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Histopathological comparison of the onset of peri-implantitis and periodontitis in rats

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Key words: bone implant interactions, periodontology, soft tissue–implant interactions

Abstract

Background and objective: There are a few experimental models that clearly describe the pathological differences in tissue destruction between periodontitis and peri-implantitis. We recently reported that the formation of immune complexes accelerates site-specific loss of attachment and alveolar bone resorption when an antigen is topically applied in the gingival sulcus of an immunized rat. We applied this model to the peri-implant tissues and compared peri-implant destruction to periodontitis without using a ligature.

Material and methods: Twenty-five rats were used in this study and were divided into five groups. Implantation was performed immediately after extraction of right first molars in rats. The left first molars were left untreated to be examined as natural teeth. The immunized group consisted of rats that had received intraperitoneal lipopolysaccharide (LPS), whereas the nonimmunized group received only phosphate-buffered saline (PBS). The untreated baseline group received only implantation. After intraperitoneal booster injection, half of each group received topical application of LPS in the palatal gingival sulcus daily for 3 days. The other half of the groups received PBS. Histopathological and histometrical findings were observed with hematoxylin and eosin staining, collagen fibers were observed with Azan staining, and formation of immune complexes was immunohistologically evaluated by C1qB expression.

Result: Peri-implant tissue destruction was greater in the immunized and LPS-applied groups than in the other groups. No periodontal destruction was observed. Formation of immune complexes was observed in the junctional epithelium and adjacent connective tissue in the immunized groups.

Conclusion: Antigen-induced peri-implant tissue destruction occurs faster than periodontal tissue destruction.

In recent years, dental implants have become increasingly popular as dental prostheses. Jung et al. (2008) reported that 96 of Bråne-mark system implants in the maxilla survived up to 10 years. However, Zitzmann and Berglundh reported that approximately 50% of all implants that had functioned in the mouth for 5 years were affected by peri-implantitis (Zitzmann & Berglundh 2008). Periodontitis and peri-implantitis are caused by bacterial infection and show similar clinical features such as inflammation of the soft tissues and defects in the alveolar bone supporting the teeth or implants (Lindhe et al. 1992). Additionally, there may be bleeding and/or pus discharge from periodontal or peri-implant pockets (Lang et al. 2004; Lang & Berglundh 2011). However, the two diseases greatly differ pathologically. Strong

connective tissue inflammation is observed near the junctional epithelium (JE) in the early phase of periodontitis, but this does not involve the bone around the tooth (Carcuac et al. 2013; Carcuac & Berglundh 2014). In contrast, peri-implantitis exhibits inflammation in the connective tissue that extends to the bone surface (Lindhe et al. 1992). Moreover, inflammation of the peri-implant tissue develops more easily than that of periodontal tissue (Berglundh et al. 2011), possibly because the sealing between implant body and gingiva is weaker than the tooth–gingival sealing (Lindhe et al. 1992; Ikeda et al. 2002). A few studies using animal models have demonstrated that peri-implantitis advances much faster than periodontitis. The presence of ligating silk or cotton thread around the tooth or implant often results in plaque accu-

Date:
Accepted 21 December 2015

To cite this article:

Takamori Y, Atsuta I, Nakamura H, Sawase T, Koyano K, Hara Y. Histopathological comparison of the onset of peri-implantitis and periodontitis in rats. *Clin. Oral Impl. Res.* 00, 2016, 1–8
doi: 10.1111/clr.12777

mulation leading to peri-implantitis or periodontitis (Lindhe & Ericsson 1978; Lindhe et al. 1992). However, ligatures do not necessarily create problems on their own. For example, when the ligature was removed before sacrifice, periodontal tissue destruction was seen to diminish (Lindhe et al. 1992), but peri-implant tissue destruction continued (Carcuac et al. 2013). Therefore, using an animal model without ligatures will aid in the comparison of onset and development of periodontitis and peri-implantitis. We recently reported that the formation of immune complexes in the gingival sulcus accelerates loss of attachment and alveolar bone resorption (Kuramoto et al. 2012). Additionally, we induced site-specific loss of attachment and alveolar bone resorption by topical application of lipopolysaccharide (LPS) in the gingival sulcus of LPS-immunized rats with elevated serum levels of anti-LPS IgG (Yoshinaga et al. 2012). Using this animal model, we attempted to compare tissue destruction in periodontitis and peri-implantitis histopathologically and histomorphometrically.

Material and methods

Implants

This study used Ti-6AL-4V screw-type implants (Sky Blue, Fukuoka, Japan) that were 2 mm in diameter and 4.5 mm in length. Prior to surgical procedure, the implants were cleaned with 100 acetone and rinsed in distilled water and 70 alcohol, as previously described (Ikeda et al. 2002).

