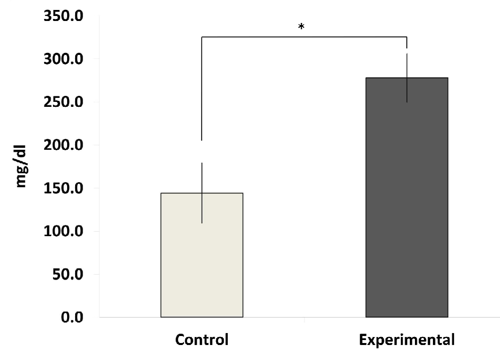

Figure 2. Blood glucose level

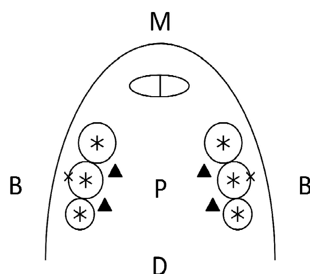

Figure 3. Schema of a rat's maxilla. (×): Ligation, (Triangle): Injecting *P. gingivalis* LPS

(DOJINDO, Kumamoto, Japan) at 4 °C for 1 month. Unwanted tissues were removed from decalcified samples. The samples were divided along the tooth axis to divide the second molar palatal root, the observation site. After washing with 0.1 M phosphate-buffered saline (PBS; pH 7.2) at 4 °C, the divided samples were paraffin-embedded in the usual manner. The 5-µm-thick sections were prepared in a range allowing observations up to the vicinity of the root apex in the buccal/palatal direction of the second molar palatal mesial root.

Immunohistochemical staining

The sliced specimens from both groups were immunohistochemically stained with anti-VEGF monoclonal antibody (sc-7269, Santa Cruz Biotechnology, USA). Before immunostaining with the anti-VEGF monoclonal antibody, the specimens were deparaffinized and then treated with pepsin adjusted to 0.4 % in 0.01 M HCl at 37 °C for 30 min to activate antigens. Endogenous peroxidase was then quenched with 0.3 % H^2O^2, and the anti-VEGF monoclonal antibody was diluted 100-fold with PBS and allowed to react with the sample at 37 °C for 60 min. The specimens were developed with 3,3 Œ-diaminobenzidine tetrahydrochloride (DAB; DakoCytomation, Glostrup, Denmark) on an EnVisionTM++system (DakoCytomation, Glostrup, Denmark). Thereafter, the specimens were subjected to nuclear staining using hematoxylin, dehydrated, and mounted. The specimens were observed under an All-in-One Fluorescence Microscope (BZ9000, KEYENCE, Tokyo, Japan).

Tartrate-resistant acid phosphatase (TRAP) staining

The rest of sections from both groups were stained with the TRAP/ALP staining kit (TRAP/ALP Stain Kit, Wako, Osaka, Japan). The adjusted TRAP staining solution was added in drops to the deparaffinized specimens, and allowed to react for 30 min at room temperature. The specimens were subjected to nuclear staining, dried, dehydrated, and mounted. The specimens were observed with an All-in-One Fluorescence Microscope (BZ9000, KEYENCE, Tokyo, Japan).

Statistical analysis

Student's t-test was used to compare body weight, blood glucose levels, and bone resorption levels between the two groups. The control and experimental groups were compared to identify significant differences ($p < 0.05$).

Results

Body weight and blood glucose level

The mean body weight in the experimental group was significantly greater than that in the control group ($p < 0.05$; Fig. 1). The mean fasting blood glucose level in the experimental group was significantly greater than that in the control group ($p < 0.05$; Fig. 2).

Micro X-ray CT findings

On micro X-ray CT images, both the experimental and control groups showed bone resorption centering around the second molar palatal alveolar bone, encompassing an area from the first molar distal root to the third molar mesial root (Fig. 6). The distance between CEJ and ABC was measured in palatal M1D, M2M, M2D, and M3M to compare bone resorption between the experimental and control groups. Bone resorption was greatest in the M2M in both groups, with a distance of 0.746 ± 0.146 mm in the experimental group, which was significantly greater than 0.593 ±

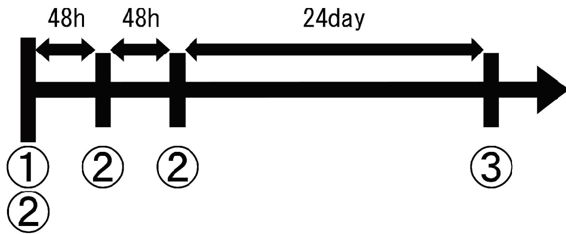

Figure 4. Time schedule. 1. Inserting silk ligatures, 2. Injecting *P. gingivalis* LPS, 3. Euthanasia.

