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The inhibitory effect of secretory leukocyte protease inhibitor (SLPI) on the alveolar bone resorption with lipopolysaccharide (LPS)-induced periodontitis in rat

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치의생명공학과

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ABBREVIATIONS

bLF: bovine lactoferrin
BSP: bone sialoprotein
CBD: cammabidiol
Col Ⅰ: collagen type Ⅰ
GCF: gingival crevicular fluid
IL-1: interleukine-1
LPS: lipopolysaccharide
M-CSF: macrophage-colony stimulating factor
Micro-CT: micro-computed tomography
NF-κB: nuclear factor-κB
NO: nitric oxide
OCN: osteocalcin
OPG: osteoprotegerin
PAMP: pathogen-associated molecular pattern
PCO: polycystic ovary
PCOS: polycystic ovary syndrome
PDL: periodontal ligament
RANKL: receptor activator of nuclear factor-κB ligand
ROI: region of interest
Runx2: runt-related factor 2
SCW: streptococcal cell wall
SLPI: secretory leukocyte protease inhibitor
TLR: toll-like receptor
TNF-α: tumor necrosis factor-α

ABSTRACT IN KOREAN

흰쥐에서 LPS로 유도된 치주염에 동반된 이틀뼈 흡수에 대한 SLPI의 억제 효과

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치주염은 주로 치면세균막에 존재하는 그람음성 세균들에 의해 유도되는 감염성 질환이다. 그람음성세균의 세포벽 구성성분중 하나인 LPS는 대식세포와 같은 염증세포들을 자극해 주변 조직에 염증반응을 유도함으로써 치주낭 형성과 이틀뼈 흡수를 일으킨다. 분비백혈구단백분해효소억제제(SLPI)는 염증반응부위에서 항세균 및 항염증작용을 하는 물질로 알려져 있다. SLPI 발현은 LPS 뿐만 아니라 TNF-α와 IL-1β 같은 전염증사이토카인에 의해서도 증가된다. 염증반응에 의해 증가된 SLPI는 TNF-α와 NO분비를 억제함으로서 관절염증, 연골 및 뼈의 파괴를 감소시킨다. 따라서, 본 연구에서는 SLPI가 LPS 처리에 의해 유도된 치주염에 동반되는 치조골 흡수 억제에 대한 효과를 규명하고자 하였다.

Micro-CT를 이용한 ROI 분석에서 LPS 주사 후 SLPI를 주사한 군(LPS/SLPI군)은 LPS를 주사한 군(LPS군)에 비해 뼈 부피(bone volume)와 잔기둥뼈 개수(trabecular bone number)가 높았고, 뼈 복잡성(ratio of bone surface area to bone volume), 잔기둥뼈 사이 공간(trabecular bone space) 그리고 잔기둥뼈 형태(trabecular bone structure model index)는 낮았다. 조직학적 분석을 통해 LPS/SLPI군은 LPS군에 비해 이틀뼈 흡수가 감소하였다. 면역조직화학적 분석에서 LPS/SLPI군은 LPS군에 비해 TNF-α, IL-1β 그리고 RANKL 단백질이 낮게 발현되었으며, SLPI, OPG 그리고 Runx2 단백질은 높게 발현됐다. LPS를 처리한 MC3T3-E1 세포(MC3T3-E1/LPS)에서

는 대조군에 비해 TNF-α, IL-1β 그리고 RANKL의 mRNA와 단백질 발현이 증가됐고, OPG와 Runx2는 감소됐다. LPS/SLPI를 처리한 세포(MC3T3-E1/LPS/SLPI)에서는 MC3T3-E1/LPS에 비해 TNF-α, IL-1β 그리고 RANKL의 mRNA와 단백질 발현이 감소됐고, OPG와 Runx2는 증가됐다.

본 결과를 요약하면, Micro-CT 및 조직학적 분석 결과로 SLPI는 흰쥐의 LPS로 유도된 치주염 억제 및 이틀뼈 흡수를 감소시켰다. LPS/SLPI군의 치주조직 면역화학분석 및 MC3T3-E1/LPS/SLPI에서는 TNF-α, IL-1β, 그리고 RANKL이 감소됐고 SLPI, OPG 그리고 Runx2가 증가됐다. 따라서 SLPI는 염증성 사이토카인 및 뼈파괴세포 활성 인자를 억제하고, 뼈모세포 활성인자들을 증가시킴으로써 뼈파괴세포 활성을 억제하고, 뼈형성 유도를 조절하는 신호분자 일 것으로 생각된다.

I. INTRODUCTION

Periodontitis is an inflammatory and infectious disease induced by dental plaque and causes gingival bleeding, formation of periodontal pocket, alveolar bone resorption, destruction of tooth supporting tissue, and, in severe cases, tooth loss (Socransky et al., 1984; Petersen et al., 2005). Periodontitis in humans is known to occur by various types of gram-negative bacteria in dental plaque (Offenbacher, 1996). Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is known as an important factor that causes periodontitis (Daly et al., 1980). LPS induces the expression of tumor necrosis factor-α (TNF-α) and receptor activator of nuclear factor-κB ligand (RANKL) by activating nuclear factor-κB (NF-κB) through a toll-like receptor 4 (TLR4) (Inubushi et al., 2012). Formation and resorption of bone occur consistently in healthy alveolar bone. Bone is formed by mineralization and secretion of bone matrix of osteoblast derived from ectomesenchymal cells, and bone resorption takes place by differentiation of osteoclast from monocyte. In periodontitis, the mechanism associate with bone resorption is activated through the increasing activation of osteoclast (Harada and Rodan, 2003; Braun et al., 2011; Schett, 2011).

Macrophage-colony stimulating factor (M-CSF) and RNAKL, expressed in osteoblast, are known to be the key cytokines for differentiation of osteoclast (Yoshida et al., 1990; Yasuda et al., 1998; Kong et al., 1999). RANKL, expressed in bone marrow stromal cell and osteoblast, is one of the TNF family groups, which has been identified as the factor that leads to differentiation of osteoclast progenitor cells into osteoclast (Suda et al., 1999). RANKL expression in osteoblast is induced by activated vitamin D3 (1a, 25-dihydroxyvitamin D3), bone resorption stimulating hormone (parathyroid hormone, prostaglandin E2), Interleukine-1 (IL-1), and LPS (Yasuda et al., 1998; Suda et al., 1999; Koide et al., 2000). RANKL is an essential molecule of osteoclastogenesis, which directly induces the differentiation of monocyte/macrophage lineage cells into osteoclast (Lacey et al., 1998). In RANKL-deficient mice, the bone mass is excessively increased and there is no

formation of incisors and molar eruption (Kong et al., 1999). The lack of osteoclastogenesis and severe induction of osteopetrosis are presented in RANK-deficient mice (Dougall et al., 1999).

TNF-α is an osteoclastogenic factor similar to RANKL, which increases proliferation and differentiation of osteoclast. In addition, TNF-α stimulates not only IL-1β and chemokine production as the proinflammatory cytokines, but also prostaglandin E2 and RANKL (Bertolini et al., 1986). IL-1 is a key regulator of host response, extracellular matrix degradation, and bone resorption, which is known as a marker of periodontium destruction due the identification of the secretion and sufficient concentration in gingival crevicular fluid (GCF) of human periodontitis (Masada et al., 1990; Archana et al., 2012). Proinflammatory cytokines such as IL-1β regulate bone resorption through the acceleration of RANKL expression from stromal cell and osteoblast in gingival connective tissue near the anterior edge of alveolar bone (Nakamura et al., 2009). Osteoprotegerin (OPG) is a TNFR-related protein that decreases osteoclast differentiation in OPG transgenic mice (Simonet et al., 1997). In addition, OPG suppresses osteoclastogenesis through the blocking of RANKL-RANK binding in ST2 cell as the decoy receptor of RANKL (Yasuda et al., 1998). OPG production is decreased by bone resorption stimulating hormones, TNF-α, and IL-1 (Takahashi et al., 2002). Runt-related factor 2 (Runx2) is expressed in the early stage of bone development, and lack of bone formation presents in Runx2-deficient mice (Komori et al., 1997). Moreover, Runx2 is essential to stimulate differentiation of mesenchymal cells into osteoblast progenitor cells, and its expression is significantly decreased by LPS in osteoblast of rat mandible (Tomomatsu et al., 2009; Komori, 2011).

Secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa cysteine-rich protein, and not only affects anti-human immunodeficiency virus (HIV) action in human salivary gland and vaginal fluid but also inhibitory function of inflammation and proteolysis during skin or mucosal wound healing (McNeely et al., 1997; Ashcroft et al., 2000; Pillay et al., 2001; Angelov et al., 2004). SLPI has been identified as a protease inhibitor for regulation of excessive proteolysis by neutrophil serine proteases such as elastase and cathepsin G (Campbell et al., 1989).

SLPI expression is temporarily increased in odontoblast by LPS stimulation or wound, and it inhibits inflammatory response through decrease of NFκ-B activation (Choi et al., 2009). In addition, SLPI suppresses tissue destruction of polycystic ovary (PCO) and topically inhibits inflammatory response and excessive matrix destruction in skin wound of polycystic ovary syndrome (PCOS) rat (Park et al., 2011; Jeong et al., 2012). Furthermore, SLPI is reported not only to promote migration and invasion of KB oral carcinoma cell, but also to accelerate the migration and metastasis of gastric cancer cell through increased expression and secretion of MMP-2 and MMP-9 (Wang et al., 2011; Choi et al., 2011).

Recent studies reported that SLPI regulates formation of dentin matrix and mineralization in odontoblast as well as increasing adhesion and viability of osteoblast on titanium surface (Jeong et al., 2015a; 2015b). SLPI inhibits production of TNF-α in macrophages, and TNF-α induces expression of SLPI in epithelial cells (Jin et al., 1997; Devoogdt et al., 2006). In addition, increasing SLPI expression is due to inflammatory-inducing factors such as LPS, TNF-α, IL-6 and IL-1β, which lead to inhibiting inflammation and destruction of cartilage and bone through inhibition of TNF-α and nitric oxide production (Song et al., 1999).

Based on previous studies, SLPI is considered as a useful molecule for its inhibitory effect in bone resorption through regulation of gene expression associated with inflammatory response and osteoclastogenic factors in periodontitis. Therefore, the purpose of this study is to investigate the function of SLPI in alveolar bone resorption by LPS-induced periodontitis and in osteoblast with LPS stimulation. Accordingly, the micro-CT, histological, and immunohistochemical analyses were performed in LPS and/or SLPI injected into the molar of the maxilla in rat. In addition, alteration of gene and protein expression associated with inflammation and osteoclastogenesis were analyzed in mouse osteoblastic MC3T3-E1 cells after LPS and/or SLPI stimulation.