Experimental design

Twenty-five male 5-week-old Lewis rats were used in this study. The maxillary right first molars were extracted under general anesthesia with isoflurane, and the implants were screwed into the cavity. The left first molars were left untreated as natural teeth (NT) with normal periodontal tissue. After the surgery, 0.05 mg/kg buprenorphine (Lepetan, Otsuka Pharmaceutical, Tokyo, Japan) was injected as an analgesic, and the rats were divided into five groups, as follows: untreated baseline group (0-day group), immunized and LPS-applied group (I-LPS group), immunized and phosphate-buffered saline-applied group (I-PBS group), nonimmunized and LPS-applied group (nI-LPS group), and nonimmunized and PBS-applied group (nI-PBS). There were five rats in each group. The immunized groups received intraperitoneal injections of 0.3 ml of 150 µg *Escherichia coli* (*E. coli*) LPS (O111: B4; Sigma, St Louis, MO, USA) sus-

pending in PBS and emulsified in complete Freund's adjuvant, followed by a booster injection of LPS emulsified in incomplete Freund's adjuvant 28 days later. The nI groups received intraperitoneal injections of 0.3 ml of PBS emulsified with complete Freund's adjuvant, followed by an injection of incomplete Freund's adjuvant 28 days later (Yoshinaga et al. 2012). Three days after the booster injection, the untreated baseline group was sacrificed, and five rats from the immunized and nI groups received topical applications of *E. coli* LPS (50 µg/µl) suspended in PBS in the palatal gingival sulcus or implant sulcus of maxillary first molars with a micropipette under general anesthesia. The remaining five rats in each group received PBS only. In total, 21 µl (3 µl × seven times, with a 5-min interval between each application) of LPS or PBS was administered daily within 30 min. All the rats were sacrificed while under anesthesia using carbon dioxide gas 1 h after the third application (Fig. 1).

All rats were purchased from Charles River Japan (Tokyo, Japan) and maintained in specific pathogen-free conditions in the Biomedical Research Center, Center for Frontier Life Sciences (Nagasaki University, Nagasaki, Japan). Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University and with the approval of the Institutional Animal Care and Use Committee.

Preparation of tissues

The right and left maxillary regions were resected from each rat and fixed in 4 paraformaldehyde/PBS at 4°C for 10 h. Subsequently, the specimens were decalcified by immersion in 10 ethylenediaminetetraacetic acid (EDTA)-2Na (pH 7.4) at 4°C for 3 weeks. They were then embedded in paraffin using the acetone, methylbenzoate, and xylene (AMeX) method (Sato et al. 1986). Briefly, the specimens were first dehydrated in acetone, cleared in methyl benzoate for 30 min and xylene for 30 min, and then penetrated with

paraffin at 60°C for 2 h. The implants were removed from the specimens. Thereafter, the penetrated specimens were embedded in paraffin blocks and buccopalatal serial sections (4-µm thickness) were collected.

Indirect enzyme-linked immunosorbent assay for anti-LPS IgG detection

Blood samples were collected from the retro-orbital venous plexus of the rats just after the booster injection and the first and third topical applications. The serum levels of anti-LPS IgG were determined by indirect enzyme-linked immunosorbent assay using individual serum samples. Microtiter plates with 96 wells were coated with 100 µl/well of 2.5 µg/ml solution of *E. coli* LPS in 0.05 M carbonate buffer (pH 9.6) and then incubated for 16 h at 4°C. After being washed with 0.05 Tween 20/PBS (PBST), the wells were blocked with 0.1 bovine serum albumin/PBS at room temperature (RT). After another wash with PBST, 100 µl of the sera (1 : 1000 dilutions) to be tested was added, and the plates were incubated for 1 h at RT. After further washing with PBST, 100 µl of horseradish peroxidase-conjugated goat anti-rat IgG

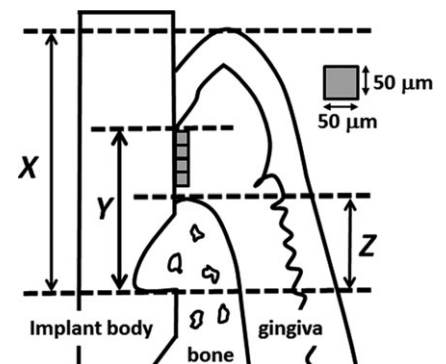


Fig. 2. Schema of rat peri-implant tissue for histometrical analysis. X: distance from the implant first thread to the crest of peri-implant mucosa. Y: distance from the implant first thread to the most apical position of the JE attached to the implant surface. Z: distance from the implant first thread to the alveolar bone crest. Square: the number of inflammatory cells in four unit areas (50 µm × 50 µm) of connective tissue adjacent to JE, junctional epithelium.

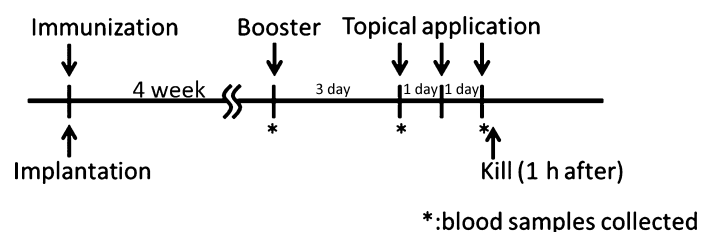


Fig. 1. The outline of the experimental schedule.