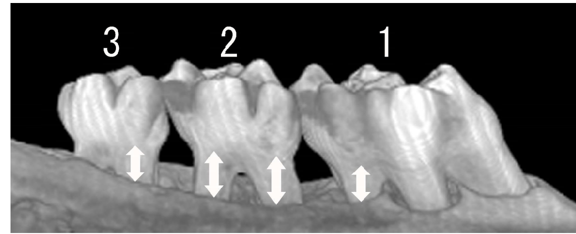

Figure 5. Measuring points

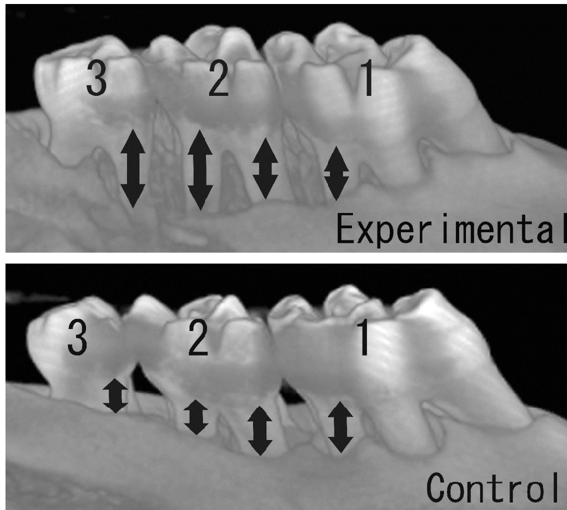

Figure 6. Micro X-ray CT images

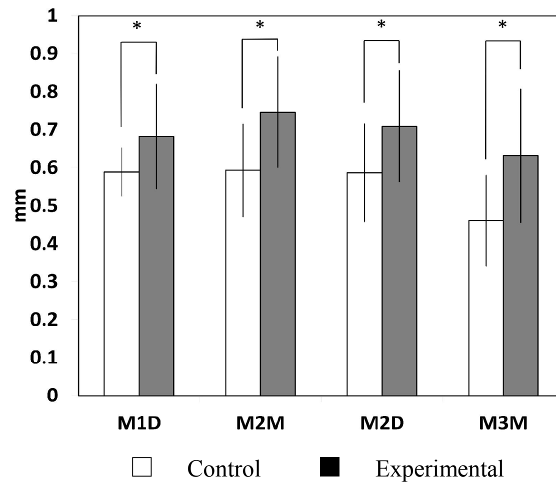

Figure 7. Amount of alveolar bone resorption

0.122 mm in the control group ($p < 0.05$). Measurements for the other sites in the control and experimental groups were 0.589 ± 0.064 mm versus 0.682 ± 0.138 mm in the M1D, 0.587 ± 0.129 mm versus 0.709 ± 0.148 mm in the M2D, and 0.461 ± 0.120 mm versus 0.632 ± 0.177 mm in the M3M, respectively. The amount of bone resorption in the experimental group was significantly greater than that in the control group for all measurement sites. ($p < 0.05$; Fig. 7).

Immunohistochemical staining findings with anti-VEGF antibody

In the experimental group, VEGF was expressed strongly along the alveolar bone surface (Fig. 8A, C and E). VEGF expression was observed on the bone surface wherever a bone resorption image was seen (Fig. 8B and F). VEGF expression was observed around microvessels in the gingival connective tissue in the vicinity of the alveolar bone (Fig. 8B and D). In the control group, VEGF expression along the alveolar bone surface, as seen in the experimental group, was hardly observed (Fig. 8G, I and K). However, bone resorption images were observed on the alveolar bone surface also in the control group (Figure J). VEGF expression in the gingival connective tissue in the vicinity of the alveolar bone, as seen in the experimental group, could not be observed (Fig. 8B).