Ⅱ. MATERIALS AND METHODS

II-1. Induction of periodontitis by LPS injection

Eighteen male rats of a 9-week old Sprague Dawley strain (Samtako Bio, Osan, KOR), weighing 350 - 400 g, were used in this experiment. The feeding environment was maintained at 23 ± 2℃ and 60 ± 10% humidity on a light and darkness of 12 h cycles. The rats were given pelleted rat chow and tap water ad libitum. The 18 rats were divided into three groups of six. Periodontitis was induced by LPS (1 mg/ml) injection into the bottom of the gingival groove at the buccal aspect of the right second molar of the maxilla every 48 h, five times. SLPI (rhSLPI, R&D systems, MN, USA) (0.1 or 1 μg/ml) was also injected into the same area after each LPS injection every 48 h, five times. Each group was divided as follows: the LPS group was injected with only LPS, the LPS/SLPI-L group was injected with SLPI (0.1 μg/ml) after LPS injection, and the LPS/SLPI-H group was injected with SLPI (1 μg/ml) after LPS injection. The left second molar of the maxilla area of each group was used as a control. All animal experiments took place with the approval of the Institutional Animal Care and Use Committee at Chosun University.

II-2. Micro-CT analysis

Male rats were anesthetized by intramuscular injection of 0.2 ml/100 g Ketamine (Yuhan, Seoul, KOR) mixed with 0.08 ml/100 g Rompun (Bayerkorea, Seoul, KOR) for extraction of periodontitis-induced maxilla. Thoracic cavity of rat was opened after anesthesia for perfusion fixation, and diethyl pyrocarbonate (DEPC)-PBS was injected slowly in the left ventricle after cutting of the vena cava from the right atrium; 10% neutral buffered formalin (BBC biochemical, WA, USA) was injected for perfusion fixation after circulation of DEPC-PBS until

complete removal of blood. Jaw bone, including maxilla, was extracted after complete circulation of fixative; it was fixed in fresh fixative at 4℃ for 16 h. The maxilla was scanned by micro-computed tomography (micro-CT) (Skyscan 1172, Micro photonics Inc., CA, USA). The set points of micro-CT in this study were that pixel size was 1,000 X 1,000; thickness of slice was 16 μm; voltage was 80 kv; and electrical current was 0.1 mA. The tomography and analysis were performed parallel with the root of second molar using by computer program (CTAn, 1.12.00, CA, USA). The region of interest (ROI, 0.5 mm X 0.5 mm) in alveolar bone of second molar root furcation was analyzed and measured using computer program (CTAn). The measured list was as follows: bone volume (BV / TV, %); ratio of bone surface area to bone volume (BS / BV, mm^{-1}); trabecular bone thickness (Tb Th, mm); trabecular bone number (Tb N, mm^{-1}); trabecular bone space (Tb Sp, mm); trabecular bone structure mode index (SMI), and trabecular bone pattern factor (Tb Pf, mm^{-1}). To represent degree of alveolar bone resorption in ROI, the degree of resorption was divided into 1+ (slight), 2+ (medium), and 3+ (severe), respectively.

II-3. Tissue preparation and hematoxylin & eosin staining

The maxilla in fixative was washed in phosphate buffered saline (PBS) for 2 h and decalcified in solution of 10% EDTA supplement with 1% paraformaldehyde for 3 months. The tissues were dehydrated by sequential washes in 70%, 80%, 90%, 100% I, 100% II, 100% III, and finally 100% IV ethanol, after decalcification. The clearing process was performed using the xylene, and the tissues were embedded in paraffin. The paraffin-embedded tissue was made into a section of 12 μm thickness using the Histocut 820 (Leica, HE, GER) and drying on 37℃ slide warmer overnight after being placed onto a slide glass coated by 3-(Trimethoxysilyl) propyl methacrylate (SIGMA, MO, USA). Hematoxylin & eosin staining was performed to observe the histological change and bone resorption level.

II-4. Immunohistochemistry

The samples were deparaffinized sequentially in xylene I and II for 10 min, and then hydrated by 95% ethanol for 10 min. For detection of SLPI, TNF-α, IL-1 β, RANKL, OPG, and Runx2 protein, the samples were washed three times in TBS-T for 5 min and were incubated with proteinase K (Invitrogen, NY, USA) of 1:1,000 in Tris-HCl (pH 7.5) at 37℃ for 20 min. The samples were washed three times and primary blocked with 3% H_2O_2 in dH_2O for 10 min at room temperature. Normal goat serum (VectorLab, CA, USA) in TBS (50 μl/ml) was used for secondary blocking. After washing, the samples were incubated with 1:1,000 of anti-rabbit SLPI (Choi et al., 2009), 1:1,000 of anti-rabbit TNF-α (Abcam, Cambridge, UK), 1:1,000 of anti-rabbit IL-1β (Abcam), anti-mouse RANKL (Novus, MO, USA), anti-rabbit OPG (Abcam), and anti-rabbit Runx2 (Abcam) were diluted in fresh normal goat serum for 16 h at 4℃. Goat-anti-Rabbit or mouse IgG (1:250, Vector Lab) was used for secondary antibodies for 30 min at room temperature. After washing, the samples were incubated with an ABC reagent for 30 min at room temperature. For color development, samples were incubated with 0.05% Deaminobenzidine Tetrahydrochloride (DAB) (Vector Lab) for 3 min. After washing twice with ddH_2O for 10 min, the samples were stained with Harris hematoxylin as counterstain and then dehydration, clearing, and mounting were performed. The stained tissues were observed by optical microscope (Carl Zeiss). The intensity of SLPI, TNF-α, IL-1β, RANKL, OPG, and Runx2 protein expression in the bottom of alveolar bone at root furcation of the left and right maxillary second molar were quantified using Axiovision LE release 4.6 software (Carl Zeiss). The Runx2-positive cells were counted in the same area.

II-5. MC3T3-E1 cell culture and treatment of LPS and SLPI

MC3T3-E1 cells, the osteoblastic cell line derived from mouse calvaria, were

cultured in Alpha-modified eagle's medium (α-MEM) (Gibco BRL, USA) containing 10% fetal bovine serum (WelGENE, KOR) and 1% antibiotic antimycotic solution (WelGENE). Cells were humidified in a chamber maintained with 5% CO_2 at 37℃. LPS (200 ng/ml) was treated in MC3T3-E1 cells (MC3T3-E1/LPS), and SLPI (R&D systems) (1 μg/ml) was treated in MC3T3-E1 cells after LPS treatment (MC3T3-E1/LPS/SLPI) for 2, 4, 6, 12, and 24 h.

II-6. Extraction of total RNA and reverse transcription and polymerase chain reaction

Total RNA was extracted with Tri reagent (MRC Inc., OH, USA) according to the manufacturer's instructions. A 1 μg Total RNA was used to synthesize the complementary DNA (cDNA). The synthesis of cDNA was performed on RT Premix (GeNet Bio, Daejeon, KOR). PCR reaction was carried out in a thermo cycler (Takara) after adding 1 μl of cDNA to the PCR premix (GeneAll, Seoul, KOR). The following primers (Bioneer, Daejeon, KOR) were used for RT-PCR analysis: 1) SLPI Forward 5'-TGC TTA ACC CTC CCA ATG TC-3', Reverse: 5'-AAT GCT GAG CCA AAA GGA GA-3'; 2) TNF-α Forward 5'-ACG GCA TGG ATC TCA AAG AC-3', Reverse: 5'-CGG ACT CCG CAA AGT CTA AG-3'; 3) IL-1β Forward, 5'-CAG GCA GGC ACT ATC ACT CA-3', Reverse: 5'-GGC CAC AGG TAT TTT GTC GT-3', 4) RANKL Forward, 5'-TAT GAT GGA AGG CTC ATG GT-3', Reverse: 5'-TGT CCT GAA CTT TGA AAG CC-3'; 5) OPG Forward, 5'-CAG AGA CTA ATA GAT CAA AGG CAG G-3', Reverse: 5'-ATG AAG TCT CAC CTG AGA AGA ACC-3'; 6); Runx2 Forward, 5'-GCA GTG CCC CGA TTG AGG-3', Reverse: 5'-CAT ACR GGG ATG AGG AAT GCG-3' and 7); GAPDH Forward, 5'-CCA TGG AGA AGG CTG GG-3', Reverse: 5'-CAA AGT TGT CAT GGA TGA CC-3'. GAPDH was used as the internal control for RT-PCR.

The PCR conditions were as follows: 1) 30 cycles, 95℃ 20 sec, 60℃ 10 sec, and 72℃ 30 sec for SLPI; 2) 35 cycles, 95℃ 20 sec, 56℃ 10 sec, and 72℃ 30 sec for

TNF- α; 3) 35 cycles, 95℃ 20 sec, 58℃ 10 sec, and 72℃ 30 sec for IL-1β; 4) 36 cycles, 95℃ 20 sec, 59℃ 10 sec, and 72℃ 30 sec for RANKL; 5) 30 cycles, 95℃ 20 sec, 59.7℃ 10 sec, and 72℃ 30 sec for OPG; 6) 34 cycles, 95℃ 20 sec, 59℃ 10 sec, and 72℃ 30 sec for Runx2; 7) 30 cycles, 95℃ 20 sec, 60℃ 10 sec, and 72℃ 30 sec for GAPDH. All samples were incubated for an additional extension for 5 min at 72℃. The products were electrophoresed on a 2% agarose gel buffered with 0.5 X TBE and stained with ethidium bromide (EtBr) after amplification. The staining bands are visualized by Gel-Doc (BIO-RAD, CA, USA). The primer sets were designed only for recognition of the interest genes and expected amplification size were as follows: 283 bp for SLPI, 324 bp for TNF-α, 349 bp for IL-1β , 512 bp for RANKL, 607 bp for OPG, 507 bp for Runx2 and 199 bp GAPDH according to the nucleotide sequence of SLPI (Genbank # BC_028509), TNF-α (Genbank # NM_013693), IL-1β (Genbank # NM_008361), RANKL (Genbank # AF_019048), OPG (Genbank # AB_013898), Runx2 (Genbank # NM_001146038). The intensity of bands was measured using a Science Lab Image Gauge (FUJI FILM, Tokyo, JPN).