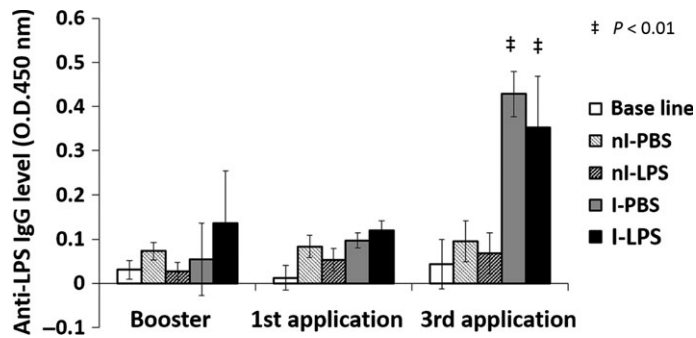


Fig. 3. Serum levels of anti-LPS IgG antibodies in the immunized, nonimmunized, and baseline groups. The immunized groups showed significant increase of serum anti-LPS IgG antibody level compared with the baseline group, ni-PBS group and ni-LPS group. Each bar represents the mean \pm SD. Mann-Whitney *U*-test, $P < 0.01$. ni-LPS, non-immunized lipopolysaccharide; PBS, phosphate-buffered saline.

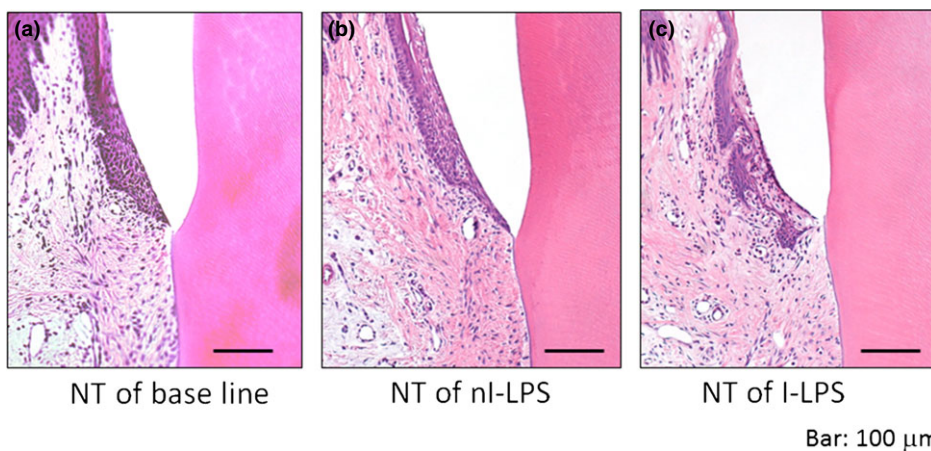


Fig. 4. Histopathological findings of the palatal side of the first molar. No loss of attachment was observed in all groups. However, in the I-LPS group, inflammatory cell infiltration was observed in the JE and adjacent connective tissue. (a) Baseline group, (b) ni-LPS group, and (c) I-LPS group. Scale bar = 100 μ m. I-LPS, immunized lipopolysaccharide; JE, junctional epithelium.

(1 : 5000 dilutions Invitrogen, Tokyo, Japan) in PBS was added and incubated for 1 h at RT. The plates were washed with PBST, and 75 μ l of the tetramethylbenzidine substrate solution (R&D Systems, Minneapolis, MN, USA) was added and incubated at RT. The enzyme reaction was stopped with 25 μ l/well of 2N H_2SO_4 , and the absorbance was read at 450 nm on a microplate reader.

Histopathological and histometrical study

Fifty serial sections obtained from each specimen were divided into five groups, that is, each containing 10 subsections. The first sections from each subsection group were stained with hematoxylin and eosin for histopathological observation. The sections were also used to measure the distance from the cemento–enamel junction (CEJ) of the tooth to the coronal portion of the JE attached to the root surface to evaluate loss of attachment and the distance from the CEJ to the alveolar bone crest to evaluate bone

destruction in the NT site. The distance between the crest of peri-implant mucosa and the first thread of the implant (Fig. 2X) was also measured. To evaluate the downward growth of JE, the distance between its most apical position on the implant surface and the first thread of the implant was measured (Fig. 2Y). The distance between the first thread of the implant and alveolar bone crest was also measured to evaluate the change of alveolar bone height (Fig. 2Z). Image analysis software (IMAGE J; US National Institutes of Health, Bethesda, MD, USA) was used to make all measurements. The number of inflammatory cells in four unit squares (50 μ m \times 50 μ m) of connective tissue under the JE was counted at a magnification of 400 \times and then averaged (Fig. 2). We tried to identify the most coronal portion of the JE to measure the peri-implant pocket depth using immunohistological method with anti-laminin-5 antibody. However, the reaction was patchy and discontinuous on

the implant surface, but continuous on the tooth surface. This could be explained by the fact that newly formed peri-implant epithelium does not attach to the titanium surface sufficiently until 4 weeks after implantation (Atsuta et al. 2005). Therefore, we were unable to measure the peri-implant pocket depth.