TRAP staining findings

In both experimental and control groups, TRAP-positive cells were observed on the alveolar bone surface (Fig. 9B, D, F, H, J and L). Bone resorption images were observed wherever TRAP-positive cells were found (Fig. 9B, D, F, H, J and L).

Discussion

In this study, we investigated how diabetes mellitus-associated VEGF expression in the periodontium affects *P. gingivalis* LPS-induced alveolar bone resorption. In a previous study, we have reported that expression of the angiogeneic factor VEGF in the periodontium is increased in rats with diabetes mellitus when compared to that in healthy rats[22]. Hyperglycemia-induced increases in levels of inflammatory cytokines, such as TNF-α[10], IL-1[11] and IL-6[11], cause progression of periodontitis in diabetes mellitus. However, how VEGF, a cytokine expressed and present in the periodontium under hyperglycemic conditions, affects bone resorption caused by periodontitis is unknown.

We, therefore, induced experimental periodontitis in GK rats by injecting *P. gingivalis* LPS into the maxillary palatal gingival and inserting silk ligatures into the second molar gingival sulcus. We then prepared samples from study animals to measure periodontitis-induced alveolar bone resorption visualized on CT images and to observe VEGF expression and osteoclast localization in the periodontium affected by periodontitis. We then

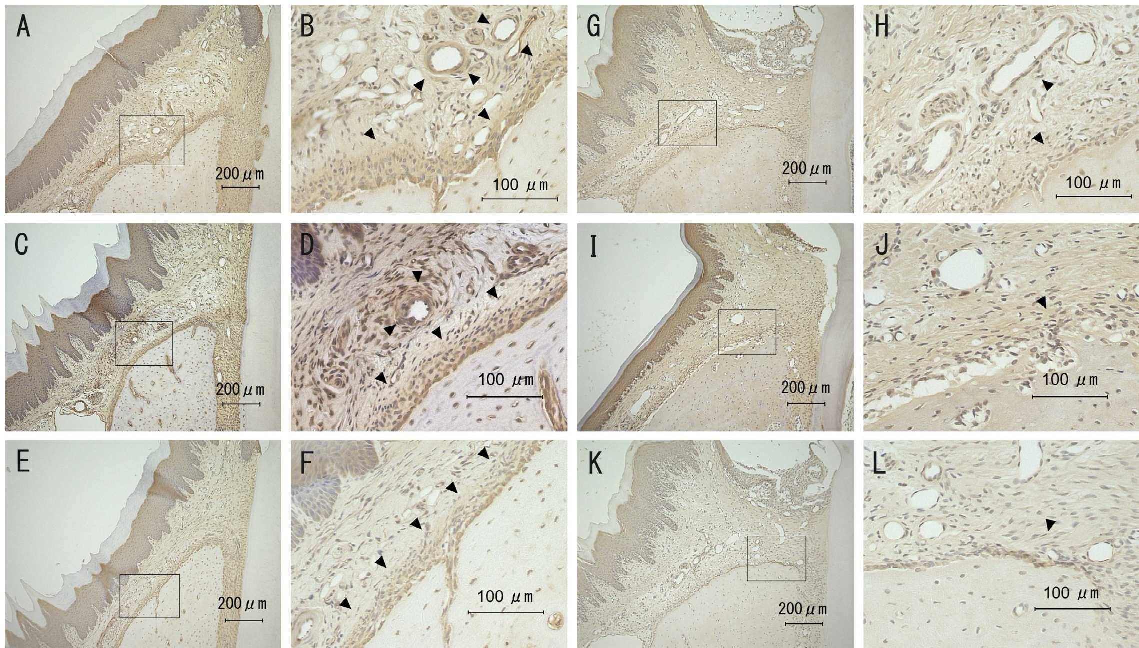

Figure 8. Immunohistochemical staining with anti-VEGF antibody.
(A) Experimental group; (B) Experimental group (High-power magnification of A); (C) Experimental group; (D) Experimental group (High-power magnification of C); (E) Experimental group; (F) Experimental group (High-power magnification of E); (G) Control group; (H) Control group (High-power magnification of G); (I) Control group; (J) Control group (High-power magnification of I); (K) Control group; (L) Control-power magnification of K). (Arrowheads)VEGF expression.