II-7. Western blotting

MC3T3-E1 cells were lysed using NP-40 lysis buffer (150 mM NaCl, 1% NP-40), 50 mM Tris-HCl (pH 7.4), 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA (pH 7.4) 0.1 μg/ml, leupeptin, and 1 μg/ml aprotinin) for extraction of protein. These lysates were incubated on ice for 30 min and to extract the total cellular protein by centrifuge at 4℃, 13,000 rpm. After the protein extraction, the concentration was determined a 30 μg using an assay kit (BIO-RAD) and electrophoresed in 10% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to PVDF membrane and blocked with either 5% non-fat dry milk or 5% bovine serum albumin (Bioshop, USA) for 1 h at room temperature. The membranes were blotted with 1:1,000 of anti-rabbit SLPI (Choi et al., 2009), anti-rabbit TNF-α (Abcam), anti-rabbit IL-1β (Abcam), anti-mouse RANKL (Novus), anti-rabbit OPG (Abcam), anti-rabbit Runx2 (Abcam), and 1:2,500 of

anti-mouse β-actin (Santa Cruz Biotechnology, TX, USA) for 16 h at 4℃. After washing, the membrane was bottled with 1:5,000 - 1:10,000 of HRP-conjugated goat anti-rabbit or mouse-IgG (SantaCruz) at room temperature for 1 h. The developing was performed using X-ray film (FUJI FILM) after detection by ECL solution (Millipore, MA, USA). The molecular weight of SLPI, TNF-α, IL-1β, RANKL, OPG, Runx2, and β-actin on the representative bans indicated 12 kDa, 23 kDa, 17/31 kDa, 21 kDa, 55 kDa, 60 kDa, and 42 kDa, respectively. The density of the expressed bands was measured using Science Lab Image Gauge (FUJI FILM).

II-8. Statistical analysis

All experiments were performed at least in triplicate. All data were reported as means and standard deviations using Excel 2010 statistical software (Microsoft, WA, USA). Significant difference ($*p<0.05$, $**p<0.01$) was determined by Student's t-test.

Ⅲ. RESULTS

III-1. Micro-CT analysis to alveolar bone in molar of the maxilla after LPS or LPS/SLPI injection

The surface of alveolar bone and first, second, and third molar were observed in 3D images of maxilla molar region; the bone resorption of alveolar bone surface was severe in the LPS group and was observed slightly in the LPS/SLPI-H group (Fig. 1a - a^3). The alveolar bone resorption was not observed in the 3D and 2D analysis area image (Box; ROI, Region Of Interest) of root furcation on the internal section of second molar as a control group, and it was observed at a degree of 3+ in the LPS group, 2+ in the LPS/SLPI-L group, and 1+ in the LPS/SLPI-H group (Fig. 1b - b^3, c - c^3). The alveolar bone and root furcation were observed in the 2D maxilla horizontal image of the control group; alveolar bone resorption was severe in the LPS group and it was observed slightly in the LPS/SLPI-H group (Fig. 1d - d^3).

III-2. The parameter of ROI analysis to alveolar bone in molar of the maxilla after LPS or LPS/SLPI injection

Ratio of the bone volume to total volume in the LPS group was 29% lower than that of the control group; LPS/SLPI-L and -H group were 6% and 20% higher than the LPS group, respectively (Fig. 2A). Ratio of the surface area to bone volume of the LPS group was 16 mm^{-1} higher than the control group; the LPS/SLPI-H group was 11 mm^{-1} lower than the LPS group (Fig. 2B). Trabecular bone thickness showed no significant difference in each experimental group, but all experimental groups were lower than the control group (Fig. 2C). The trabecular bone number of the LPS group was 2 mm^{-1} lower than the control group; LPS/SLPI-L and -H group were 0.6 mm^{-1} and 2.0 mm^{-1} higher than the LPS

group, respectively, and that of LPS/SLPI-H was similar to that of the control (Fig. 2D). Trabecular bone space of the LPS group was 40.38 μm higher than the control; the LPS/SLPI-L group was 18.08 μm lower than the LPS group, and that of the LPS/SLPI-L was similar with that of the control (Fig. 2E). Trabecular bone structure model index (SMI) of the LPS and LPS/SLPI-L group were 0.6 and 0.7 higher than the control group, respectively, and that of LPS/SLPI-H was similar with the control (Fig. 2F). Trabecular bone pattern factors of the LPS and LPS/SLPI-L group was 7 mm^{-1} and 5 mm^{-1} higher than the control group, respectively, and that of LPS/SLPI-H was similar with the control (Fig. 2G).

III-3. Histological analysis of periodontal tissue after LPS or LPS/SLPI injection

1) Histological analysis in H&E stained tissue

In the left second molar of the maxilla of the control group, density of alveolar bone of root furcation was high, and periodontal ligament (PDL) of peripheral root (box) were observed to be dense (Fig. 3a). In alveolar bone of the control group, well-developed bone matrix and osteocyte in lacuna was observed. Marginal alveolar bone was attached by PDL composed of abundant collagen showing uniform direction (Fig. 3b). In the LPS group, the alveolar bone density of root furcation (box) was very low, and PDL of peripheral root (box) was observed to be loose (Fig. 3a^1). Intact bone matrix and osteocyte in lacuna was observed in alveolar bone, except in the marginal region. Osteocytes separated from bone matrix in interstitial space of PDL were observed in the marginal region with resorption of alveolar bone and destructed lacuna. Marginal PDL was dispersed very irregularly by destruction of attachment and the groups of cells similar to inflammatory cells; osteoclasts and fibroblasts were observed in that area (Fig. 3b^1). The bone density of the LPS/SLPI-L group was higher than the LPS group in alveolar bone of root furcation, and PDL of peripheral root was observed very densely in the LPS/SLPI-L group (Fig. 3a^2). Intact bone matrix and osteocytes in

lacuna were observed in alveolar bone, except that of the marginal region; the bone resorption area was little, and matrix of new bone partially was observed in marginal alveolar bone compared to the LPS group. The collagen fiber was redistributed in the area of PDL-destructed marginal alveolar bone observed in the LPS group; in addition, the groups of cells similar with inflammatory cells and osteoclasts markedly decreased (Fig. 3b^{2}). Alveolar bone of root furcation of the LPS/SLPI-H group (box) was markedly high in bone density compared to that of the LPS/SLPI-L group; PDL of peripheral root (box) was attached very densely to marginal alveolar bone (Fig. 3a^{3}). In the LPS/SLPI-H group, the region of bone resorption was little or observed partially in the area of marginal alveolar bone compared to that of the LPS/SLPI-L group; collagen of PDL was represented as similar to control group in attachment and uniform direction (Fig. 3b^{3}).

2) Immunohistochemical analysis of SLPI

SLPI protein was expressed in PDL between alveolar bone and root furcation (box) of the control group. SLPI was not expressed in alveolar bone matrix, but it was identified in several osteoblast and cells located in intercellular space of PDL. It was also expressed in junctional region between root and PDL fiber (Fig. 4Aa, b). The expression of SLPI in the LPS group was 1.5 times higher than that of control group in PDL between alveolar bone and root furcation (box). SLPI was expressed strongly in alveolar bone matrix, osteoblasts, dispersed collagen, and connective tissue cells in PDL compared to that of the control (Fig. 4Aa^{1}, b^{1}, B). The expression of SLPI in the LPS/SLPI-L group was 1.1 times higher than in the LPS group in PDL between alveolar bone and root furcation (box). SLPI was not expressed in alveolar bone matrix but expressed strongly in almost all cells in PDL compared to that of the LPS group. In addition, it was expressed weakly in part of collagen fibers in PDL compared to that of the LPS group (Fig. 4Aa^{2}, b^{2}, B). The expression of SLPI in the LPS/SLPI-H group was similar to the LPS group in PDL between alveolar bone and root furcation (box). The expression of SLPI was not observed in alveolar bone matrix, and it was weak in several cells

in marginal alveolar bone and cells of intercellular space in PDL (Fig. 4Aa^3, b^3).

3) Immunohistochemical analysis of TNF-α

TNF-α protein was rarely expressed in PDL between alveolar bone and root furcation (box) of the control group (Fig. 5Aa, b). The expression of TNF-α in the LPS group was 4.4 times higher than that of the control group in PDL between alveolar bone and root furcation (box). TNF-α was expressed strongly in collagen fibers attached to the marginal region of alveolar bone and the group of collagen and cells near the marginal region (Fig. 5Aa^1, b^1, B). The expression of TNF-α in the LPS/SLPI-L group was 0.3 times lower than LPS group in PDL between alveolar bone and root furcation (box). TNF-α was expressed weakly in several cells and collagen fibers in PDL compared to that of the LPS group (Fig. 5Aa^2, b^2, B). The expression of TNF-α in the LPS/SLPI-H group was 0.4 times lower than in the LPS group in PDL between alveolar bone and root furcation. In addition, TNF-α was expressed in collagen fibers partially attached to the marginal region (Fig. 5Aa^3, b^3, B).

4) Immunohistochemical analysis of IL-1β

IL-1β protein was rarely expressed in PDL between alveolar bone and root furcation (box) of the control group (Fig. 6Aa, b). The expression of IL-1β in the LPS group was 3.4 times higher than in the control group PDL between alveolar bone and root furcation (box). IL-1β was expressed in the entire PDL, and it was expressed strongly in collagen fibers attached to the margin of alveolar bone and group of cell (Fig. 6Aa^1, b^1, B). The expression of IL-1β in the LPS/SLPI-L group was 0.3 times lower than the LPS group in PDL between alveolar bone and root furcation (box). IL-1β was expressed in a part of the marginal alveolar bone and peripheral collagen fibers and cells of marginal region (Fig. 6Aa^2, b^2, B). The expression of IL-1β in the LPS/SLPI-H group was similarly low to that of the control group in PDL between alveolar bone and root furcation (box) (Fig. 6Aa^3, b^3, B).

5) Immunohistochemical analysis of RANKL

RANKL protein was expressed weakly in PDL between alveolar bone and root furcation (box) of the control group. RANKL was expressed weakly in almost all cells in PDL (Fig. 7Aa, b). The expression of RANKL in the LPS group was 3.3 times higher than that of the control group in PDL between alveolar bone and root furcation (box). RANKL was expressed strongly in presenting cells and collagen fibers located in the resorbed region of alveolar bone (Fig. 7Aa[1], b[1], B). The expression of RANKL in the LPS/SLPI-L group was 0.2 times lower than in the LPS group in PDL between alveolar bone and root furcation (box). RANKL was expressed in cells and collagen fibers localized in intracellular space of PDL attached to marginal alveolar bone (Fig. 7Aa[2], b[2], B). The expression of RANKL in the LPS/SLPI-H group was 0.7 times lower than in the LPS group in PDL between alveolar bone and root furcation (box). RANKL was expressed weakly in cells of intercellular space and collagen fiber in PDL attached to marginal alveolar bone (Fig. 7Aa[3], b[3], B).

6) Immunohistochemical analysis of Runx2

Runx2 protein was expressed weakly in PDL between alveolar bone and root furcation (box). Runx2 was expressed in osteocytes and adjacent cells in alveolar bone (Fig. 8Aa, b). The expression of Runx2 in the LPS group was 0.8 times lower than that of the control group in PDL between alveolar bone and root furcation (box). Runx2 was expressed in several osteocytes in alveolar bone as well as osteocytes in the region of destructed bone matrix (Fig. 8Aa[1], b[1], B). The expression of Runx2 in the LPS/SLPI-L group was 6.4 times higher than in the LPS group in PDL between alveolar bone and root furcation (box). Runx2 was expressed strongly in osteocytes in alveolar bone and resorbed bone matrix; in addition, it was expressed in several cells of PDL (Fig. 8Aa[2], b[2], B). The expression of Runx2 in the LPS/SLPI-H group was 7.3 times higher than in the LPS group in PDL between alveolar bone and root furcation (box). Runx2 was expressed strongly in osteocytes of alveolar bone and cells attached to marginal

alveolar bone (Fig. 8Aa[3], b[3], B).