To identify the osteoclasts, the second sections from each subsection were stained with tartrate-resistant acid phosphatase (TRAP) (Katayama et al. 1972). Briefly, a staining solution was made by mixing 0.5 ml of pararosaniline solution (1 g of pararosaniline in 20 ml of distilled water and 5 ml of concentrated hydrochloric acid), 0.5 ml of 4 sodium nitrite solution, 10 ml of 0.1 M acetate buffer with pH 5.0, and 10 mg of naphthol AS-BI phosphate (Sigma) dissolved in 8 ml of distilled water. The mixture was adjusted to pH 5.0 using concentrated 1 M NaOH and filtered through Whatman Grade 1 filter paper (Whatman Fisher Scientific, Houston, TX, USA). After adding 75 mg of L-(+)-tartaric acid to a 10-ml aliquot of the mixture solution so as to give a final tartrate concentration of 0.05 M, the solution was adjusted to pH 5.0 with concentrated NaOH. After incubation in the stain solution for 30 min at 37 $^{\circ}$ C, the subsections were counterstained with hematoxylin. The number of TRAP-positive multinuclear cells in an area of 300 μ m width on the surface of the alveolar bone crest was counted.

The third sections from each subsection were subjected to Azan staining. They were deparaffinized and immersed in 10 potassium dichromate solution containing 10 trichloroacetic acid for 10 min for metachroming and then stained with 0.1 azocarmine G (Wako Pure Chemical Industries, Osaka, Japan) for 3 h. The sections were soaked in 0.1 aniline in 95 ethanol for a few seconds and then in 0.1 acetic acid in 95 ethanol for 1 min. After washing with water, they were immersed in 5 phosphotungstic acid solution for 1 h and then stained with aniline blue–orange G solution (Wako Pure Chemical Industries) for 1 h.

Immunohistological staining

The fourth sections from each subsection were used for immunohistological staining. C1qB was immunohistologically stained to detect immune complexes, per previous reports (Kuramoto et al. 2012; Yoshinaga et al. 2012; Nagano et al. 2013; Nakatsu et al. 2014). After the fourth subsections were deparaffinized, endogenous peroxidase activity was blocked with 0.3 H_2O_2 /metha-

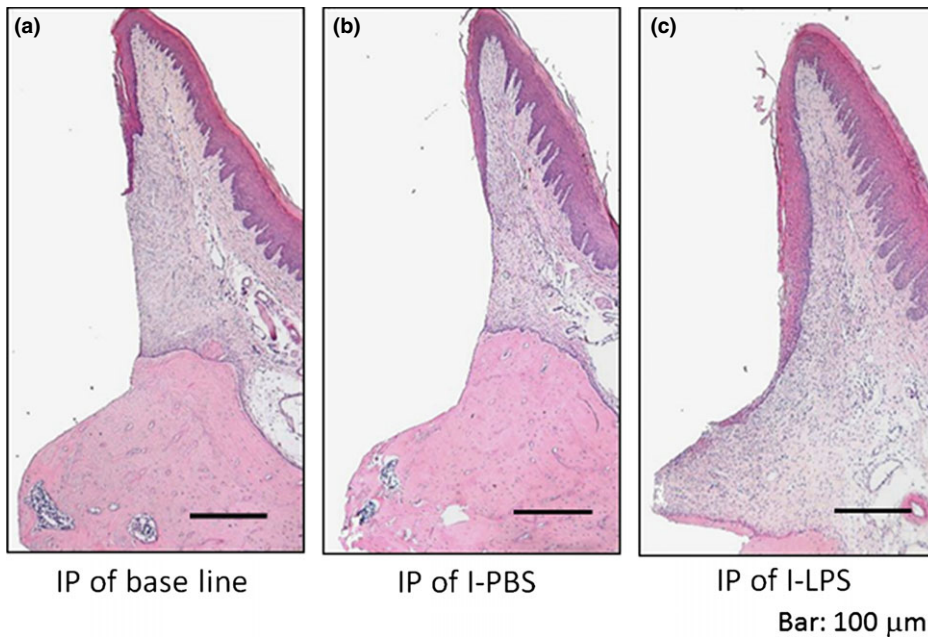


Fig. 5. Histopathological findings of the palatal side of the peri-implant tissue. Apical migration of JE attached to the implant surface and extensive infiltration of inflammatory cells into JE and adjacent connective tissue were observed in peri-implant tissue of I-LPS group. Furthermore, distinct alveolar bone resorption was observed in the I-LPS group. (a) Baseline group, (b) I-PBS group, and (c) I-LPS group. Scale bar = 100.