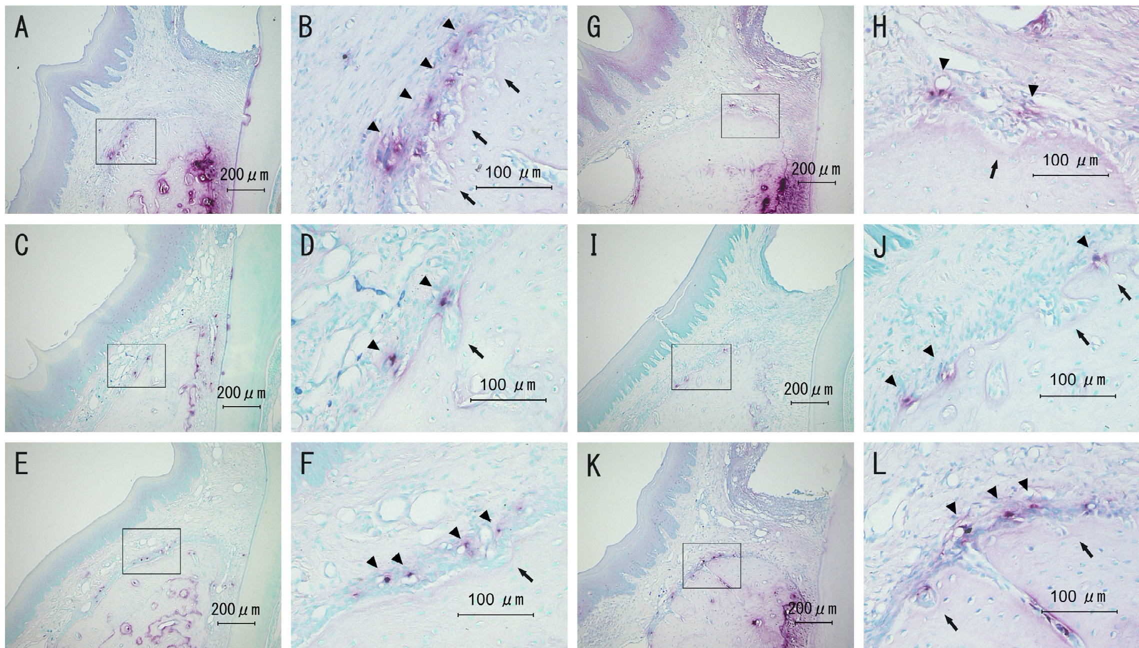

Figure 9. TRAP staining.
(A) Experimental group; (B) Experimental group (High-power magnification of A); (C) Experimental group; (D) Experimental group (High-power magnification of C); (E) Experimental group; (F) Experimental group (High-power magnification of E); (G) Control group; (H) Control group (High-power magnification of G); (I) Control group; (J) Control group (High-power magnification of I); (K) Control group; (L) Control group (High-power magnification of K). (Arrowheads) TRAP-positive cell, (Arrow) resorption lacunae

examined the effects of VEGF expressed in the periodontium due to diabetes mellitus on alveolar bone resorption in periodontitis.

In this study, the mean preoperative blood glucose level in the experiment group of GK rats was 278.0 mg/dl, which was significantly different from the mean blood glucose level of 144.6 mg/dl observed in the control group. This indicates that GK rats used as the experimental group in this study had diabetes mellitus, with a more pronounced hyperglycemic state when compared to the control group. The mechanism underlying *P. gingivalis* LPS-triggered alveolar bone resorption involves *P. gingivalis* LPS binding to the Toll-like receptor 2[27] and 4[28] on osteoblasts, which stimulates secretion of RANKL; this binds to RANK on pre-osteoclast and thereby promotes differentiation into osteoclasts[27]. Furthermore, *P. gingivalis* LPS serves as a bacterial antigen and activates macrophages and T lymphocytes to induce the release of inflammatory cytokines, such as TNF-α, IL-1, and IL-6[29]. These inflammatory cytokines act directly on osteoclasts and promote their differentiation and activation[30]. In this study, an injection of *P. gingivalis* LPS to the second molar palatal gingival and insertion of the silk ligature to the gingival sulcus induced alveolar bone resorption observed primarily in the palatal side of the second molar.

As described above, the observed alveolar bone resorption is attributable to induction of RANKL expression from osteoblasts by *P. gingivalis* LPS injected to the rat palatal gingival and immune response to the LPS antigen that enhanced secretion of inflammatory cytokines from macrophages and neutrophils.