III-4. mRNA expression of SLPI, TNF-α, IL-1β, RANKL, OPG, and Runx2 in LPS- or LPS/SLPI-treated MC3T3-E1 cells

In LPS-treated MC3T3-E1 cells (MC3T3-E1/LPS) and MC3T3-E1 cells treated with SLPI after LPS treatment (MC3T3-E1/LPS/SLPI), the expression of SLPI in MC3T3-E1/LPS/SLPI was 1.3 times higher at 2 h and 4 h, and 1.1 times higher at 12 h than that of MC3T3-E1/LPS; furthermore, it was strong until 24 h. TNF-α expression in MC3T3-E1/LPS/SLPI was 0.3 times lower than MC3T3-E1/LPS at 2 h, and it was decreased remarkably at 4 h. IL-1β expression in MC3T3-E1/LPS/SLPI was 0.7 and 0.6 times lower than MC3T3-E1/LPS at 4 h and 6 h, respectively, and it was expressed weakly until 24 h. RANKL expression in MC3T3-E1/LPS/SLPI was 0.5 and 0.7 times lower than MC3T3-E1/LPS at 2 h and 4 h, respectively, and it was expressed weakly until 24 h. OPG expression in MC3T3-E1/LPS/SLPI was 2.2 and 3.0 times higher than MC3T3-E1/LPS at 2 h and 4 h, respectively and it was expressed strongly until 6 h. Runx2 expression in MC3T3-E1/LPS/SLPI was 1.7 and 1.5 times higher than MC3T3-E1/LPS at 4 h and 6 h, respectively, and Runx2 expression was increased time dependently (Fig. 9 A - C).

III-5. Protein expression of SLPI, TNF-α, IL-1β, RANKL, OPG, and Runx2 in LPS- or LPS/SLPI-treated MC3T3-E1 cells

In LPS-treated MC3T3-E1 cells (MC3T3-E1/LPS) and MC3T3-E1 cells treated with SLPI after LPS treatment (MC3T3-E1/LPS/SLPI), the expression of SLPI in MC3T3-E1/LPS/SLPI was 1.3 times higher than that of MC3T3-E1/LPS at 2 h, 4 h, and 24 h. TNF-α expression in MC3T3-E1/LPS/SLPI was 0.7 and 0.9 times

lower than MC3T3-E1/LPS. IL-1β expression in MC3T3-E1/LPS/SLPI was 0.7, 0.6, 0.5, and 0.6 times lower than MC3T3-E1/LPS at 4 h, 6 h, 12 h, and 24 h, respectively. RANKL expression in MC3T3-E1/LPS/SLPI was 0.9 times lower than MC3T3-E1/LPS at 2 h, 4 h, and 6 h as well as 0.6 and 0.2 times lower at 12 h and 24 h, respectively. OPG expression in MC3T3-E1/LPS/SLPI was 1.3, 4, 3.4, 2.7, and 10 times higher than MC3T3-E1/LPS at 2 h, 4 h, 6 h, 12 h, and 24 h, respectively. Runx2 expression in MC3T3-E1/LPS/SLPI was 6.2, 9.9, 39, and 6.9 times higher than MC3T3-E1/LPS at 4 h, 6 h, 12 h, and 24 h, respectively (Fig. 10 A - C).

III-6. Diagram on the possible role of SLPI in periodontitis in rat

Increased TNF-α, IL-1β, and RANKL secreted from LPS-stimulated preosteoblast differentiates mature osteoclast into active form osteoclast, and decreased OPG and Runx2 inhibits osteoblast function associated with bone formation. SLPI inhibits the secretion of TNF-α and IL-1β, which reduces the inflammation and RANKL to suppress the formation of active osteoclast and increases OPG and Runx2 to stimulate osteoblast function in LPS-stimulated preosteoblast (Fig. 11).

Ⅳ. DISCUSSION

Periodontitis is the chronic inflammation of the structure attached in tooth such as alveolar bone, PDL and gingival connective tissue. Periodontitis is initialized and prolonged by mainly colonized gram-negative anaerobic or microaerobic bacteria in subgingival area. In addition, the main characteristic of periodontitis is destruction of periodontal tissue and the most common bacterial cause is *Porphyromonas gingivalis.* LPS is a main component of outer cell membrane in this bacterium, which is a toxin that induces inflammatory response. LPS is known as the pathogen of periodontitis and induces the production of inflammatory cytokines such as TNF-α, IL-8, and IL-6 in monocytes or macrophages. These cytokines induce lesions in periodontal tissue. Baicalin is one of the molecules effective for inhibition or treatment of alveolar bone resorption by periodontitis, which is purified from Scutellaria baicalensis, known as the medicinal plant. Baicalin has been reported to suppress the secretion of inflammatory response and increase of bone resorption factors such as TNF-α, IL-1β, and IL-6 (Lin et al., 1996).

Alveolar bone resorption in LPS-injected rat periodontal tissue was revealed in Micro-CT study through the highly resorption of bone surface and trabecular bone space with reduction of bone density and trabecular bone number (Chen et al., 2008). In this study, Micro-CT analysis for the LPS-induced periodontitis tissue showed results identical to the previous study. In addition, the reduction of alveolar bone resorption has been reported in a baicalin (0.1 and 1 mg/ml)-injected group after LPS injection, which is confirmed by raised bone density and trabecular bone number and narrowed trabecular bone space compared to LPS-injected group (Chen et al., 2008). Similarly, the present study also identified the reduction of alveolar bone resorption by SLPI in the LPS/SLPI group compared to that of the LPS group by lowered surface resorption of alveolar bone and narrowed trabecular bone space and raised trabecular bone number as well as closed to plate-like structure of trabecular bone. Therefore, SLPI may be a molecule that inhibits alveolar bone

resorption in LPS-induced periodontitis of rat.

Simvastatin is the medicine for reduction of cholesterol concentration in blood and has been reported to inhibit the expression of factors associated with osteoclastogenesis in rat with disturbances of metabolism, such as obesity. In the histological analysis using H&E staining, alveolar bone resorption and infiltration of leukocyte are inhibited markedly in LPS/simvastatin group compared to in LPS group of LPS-induced alveolar bone resorption in fat or lean rat (Jin et al., 2014). In the present study, the inflammatory cells were reduced as well as reduction of alveolar bone resorption in LPS/SLPI group compared to that of LPS group. Therefore, SLPI may reduce the alveolar bone resorption, through it suppresses the expression of factors associated with periodontal inflammatory response and osteoclastogenesis.

Human SLPI is a non-glycosylated protein, 11.7 KDa molecular weights found in epithelium of mucosal surface. SLPI protects tissues from secreted protease in the inflammatory region and it has been reported that it has a function for suppression of HIV infection, anti-bacterial and anti-fungal functions, as well as promotion of wound healing and cell proliferation (Williams et al., 2006; Hiemstra, 2002). SLPI production is increased from LPS-stimulated macrophages and inhibits activity of proinflammatory-related molecules such as NF-κB and nitric oxide (NO) induced by LPS-stimulated macrophages (Jin et al., 1998; Son et al., 2007). In odontoblasts, temporal increase in expression of SLPI by LPS stimulation inhibits the NF-κB activation to induce the expression of proinflammatory cytokines (Choi et al., 2009). SLPI mRNA expression is increased in peritoneal macrophages of streptococcal cell wall (SCW)-induced inflammatory polyarthritis rat and increase of SLPI protein expression is also identified in the arthritic area compared to that of control through immunohistochemical analysis. In addition, the result of radiographic analysis showed that edema is significantly reduced in an SCW/SLPI (0.1 mg) group compared with an SCW group. Thus, this study suggested that SLPI will be available to use for the therapy on the various inflammatory destructive diseases (Song et al., 1999). In this study, from the result of immunohistochemistry, SLPI protein expression of the LPS group was also increased compared to that of the

control group, and that of the LPS/SLPI-L group was more increased compared to that of LPS group in LPS-induced periodontitis tissue. In addition, mRNA and protein expression of SLPI were more increased in MC3T3-E1/LPS/SLPI than MC3T3-E1/LPS. Therefore, SLPI may suppress the inflammatory response in LPS-induced inflammatory periodontal tissue and osteoblast.

TNF-α, mainly secreted from activated macrophage, is a cytokine associated with systemic inflammation and stimulates the acute phases inflammatory response. TNF-α can increase the production of SLPI from epithelial cells, and increased SLPI inhibits TNF-α secretion of macrophages (Sallenave et al., 1994; Song et al., 1999). In addition, SLPI decreases the expression of TNF-α by inhibition of NF-κB activation in LPS-treated monocytes (Lentsch et al., 1999; Taggart et al., 2002). LPS increases the expression of mRNA and protein secretion of TNF-α and IL-1β in osteoblast (Die et al., 2012). Increased production of TNF-α and IL-1β induces bone resorption by osteoclasts through the stimulation of RANKL production, which is key mediator of osteoclast differentiation (Yamano et al., 2010). The destruction of alveolar bone is accelerated by injection of IL-1β protein into the ligature-induced periodontitis region and supplementation of antagonists for IL-1β and TNF-α in the infectious periodontitis region suppress the alveolar bone resorption (Koide et al., 1995; Assuma et al., 1998). Bovine lactoferrin (bLF) is known to regulate the production of proinflammatory cytokines including TNF-α and alveolar bone destruction associated with periodontitis. A bLF suppresses the LPS-induced TNF-α production by inhibition of NF-κB binding to the TNF-α promoter (Haversen et al., 2002). TNF-α mRNA expression is decreased in LPS-stimulated ST2 cells by bLF treatment; TNF-α protein expression is also decreased in bLF-treated gingival tissue and PDL region compared to that of LPS-treated group. In addition, a bLF inhibits alveolar bone resorption by reduction of TNF-α production from macrophages (Yamano et al., 2010). Similar to previous studies, in the present study, TNF-α and IL-1β protein expression was significantly decreased in cells of intercellular space in PDL and collagen fibers in the LPS/SLPI group compared to that of the LPS group. In addition, mRNA and protein expression of TNF-α and IL-1β were significantly decreased in

MC3T3-E1/LPS/SLPI compared to that in MC3T3-E1/LPS. Therefore, SLPI may suppress the inflammation of periodontal tissue through reduction of TNF-α and IL-1β production by inhibition of NF-κB activation in LPS-stimulated osteoblasts.