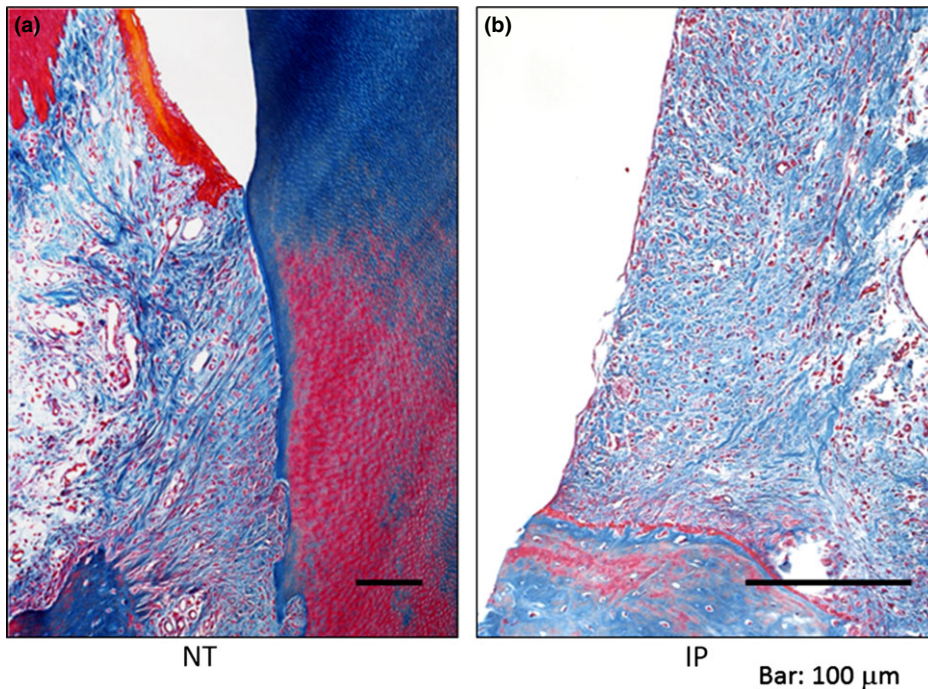


Fig. 6. Histopathological findings of the palatal side of the first molar and implant of baseline group. Oriented collagen fibers were observed in the NT side but were not seen clearly in the implant side. (a) NT side and (b) implant side. Scale bar = 100 μm. Azan staining, implant, implant; NT, natural teeth.

nol for 30 min, followed by incubation in normal goat serum for 30 min at RT. These sections were then immersed overnight in rabbit polyclonal anti-C1qB (AVIVA Systems Biology, San Diego, CA, USA). Thereafter, they were incubated with biotinylated goat

anti-rabbit polyclonal immunoglobulin (Dako, Glostrup, Denmark) for 30 min and peroxidase-conjugated streptavidin (Dako) for 30 min and then placed in diaminobenzidine tetraoxide solution and counterstained with hematoxylin.

Statistical analysis

All statistical analyses were carried out using StatMate IV (ATMS Co., Ltd, Tokyo, Japan). Differences between the nI rats and the immunized rats were evaluated using the Mann–Whitney *U*-test. Level of statistical significance was set at $P < 0.01$. Differences between the three groups were evaluated using a Kruskal–Wallis nonparametric analysis of variance (ANOVA) and Tukey-type multiple comparisons. Level of statistical significance was set at $P < 0.05$ and $P < 0.001$.

Result

Anti-LPS IgG level in the serum

The serum level of anti-LPS IgG was quite low in the nI groups. In the immunized groups, the serum levels of anti-LPS IgG were elevated after the third topical application and were significantly higher than that of the nI groups ($P < 0.01$; Fig. 3).

Histopathological findings

The most coronal portion of the JE was located at the CEJ, and no loss of attachment was observed on the NT side of all groups. In the I-LPS group, slight inflammatory cell infiltration was observed in the connective tissue adjacent to the JE (Fig. 4c), while no remarkable changes was observed in the other groups (Fig. 4a,b). No osteoclasts were detected on the alveolar bone surface, and there was slight infiltration of inflammatory cells in the JE and adjacent connective tissue. The implant side of all groups except the I-LPS group showed absence of alveolar bone resorption and few osteoclasts (Fig. 5a,b). In contrast, apical migration of JE and extensive infiltration of inflammatory cells were observed in the peri-implant connective tissue of I-LPS group (Fig. 5c). Furthermore, severe bone resorption was detected accompanied by many osteoclasts.

Azan staining revealed orientated dense collagen fibers around the tooth in the NT sites (Fig. 6a), but these were seen to lose their orientation on the implant side (Fig. 6b).