In comparison of the bone resorption measurements (CEJ-ABC) between diabetes mellitus and normal rats, the mean value for the second molar mesial root in the group of diabetes mellitus rats was 0.746 mm, showing a significant difference from 0.607 mm in the group of normal rats ($p < 0.05$). In addition, for the first, second, and third molar distal roots, the amount of bone resorption in diabetes mellitus rats was greater than in normal rats (M1D, M2D, M3M; $p < 0.05$).

Hyperglycemia caused by diabetes mellitus has some detrimental effects. Hyperglycemia causes formation and accumulation of irreversible advanced glycation end products (AGEs) in the body[31] and induces expression of their primary receptor RAGE[32]. Interactions between the AGEs and RAGE result in the dysfunction of immune cells[6,7] and functional alterations of various cells[8,9], causing cytokine imbalance associated with inflammatory cytokine increases[10,11]. Furthermore, hyperglycemia shifts the balance of the RANKL/OPG interaction in the direction of tissue destruction through AGEs/RAGE interaction[33,34]. This study also indicated that the level of bone resorption due to experimental periodontitis was prominent in rats with diabetes mellitus when compared to that in normal rats.

We hypothesize that this is because immune dysfunction, cellular stress, and cytokine imbalance occurred in the periodontium due to hyperglycemia in rats with diabetes mellitus; thus, periodontium destruction in the affected site worsened, as described previously. In cytokine imbalance caused by hyperglycemia, it has been extensively reported that inflammatory cytokines, such as TNF-α[10], IL-1[11], and IL-6[11], increase. These inflammatory cytokines both directly and indirectly promote differentiation and activation of osteoclasts[13]. Meanwhile, cellular stress caused by AGEs, which are formed and accumulated in the body because of hyperglycemia, has been known to promote VEGF expression from vascular endothelial cells[35]. VEGF is a cytokine involved in normal angiogenesis, and it is also deeply involved in development of diabetic retinopathy, one of the three major complications of diabetes mellitus.

Diabetic retinopathy is a severe vision disorder caused by various retinal blood vessel disorders[36]. This disease is caused by disorders of endothelial cells and pericytes of retinal microvessels, and VEGF has been strongly suggested to be involved in its development[37]. In addition to periodontitis, TNF-α is deeply involved in disease progression of rheumatoid arthritis, a chronic inflammatory disease[38]. There is a report showing that the VEGF levels in joints of patients with rheumatoid arthritis is approximately 10-times higher than those in joints of healthy individuals[39]. Thus VEGF is a cytokine, similar to inflammatory cytokines, such as TNFα, that is deeply involved in diabetes mellitus complications caused by vascular disorders and inflammatory diseases. In a previous study, we have reported that VEGF expression is enhanced in the periodontium in rats with diabetes mellitus when compared to normal rats[22]. In this study, in the experimental group, VEGF was expressed strongly on the alveolar bone surface and around microvessels in the gingival connective tissue compared with the control group. In addition, localization of TRAP-positive cells was observed on the alveolar bone surface in both experimental and control groups, and resorption lacunae were observed on the bone surface. VEGF promotes the RANKL expression through binding to VEGF receptor1 on osteoblasts, and has also been reported to promote osteoclast differentiation and activation through binding to VEGF receptor1 on osteoclasts[40]. There is a report showing that in patients with rheumatoid arthritis, the RANKL levels in synovial fluid correlates positively with the VEGF levels. There is also a report indicating that, even in the absence of RANKL, VEGF has induced differentiation of osteoclasts from monocytes[41]. Thus, it is likely that VEGF expressed around microvessels and on bone surfaces had some effects on differentiation and activation of osteoclasts on the alveolar bone surface in this study. The results from the present study demonstrated that bone resorption worsened in diabetes mellitus rats with LPS-induced experimental periodontitis compared with the normal rats. A plausible cause of increased bone resorption is that hyperglycemia-associated VEGF expression in the periodontium affected differentiation and activation of

osteoclasts.

In the future, to elucidate the mechanism underlying the exacerbation of periodontitis due to diabetes mellitus, a better understanding of the role of VEGF is as important as understanding of effects of inflammatory cytokines on destruction of the periodontium.

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Conflict of Interest

The authors have declared that no COI exists

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