RANKL belong to TNF-α family member that directly induces the osteoclastogenesis through binding to RANK of osteoclast precursor cell, and it increases bone resorption by activation of signal transduction pathway leading to maturation of osteoclast (Kong et al., 1999; Taubman et al., 2005; Jimi et al., 1999). Pathogen-associated molecular patterns (PAMPs), including LPS bound to toll-like receptors (TLRs) on surface of inflammatory cell, activate intercellular NF-κB. Activated NF-κB acts as the transcription factor and induces the expression of proinflammatory cytokines including RANKL (Matsumoto et al., 2012). RANKL and TNF-α expression are promoted in LPS-stimulated osteoblast through the activation of NF-κB (Inubushi et al., 2012). Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 secreted from LPS-stimulated macrophage increase RANKL expression in osteoblasts (Taubman et al., 2005). In addition, LPS can directly increase RANKL mRNA expression in osteoblasts (Tang et al., 2011). SLPI suppresses cartilage resorption and bone destruction by acute and chronic arthritis through remarkable reduction of blood TNF-α concentration and NF-κB activation in joint inflammation of inflammatory polyarthritis region (Song et al., 1999). Immunohistochemical analysis on the periodontal tissue of ligature-induced periodontitis in rat after cammabidiol (CBD) injection identified that RANKL protein expression is increased in a ligature group compared to that of a control, and it decreased in a ligature/CBD group compared to that of the ligature group (Napimoga et al., 2009). In this study, RANKL protein expression of the LPS/SLPI group was also significantly decreased compared to that of the cells of intercellular space and collagen fiber in PDL attached to marginal alveolar bone in the LPS group. In addition, expression of RANKL mRNA and protein was remarkably decreased in MC3T3-E1/LPS/SLPI compared to that in MC3T3-E1/LPS. Therefore, SLPI may lead to the reduction of bone resorption through decrease of RANKL expression by reducing TNF-α and IL-1β expression or directly decreasing RANKL in LPS-stimulated osteoblast.

OPG is one of the TNF receptor family, which is a key molecule involved in bone reconstruction. OPG is produced in osteoblasts, and it inhibits the osteoclastogenesis by block the binding of interaction between RANK and RANKL in haemopoietic precursor cells (Simonet at al., 1997). OPG mRNA expression is remarkably decreased in LPS-treated ST2 cells and is significantly increased by bLF treatment (Yamano et al., 2010). In OPG-deficient mice, the porosity of trabecular and compact bone are severely increased as well as osteoclastic bone resorptions. This suggests that OPG is the physiological regulator of bone resorption and that balance in expression between RANKL and OPG in bone is very important for regulation of bone resorption (Yano et al., 2003; Mizuno et al., 1998; Suda et al., 2004). On the contrary, in OPG overexpressed mice, the result of radiograph and histological analysis demonstrated that bone density of long bone is increased clearly compared to a control, and osteosclerosis was induced. Therefore, OPG can regulate the bone density locally and systemically by regulation of osteoclast maturation (Simonet et al., 1997). In this study, OPG mRNA and protein expression were increased significantly in MC3T3-E1/LPS/SLPI compared to in the control. Therefore, SLPI may suppress the alveolar bone resorption through inhibition of osteoclast activation by increase of OPG expression to block the RANK - RANKL binding interaction in LPS-stimulated osteoblast.

Runx2 is a major transcription factor to regulate bone development and differentiation, and stimulates the expression of bone matrix components such as bone sialoprotein (BSP), osteocalcin (OCN), and collagen type I (Col I) and is essential for osteoblast differentiation and formation of bone matrix (Komori, 2011; Banerjee et al., 1997). Runx2 as a key molecule is expressed in mesenchymal cell to differentiate into osteoblast precursor cell at earliest stage in bone development. Runx2-deficient mice present deficiency of bone formation (Komori et al., 1997; 2010). LPS strongly suppresses essential transcription factors such as Sp7 and ATF4, including Runx2, for osteoblast differentiation (Bandow et al., 2010). LPS markedly decreases Runx2 mRNA expression caused by the hypermethylation of the Runx2 gene promoter region in human PDL fibroblast; however, treatment of 5Aza as an inhibitor for methylation increases the Runx2 mRNA expression

compared to that of the LPS group (Uehara et al., 2014). The expression of SLPI is identified at cap to bell stage during tooth development. In addition, treatment of SLPI significantly increases BSP, OCN, and Col I regulated by Runx2 to promote differentiation and mineralization of secreted matrix during odontoblast differentiation (Jeong et al., 2015). In the present study, Runx2 protein expression in LPS group was decreased markedly compared to that of osteocytes in alveolar bone; in addition, cells' intercellular space in PDL in the control group was increased significantly in the LPS/SLPI group compared to in the LPS group. In addition, Runx2 mRNA and protein expression were increased significantly in MC3T3-E1/LPS/SLPI compared to that of MC3T3-E1/LPS. Therefore, SLPI increases the Runx2 expression in osteocyte and osteoblast in alveolar bone, which leads to bone formation through osteoblast differentiation and bone matrix synthesis in LPS-induced periodontal inflammatory tissue.

In summary, the results of Micro-CT and histological analysis showed that SLPI inhibits periodontitis and decreases alveolar bone resorption in LPS-induced rat. The expression of TNF-α, IL-1β, and RANKL is decreased, and that of SLPI, OPG, and Runx2 are increased in periodontal tissue of LPS/SLPI group on the immunohistochemical analysis and in MC3T3-E1/LPS/SLPI. Therefore, SLPI might be the signal molecule that can inhibit the osteoclast activity and induce bone formation through the inhibition of osteoclast activation and increase of osteoblast activation. Based on the present results, further study will be needed on how directly SLPI regulates the molecules associated with differentiation of osteoblast and osteoclast.

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FIGURE LEGENDS

Figure 1. Micro-CT images of the Alveolar bone resorption in LPS- or LPS/SLPI-injected periodontal tissue.

The resorption of alveolar bone surface and root furcation was observed weakly in the LPS/SLPI group according to dose dependent of SLPI compared to that of the LPS group. 1, first molar; 2, second molar; 3, third molar; M, mesial; D, distal; L, left; R, right. All scale bars: 1 mm

Figure 2. The parameters of ROI analysis.

(A) - (G) The bone volume and trabecular bone number were high in LPS/SLPI-H group compared to those of the LPS group, and ratio of surface area to bone volume, trabecular bone space, and trabecular bone structure model index were low. (*$p<0.05$, **$p<0.01$)

Figure 3. Histological change in LPS- or LPS/SLPI-injected periodontal tissue.

Alveolar bone resorption and PDL destruction were decreased markedly in the LPS/SLPI-H group compared to the LPS group. Con, control; Scale bars: 500 μm (a - a^3), 50 μm (b - b^3)

Figure 4. SLPI expression in LPS- or LPS/SLPI-injected periodontal tissue.

(A)(B) SLPI expression in alveolar bone and PDL tissue was high in the LPS/SLPI-L group compared to that of the LPS group. Con, control; Scale bars: 500 μm (A, a - a^3), 50 μm (A, b - b^3); (**$p<0.01$)

Figure 5. TNF-α expression in LPS- orLPS/SLPI-injected periodontal tissue.

(A)(B) TNF-α expression in cells of intercellular space in PDL and collagen fibers was high in the LPS group compared to that of the control and it was low in the LPS/SLPI group according to dose dependent of SLPI compared to that of LPS group. Con, control; Scale bars: 500 μm (A, a - a^3), 50 μm (A, b - b^3); (**$p<0.01$)

Figure 6. IL-1β expression in LPS- or LPS/SLPI-injected periodontal tissue.
(A)(B) IL-1β expression in cells of intercellular space and collagen fiber in PDL attached to marginal alveolar bone was high in the LPS group compared to that of the control, and it was low in the LPS/SLPI group according to dose dependent of SLPI compared to that of the LPS group. Con, control; Scale bars: 500 μm (A, a - a^3), 50 μm (A, b - b^3); (**p<0.01)

Figure 7. RANKL expression in LPS- or LPS/SLPI-injected periodontal tissue.
(A)(B) RANKL expression in cells of intercellular space and collagen fiber in PDL attached to marginal alveolar bone was high in the LPS group compared to that of the control, and it was low in the LPS/SLPI group according to dose dependent of SLPI compared to that of the LPS group. Con, control; Scale bars: 500 μm (A, a - a^3), 50 μm (A, b - b^3); (**p<0.01)

Figure 8. Runx2 expression in LPS- or LPS/SLPI-injected periodontal tissue.
(A) Runx2 expression in osteocytes of alveolar bone and cells in PDL was low in the LPS group compared to that of the control, and it was high in the LPS/SLPI group according to dose of SLPI compared to that of the LPS group. (B) Runx2 expression and positive cells were high in the LPS/SLPI group according to dose dependent of SLPI compared to that of the LPS group. Con, control; Scale bars: 500 μm (A, a - a^3), 50 μm (A, b - b^3); (**p<0.01)

Figure 9. The mRNA expression of SLPI, TNF-α, IL-1β, RANKL, OPG, and Runx2 in LPS- or LPS/SLPI-treated MC3T3-E1 cells.
(A)(B) The expression of TNF-α, IL-1β, and RANKL was decreased in MC3T3-E1/LPS/SLPI compared to that of MC3T3-E1/LPS, and SLPI, OPG and Runx2 were increased. (C) Quantification of the mRNA expression was calculated from result of (A) and (B). C, control; (*p<0.05, **p<0.01)

Figure 10. The protein expression of SLPI, TNF-α, IL-1β, RANKL, OPG, and Runx2 in LPS- or LPS/SLPI-treated MC3T3-E1 cells.