Histometrical analysis

After the third application, the distance from the gingival crest to the first thread of the implant in the I-LPS group was slight but significantly shorter compared to base line groups (Fig. 7). The distance from the first thread of the implant to the most apical position of the JE was significantly shorter in the I-LPS group than the all other groups except

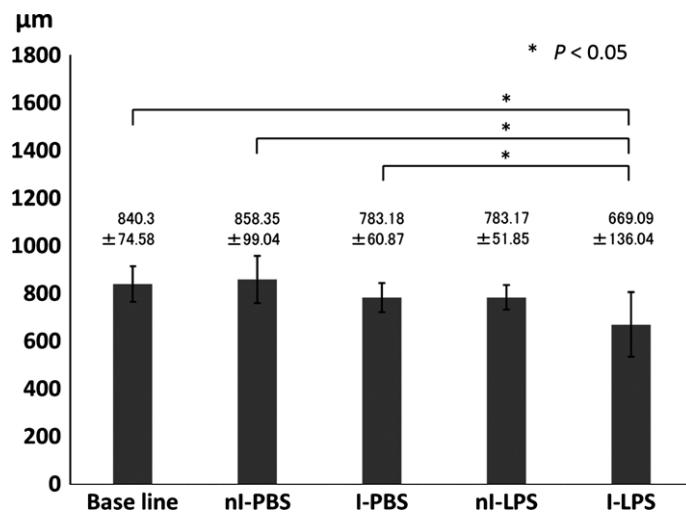


Fig. 7. Distance from the first thread of implant to gingival crest. In the I-LPS group, the distance from the first thread of implant to gingival crest was significantly shorter than base line groups. Statistical significance was analyzed by Kruskal–Wallis. Each bar represents the mean \pm SD. $P < 0.05$.

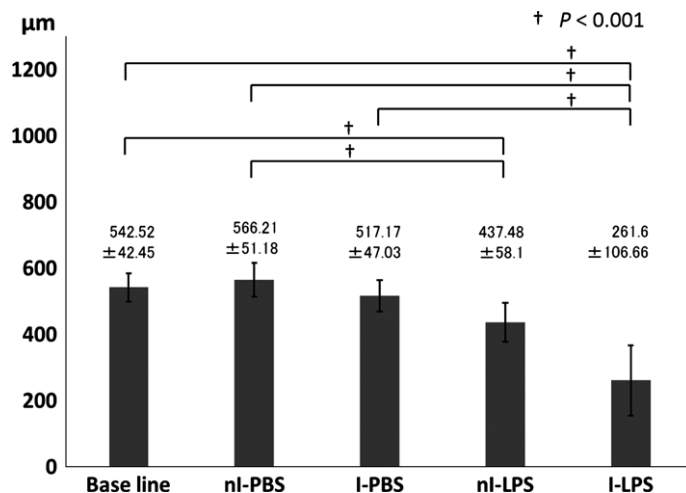


Fig. 8. Distance from the first thread of implant to the most apical position of JE. In the I-LPS group, the distance from the first thread of implant to the most apical position of JE was significantly shorter than in the other groups except nl-LPS group. Statistical significance was analyzed by Kruskal–Wallis. Each bar represents the mean \pm SD. $P < 0.001$.

the nl-LPS group (Fig. 8). The distance from the implant first thread to the alveolar bone crest was significantly shorter in the I-LPS group than the other groups (Fig. 9). The number of inflammatory cells was significantly higher in the I-LPS group than the all other groups except the nl-LPS group (Fig. 10). The number of osteoclasts in the I-LPS group was significantly greater than the other groups (Fig. 11).

Immunohistological findings of C1qB

C1qB was detected only in the JE in the NT site of the I-LPS group but was present in the JE and adjacent connective tissue in the implant sites (Fig. 12).

Discussion

The differences between inflammatory lesions of peri-implantitis and periodontitis have been previously discussed in the seventh EuroPerio workshop conducted in 2011 (Berglundh et al. 2011). However, most of the studies referred to used ligature models to induce inflammation in the soft tissue and bone surrounding the tooth or implant. Experimental peri-implantitis and periodontitis induced in this manner may not necessarily replicate naturally occurring inflammation. Moreover, the pathological progression of peri-implantitis differs from periodontitis. For example, destruction of peri-implant tissues induced by

ligature continued progressing for 6 months after ligature removal while periodontal destruction stopped immediately after (Berglundh et al. 2011; Carcuac et al. 2013). This made it difficult to compare inflammation occurring in natural tooth and ligated implant models. The current study used a new experimental model of peri-implantitis without ligature that made comparison with an experimental periodontitis model in NT possible. Based on our previous experimental periodontitis model (Yoshinaga et al. 2012), LPS was administered with a microsyringe into the peri-implant or gingival sulcus of rat a preimmunized with LPS. As a result, in this experimental period, inflammation has been induced but not periodontitis. This model enables elimination of the mechanical stimulation caused by ligature and helps us to consider the differences in resistance to foreign antigens between periodontal and peri-implant tissues. Our study also showed bone resorption, under-migration of JE, and intense inflammatory cell infiltration, neutrophils in particular, in the connective tissue of the implant site, while only a few inflammatory cells were observed in the JE of the NT site. These results are consistent with other studies that induced peri-implantitis with ligature placement and subgingival plaque formation (Lindhe et al. 1992; Carcuac et al. 2013). In our previous study (Yoshinaga et al. 2012), it took 10 days to promote periodontal destruction by topical application of LPS in LPS-immunized rats, suggesting that antigen could penetrate and spread into the peri-implant tissue more easily than periodontal tissue. The experimental peri-implantitis model used in this study allowed us to examine the onset and progression of the disease by changing the concentration of stimuli and/or duration of the experiment.

It was surprising that the severity of tissue destruction in the peri-implant tissue was clearly greater than that in the periodontal tissue. In general, peri-implant tissue is considered to be sensitive to bacterial challenges (Lindhe & Ericsson 1978; Ikeda et al. 2002), possibly due to the orientation of collagen fibers. Dento-gingival fibers, dento-periosteal fibers, and circular fibers in the periodontal tissue were observed in the NT site, while oriented fibers appeared only in the marginal bone of implant site, in accordance with previous reports (Berglundh et al. 1991). It has been reported previously that traumatic occlusion accelerated the onset of experimental periodontitis in rats immunized with LPS by causing degeneration and decrease of collagen fibers in the periodontal tissue

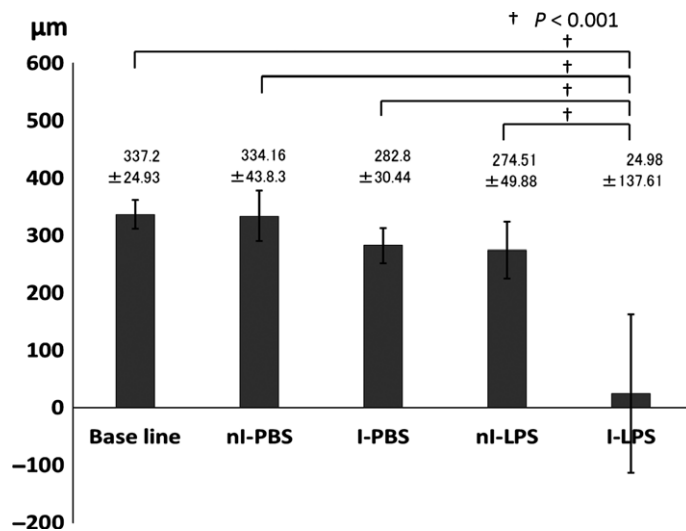


Fig. 9. Distance from the first thread of implant to alveolar bone crest. In the I-LPS group, distance from the implant first thread to the alveolar bone crest was significantly greater than the other four groups. Statistical significance was analyzed by Kruskal–Wallis. Each bar represents the mean ± SD. $P < 0.001$.

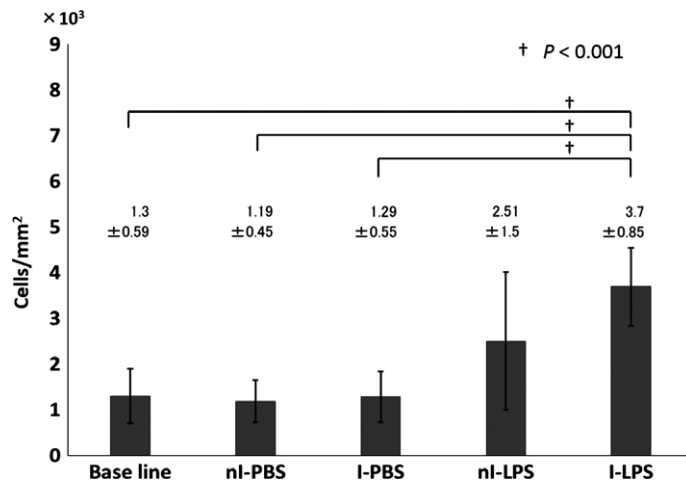


Fig. 10. The number of inflammatory cells in the peri-implant junctional epithelium. In the I-LPS group, the number of inflammatory cells was significantly larger than the other four groups. The nl-LPS group also showed significantly larger numbers than the baseline, nl-PBS, and I-PBS groups. Statistical significance was analyzed by Kruskal–Wallis. Each bar represents the mean ± SD. $P < 0.001$.

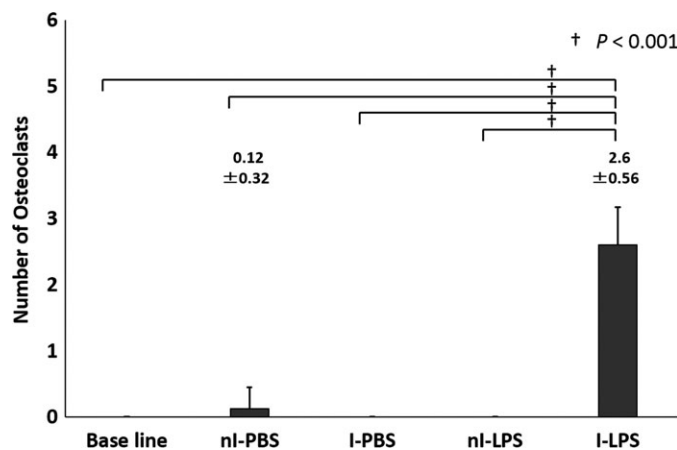


Fig. 11. The number of osteoclasts on the peri-implant alveolar bone surface. The number of the osteoclasts in the I-LPS group was significantly greater than the other groups. Statistical significance was analyzed by Kruskal–Wallis. Each bar represents the mean ± SD. $P < 0.001$.