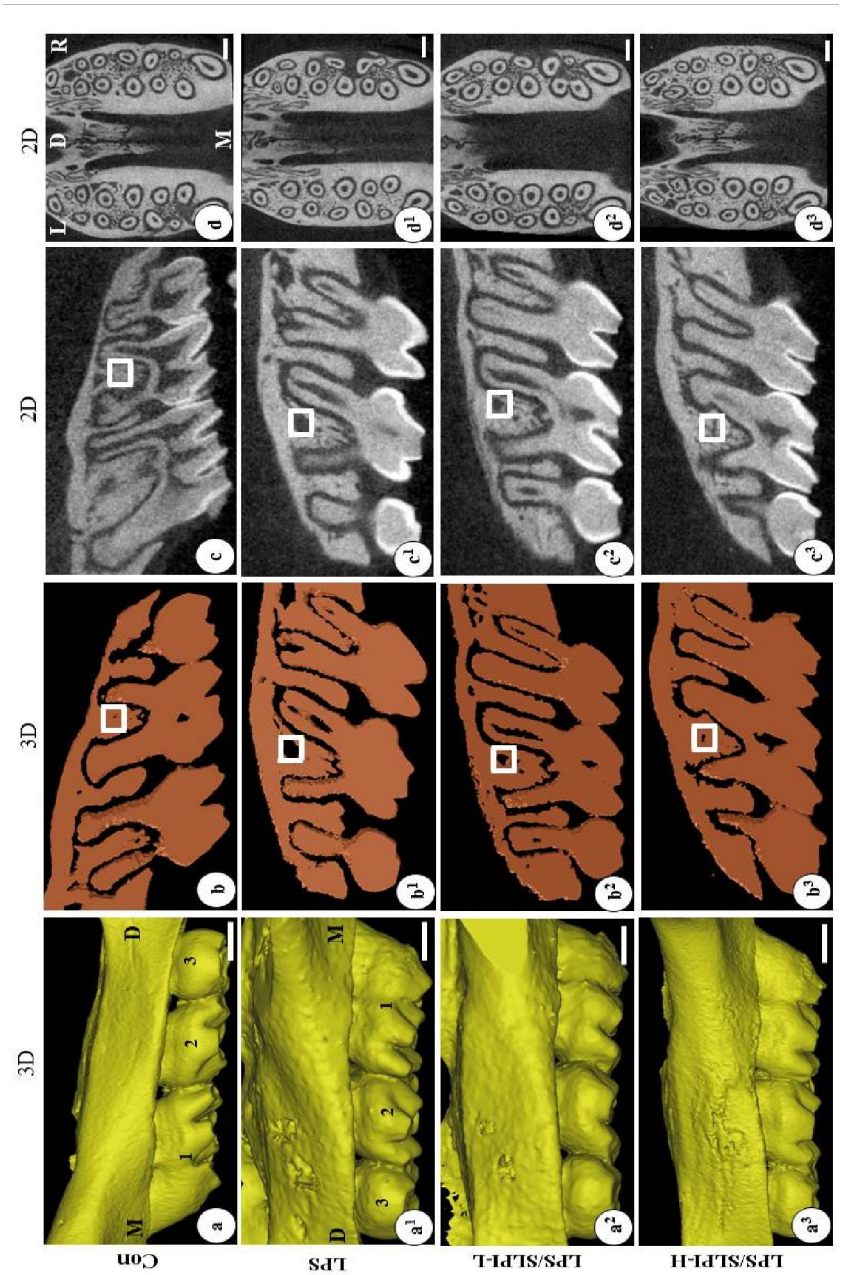
(A)(B) The expression of TNF-α, IL-1β, and RANKL was decreased in MC3T3-E1/LPS/SLPI compared to that of MC3T3-E1/LPS, and SLPI, OPG, and Runx2 were increased. (C) Quantification of the protein expression was calculated from result of (A) and (B). C, control; (*$p<0.05$, **$p<0.01$)

Figure 11. Diagram on the possible role of SLPI in periodontitis in rat.

LPS-stimulated preosteoblast increases the secretion of TNF-α, IL-1β, and RANKL. Increased TNF-α, IL-1β, and RANKL differentiates mature osteoclast into active form osteoclast, and decreased OPG and Runx2 inhibits osteoblast function. SLPI inhibits LPS-induced TNF-α, IL-1β, and RANKL secretion in preosteoblast, which leads to inhibition of the formation of active osteoclast, and increased OPG and Runx2 activates osteoblast function.

FIGURES

Figure 1.



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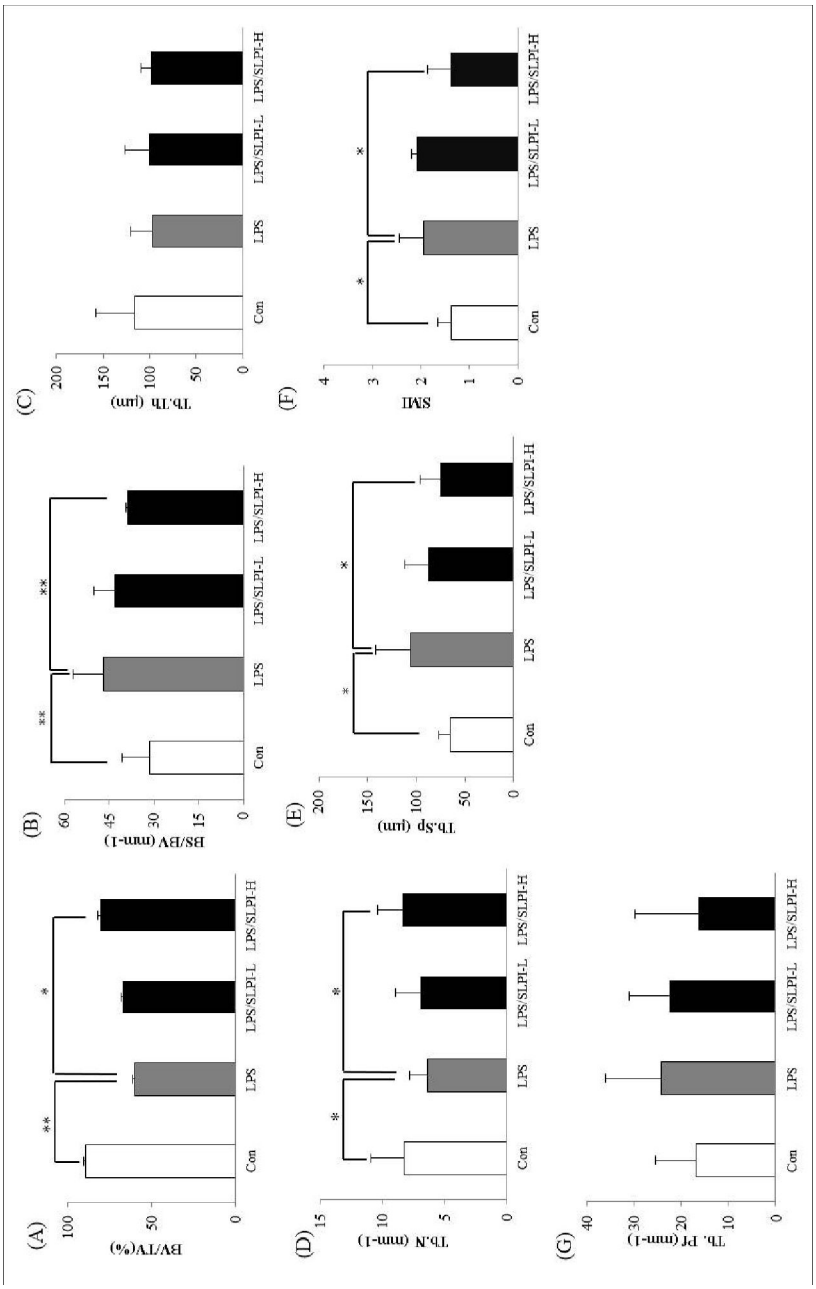

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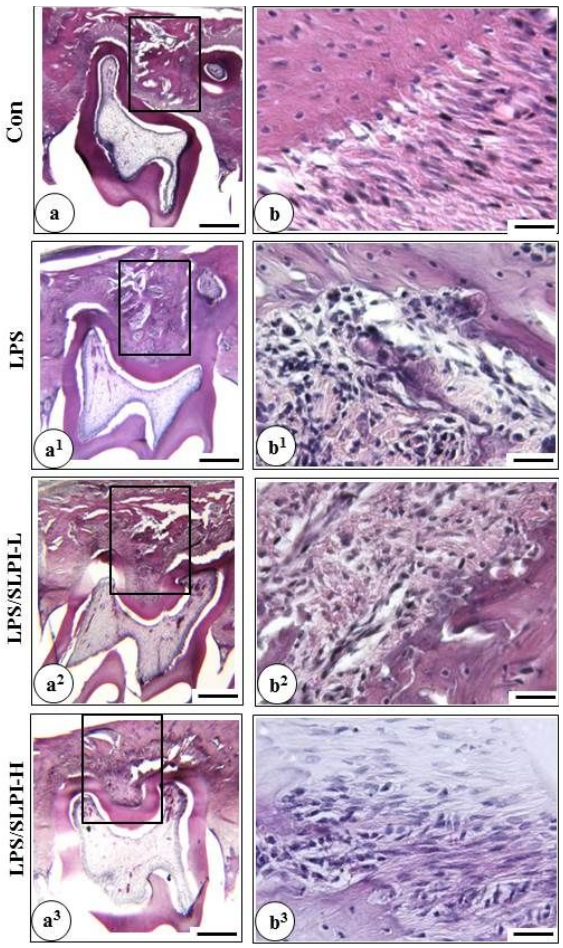

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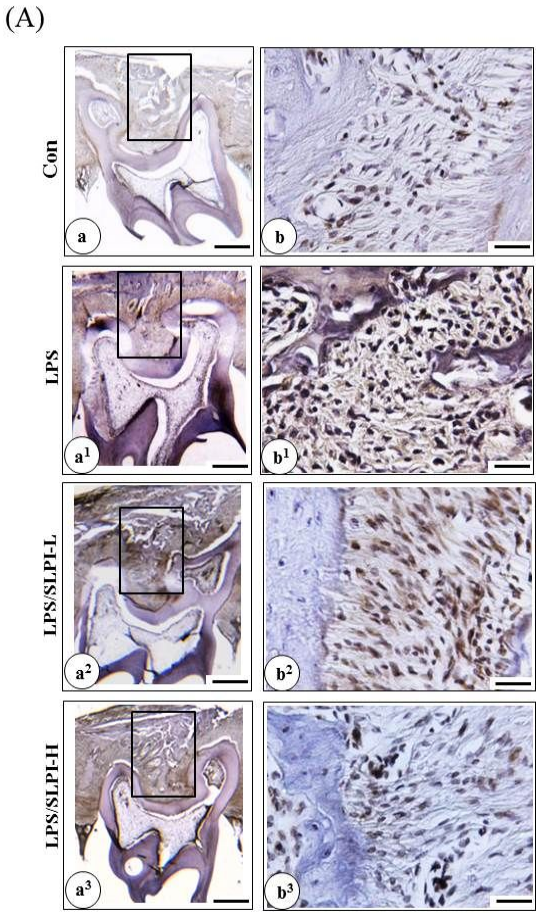