(Nakatsu et al. 2014). In this study, the collagen fibers were not oriented regularly to the implant surface. It has been suggested that sparse collagen fibers around the implant may allow higher permeability to antigens than those around normal teeth and also expand antigens by immune-complex formation. This results in acceleration of the inflammatory response. Lindhe et al. (1992) reported that the size of the inflammatory lesion in soft tissues was larger around implants than around teeth. Histopathological findings revealed that the inflammatory cells increased in number close to the bone surface in the implant site of the I-LPS group. Specific binding of antigen to antibody results in the formation of an immune complex. The activation of the classical complement pathway is initiated by binding of the complement factor C1 to the Fc region of immunoglobulin. C3a and C5a, produced during activation of the complement system, are known to be anaphylatoxins of neutrophil. Infiltrated neutrophils release pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1 β , which accelerate inflammatory response further. Moreover, reactive oxygen species released by neutrophil directly cause tissue destruction (Janoff 1985). This may explain the destruction of the JE and connective tissue observed in this study.

We also observed resorption of the alveolar bone. It has been reported that C3a and C5a directly induce the formation of osteoclasts from peripheral blood mononuclear cells (Ignatius et al. 2011). Anaphylatoxins produced by the activated complement system may be responsible for the formation of osteoclasts. Additionally, it is considered that T cells also play an important role in bone destruction and osteoclastogenesis. It has been reported previously that activation of the immune system is necessary for T-cell-related osteoclastogenesis (Ozaki et al. 2009). Activated T cells isolated from LPS-injected mice accelerate osteoclast formation in the presence of LPS. These T cells and their inflammatory cytokines may explain the accelerated alveolar bone resorption observed in the I-LPS group. Further studies are required to elucidate this hypothesis.

In this study, the healing period is shorter than those in the other studies (Lindhe et al. 1992; Carcuac et al. 2013). However, several studies reported that osseointegration between titanium implants and the alveolar bone can be established at approximately 1 month postimplantation in rat model (Karimbux et al. 1995; Fujii et al. 1998), sup-

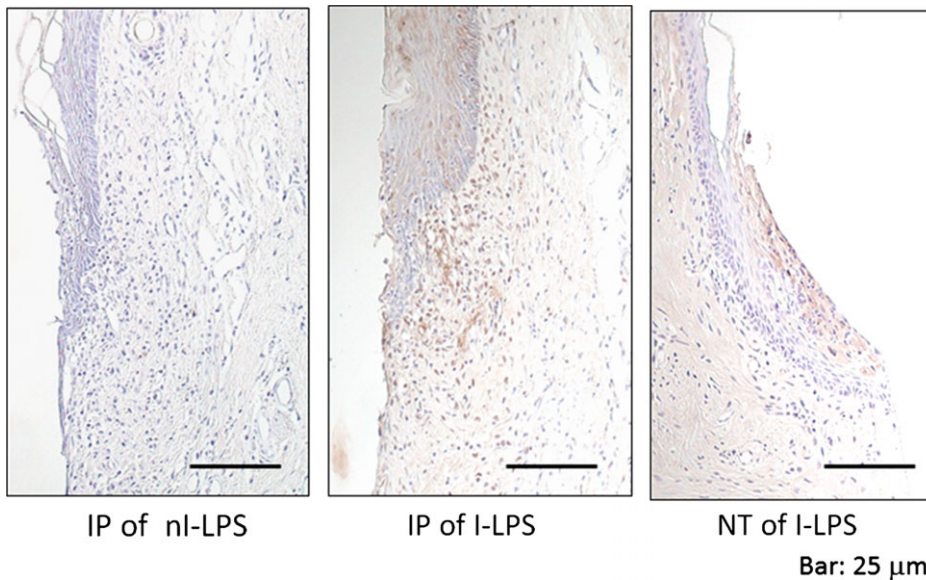


Fig. 12. Immunohistological localization of C1qB. C1qB stained with dark red color was observed in the junctional epithelium and adjacent connective tissue surrounding natural tooth and implant in the I-LPS group. Scale bar = 25 μ m.

porting the validity of our experimental model.

It has been suggested that when antigens invade the periodontal tissue of immunized patients, specific antibodies bind with them

and form immune complexes leading to the destruction of periodontal tissue [Kuramoto et al. 2012; Nagano et al. 2013]. Patients with a history of periodontitis are thought to be already immunized with periodontopathic

bacteria or their components and, therefore, are more likely to develop periodontal destruction because immune complexes are easily formed in the gingival tissue. Implantation in such patients would have the risk of early recurrence and severe progression of peri-implantitis by the same mechanism seen when accumulated plaque acts as an antigen. Although dental implants are one of the most important treatment options for periodontal patients, it is necessary to emphasize this point to achieve appropriate periodontal treatment. Further studies using this model are required to understand the mechanism of peri-implantitis better so as to aid prevention and treatment.

Acknowledgements: This study was supported by grants (24593127) for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflict of interests

The authors report no conflicts of interest related to this study.

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