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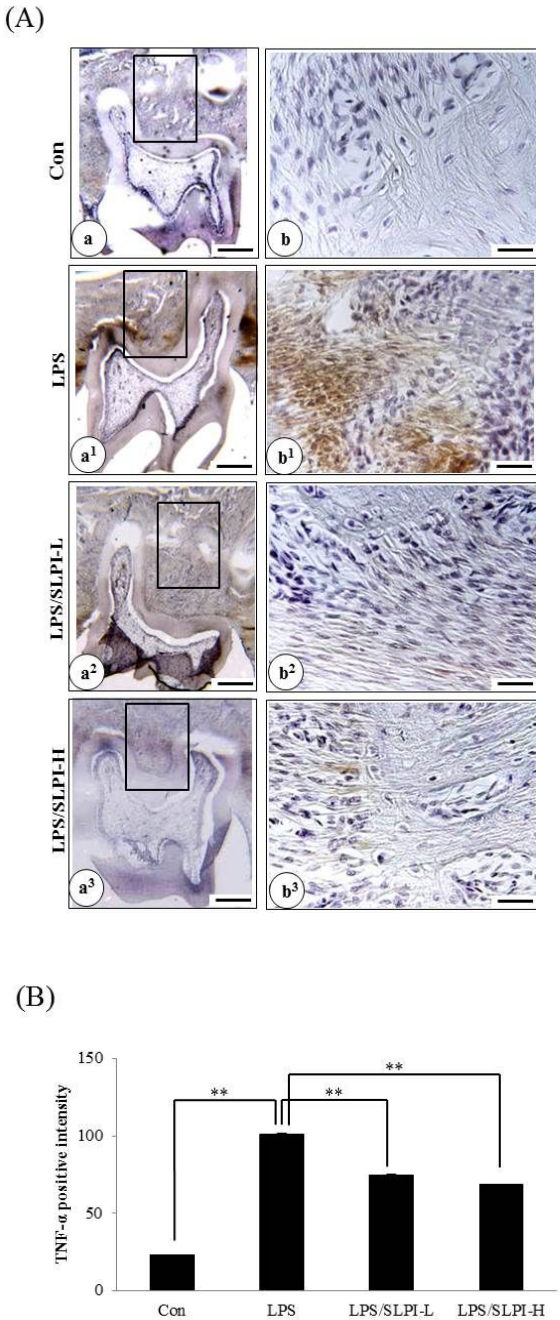

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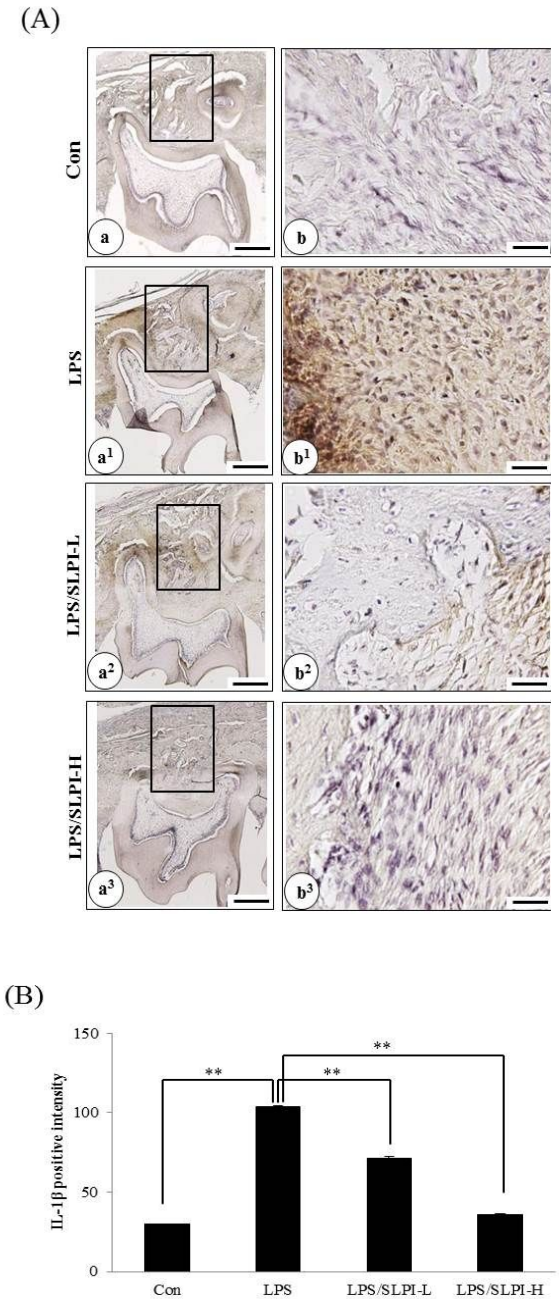

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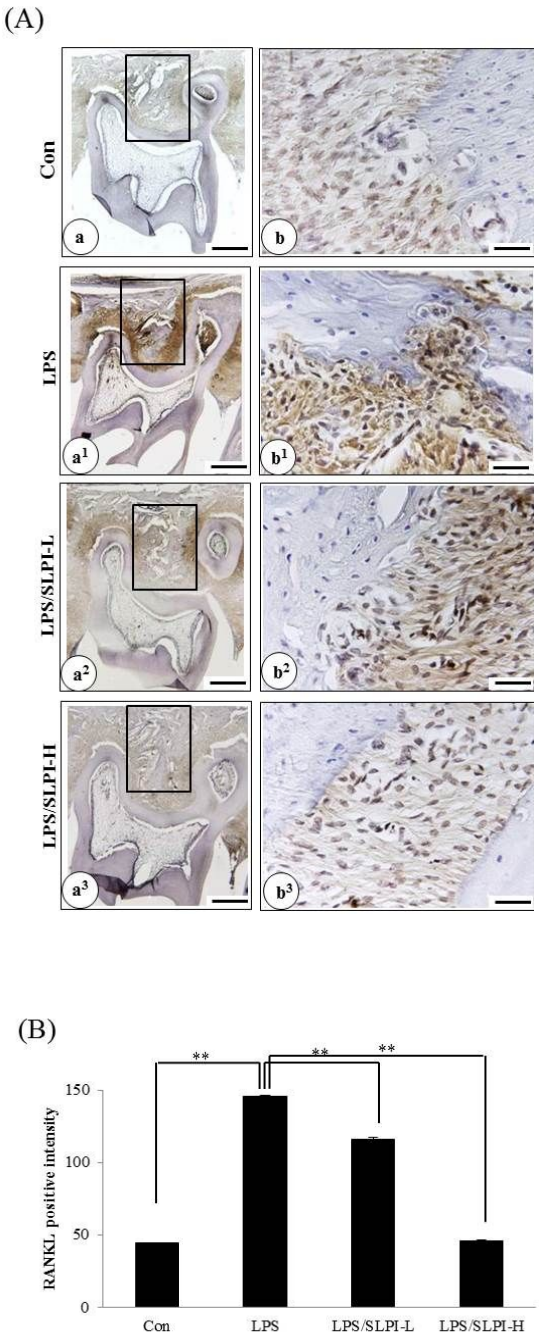

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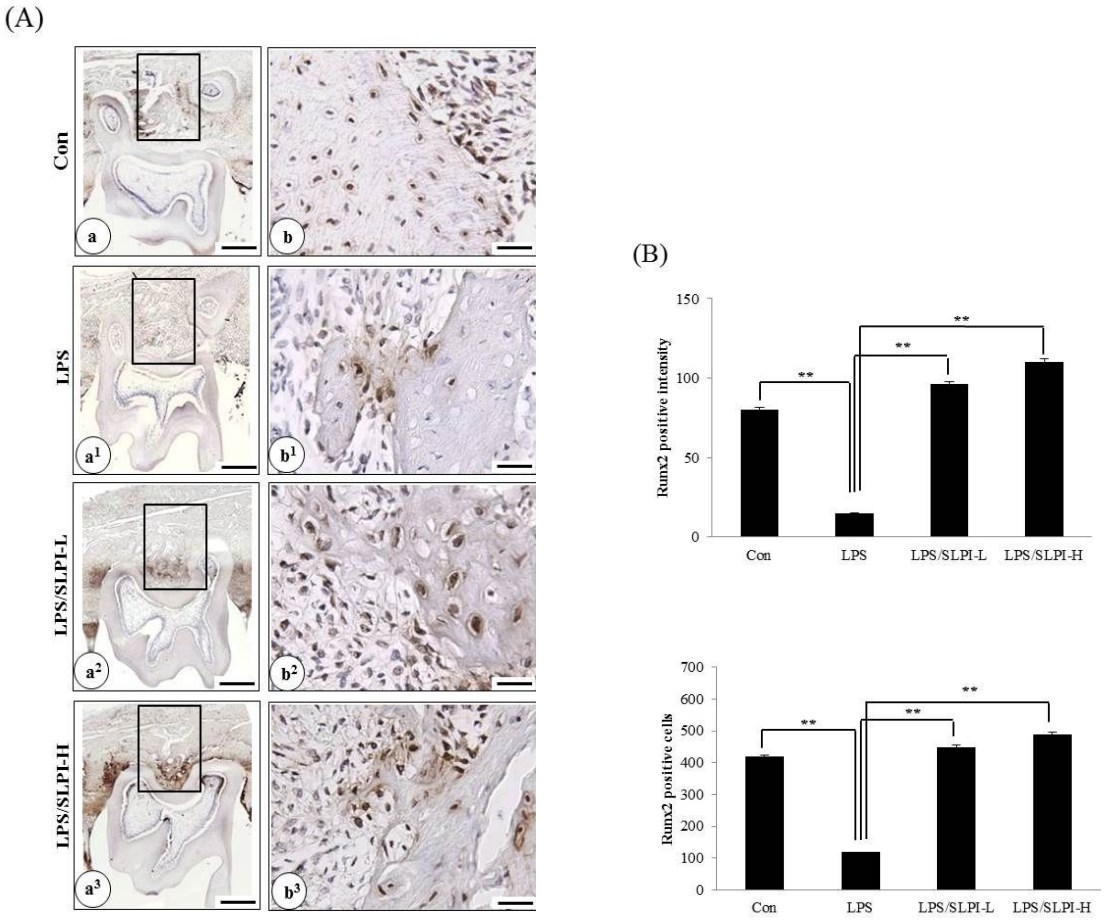

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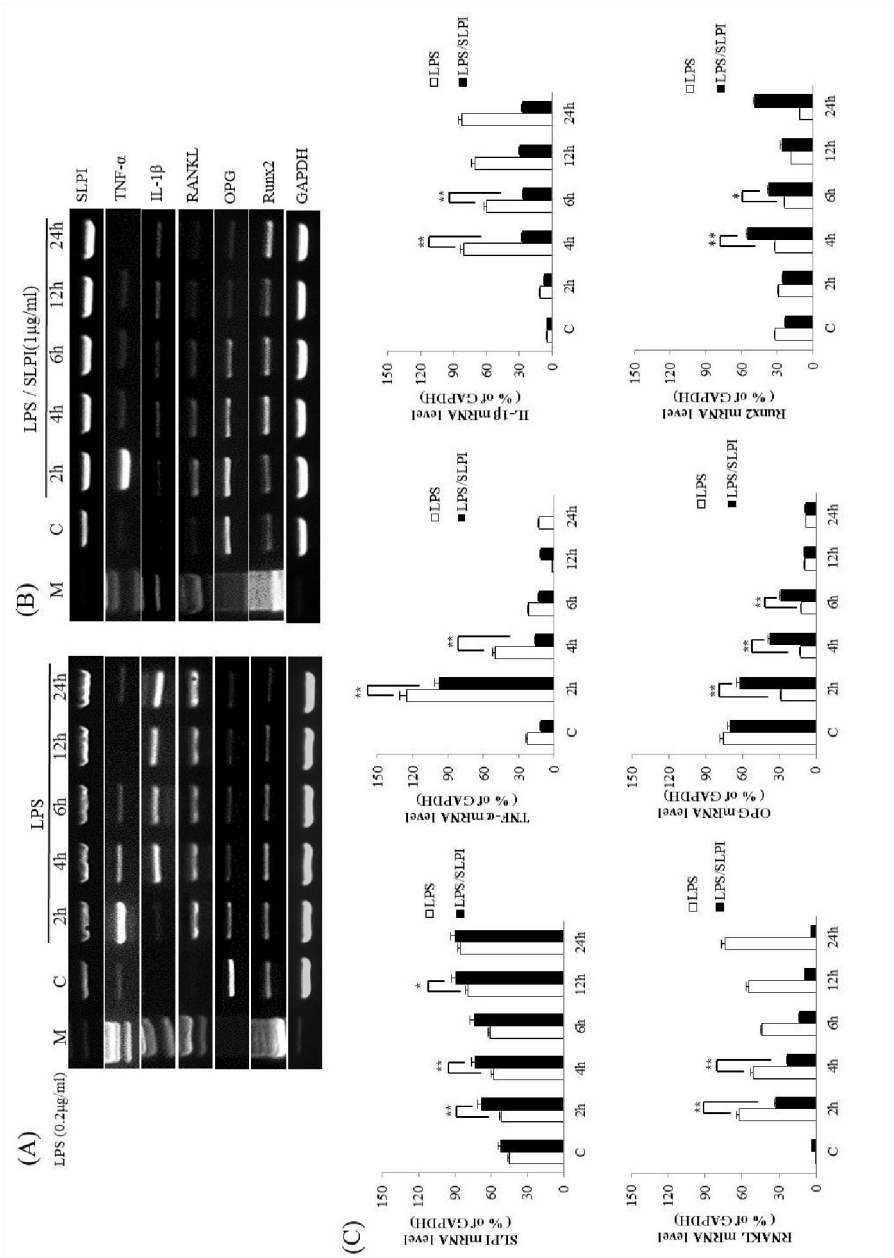

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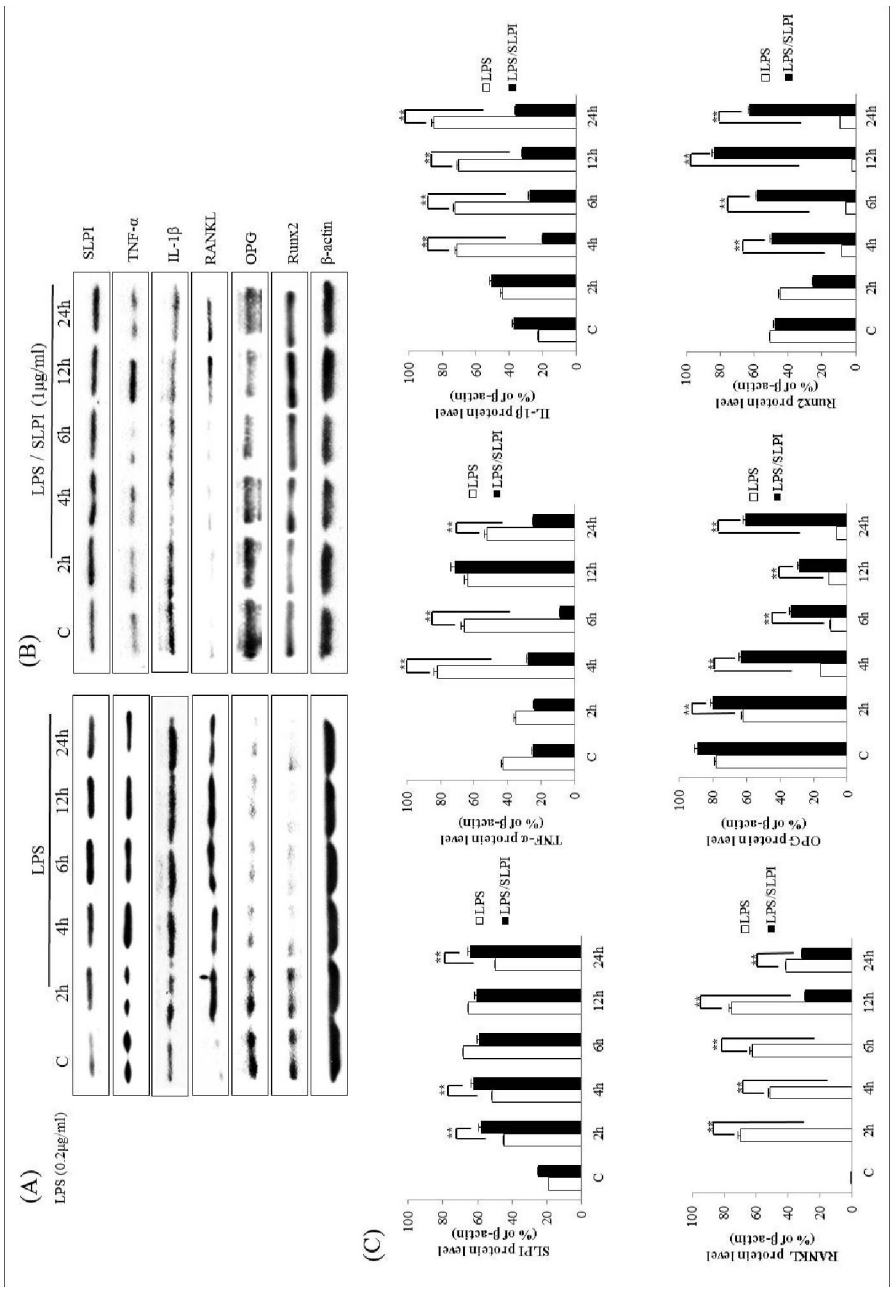

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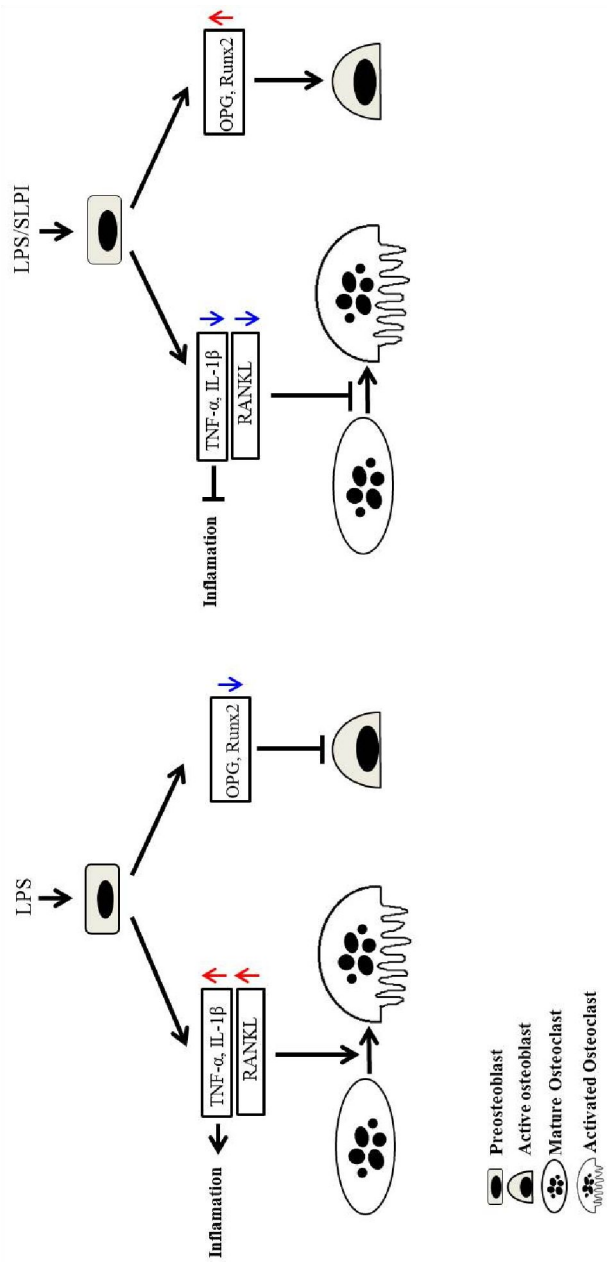

ABSTRACT

The inhibitory effect of secretory leukocyte protease inhibitor (SLPI) on the alveolar bone resorption with lipopolysaccharide (LPS)-induced periodontitis in rat

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Periodontitis is an infectious disease induced by gram-negative bacteria in dental plaque. Lipopolysaccharide (LPS), one of the cell wall components of gram-negative bacteria, induces the inflammatory response around tissue due to stimulation with inflammatory cells such as macrophage and thereby, it causes formation of periodontal pocket and alveolar bone resorption. Secretory leukocyte protease inhibitor in (SLPI) is known as anti-bacterial and anti-inflammatory cytokine inflammatory response. In addition, the expression of SLPI is increased by LPS as well as proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukine-1β (IL-1β) secreted from LPS-activated inflammatory cells. Increased SLPI by inflammatory response decreased arthritis and destruction of cartilage and bone through inhibition of TNF-α and nitric oxide secretion. Therefore, this study investigates whether SLPI inhibits inflammatory response and alveolar bone resorption in LPS-induced periodontitis.

In the SLPI-injected group after LPS injection (LPS/SLPI), the bone volume and trabecular bone number were higher, but the ratio of bone surface area to bone volume, trabecular bone space, and trabecular bone structure model index were

lower than those of the LPS-injected (LPS) group in analysis of the ROI parameter using micro-CT. The alveolar bone resorption in the LPS/SLPI group was decreased compared to that of the LPS group through histological analysis. In the LPS/SLPI group, protein expression of TNF-α, IL-1β and RANKL was decreased compared to that of the LPS group but SLPI, OPG, and Runx2 were increased in the tissues by immunohistochemical reaction. In LPS-treated MC3T3-E1 cells (MC3T3-E1/LPS), the mRNA and protein expression of TNF-α, IL-1β, and RANKL were increased compared to those of the control, but OPG and Runx2 were decreased. In LPS/SLPI-treated MC3T3-E1 cells (MC3T3-E1/LPS/SLPI), the mRNA and protein expression of TNF-α, IL-1β, and RANKL were decreased compared to those of MC3T3-E1/LPS but SLPI, OPG, and Runx2 were increased.

In summary, Micro-CT and histological analysis showed that SLPI inhibits inflammation and decreases alveolar bone resorption in LPS-induced periodontitis rat. The expression of TNF-α, IL-1β, and RANKL is decreased, and that of SLPI, OPG, and Runx2 is increased in periodontal tissue of LPS/SLPI group and in MC3T3-E1/LPS/SLPI. Therefore, SLPI might be the signal molecule that can inhibit osteoclast activity and induce bone formation through the inhibition of osteoclast activation and increase of osteoblast activation.

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끝으로 항상 저를 지지해주시고, 믿어주신 부모님께 진심으로 감사드리고, 동생에게도 고마운 마음을 전합니다.