

The effect of lipopolysaccharide on the migration of osteoclast precursors

Hee Young Lee¹, Dae-Sil Lee³, Jeong-Heon Cha², Yun-Jung Yoo^{2*}

1. Department of Oral Biology, College of Dentistry, Yonsei University

2. Department of Oral Biology, Brain Korea 21 Project and Oral Science Research Center, College of Dentistry, Yonsei University

3. Genome Research Center, Korea Institute of Bioscience & Biotechnology

I. INTRODUCTION

An osteoclast is a bone-resorbing cell which originates from hematopoietic precursors of the monocyte-macrophage lineage that reside within the bone marrow and peripheral circulation^{1,2}. Bone resorption is divided into two stages. The first stage is migration of osteoclast precursors (pre-OCs) to the bone surface from circulation and the second stage is differentiation of the pre-OCs into osteoclasts. The chemotaxis and the degradation of the basal lamina and the extracellular matrix play an important role in the migration of pre-OCs. Chemokines are the major regulators of the mobilization and homing of the cells to specific organs or tissues³. Chemokines can be divided into four classes

defined by the spacing of the first two conserved cysteine residues: C, CC, CXC, and CX3C. The chemokine for pre-OCs is stromal cell-derived factor 1 (SDF-1), which is CXC class⁴. SDF-1 is constitutively expressed in pre-osteoblastic stromal cells⁵. A receptor for SDF-1 is CXCR4, which is constitutively expressed on pre-OCs that can give rise to osteoclasts^{2,7}. SDF-1 recruits pre-OCs by inducing chemotaxis, expression of the matrix metalloproteinase (MMP)-9, and collagen transmigration^{2,6,7}.

The MMPs are a family of structurally and functionally related enzymes that are responsible for the proteolytic degradation of the extracellular matrix components⁸. These proteins can be classified into the following subgroups

*Corresponding author : Yun-Jung Yoo, Department of Oral Biology, Oral Science Research Center and Brain Korea 21 Project, College of Dentistry, Yonsei University, Shinchondong 134, Seodaemoongu, Seoul, 120-752, Korea (E-mail : yu618@yumc.yonsei.ac.kr)

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based on their substrate specificities and structural homologies: collagenase (MMP-1, -8, and -3), gelatinase (MMP-2, -9), stromelysin (MMP-3, -10, -11), and membrane-type MMPs (MMP-14, -17, -23, -24, -25). MMPs are involved in bone resorption which includes the initiation of bone resorption⁹⁾ and the migration of pre-OCs^{2,6)} and osteoclasts¹⁰⁾. Pre-OCs expressed MMP-9 and its expression was increased by SDF-1^{2,6)}. This suggests that SDF-1 increases recruitment of pre-OCs by up-regulation of MMP-9.

Periodontitis, which affects tissues that surround and support the teeth, is an inflammatory disorder and alveolar bone destruction is one of its clinical characteristics. Gram-negative bacteria such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Treponema* are representative periodontopathogens. Gram-negative bacteria contain lipopolysaccharide (LPS) in the outer membrane of their cell wall.

Therefore, an understanding of the role of LPS in alveolar bone destruction in periodontitis is important. LPS of *A. actinomycetemcomitans* induced osteoclastogenesis via induction of PGE₂ and IL-1¹¹⁾. LPS of *E. coli* stimulated osteoclastogenesis through enhancement of RANKL expression and suppression of OPG expression¹²⁾. Thus, although the mechanism of LPS to induce osteoclastogenesis is relatively well understood, very little is known about the effect of LPS on the migration of pre-OCs. Therefore, we observed the role of LPS of *E. coli* or *A. actinomycetemcomitans* in the migration of pre-OCs. LPS of each bacteria increased the migration of pre-OCs via enhancement of MMP-9 and SDF-1 α expression.

II. MATERIALS AND METHODS

1. Isolation of LPS

A. actinomycetemcomitans (ATCC29522) was cultured at 37°C in 10% CO₂ in brain heart infusion (BHI) medium for 2 days. Bacteria were harvested by centrifugation at 5000 \times g for 10 min, washed twice in phosphate buffered saline (PBS), and were resuspended in PBS. LPS was purified by use of a LPS Extraction Kit (Intron Biotechnology, USA) and suspended in a small volume of distilled water. After the LPS was heated at 90°C for 30 minutes, it was dried by lyophilization, and the weight was measured. The purified LPS preparation from *A. actinomycetemcomitans* showed a typical ladder-like band pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 14% polyacrylamide gel) after staining with silver nitrate (Amersham Biosciences, USA).

2. Culture of RAW 264.7 Cells

Mouse macrophage RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (GibcoBRL, USA) containing 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% antibiotic/antimycotic solution in 10 cm tissue culture dishes and were grown to confluence. The cells were then detached from the culture dish with trypsin-EDTA. The cells (1.5×10^5) were seeded into 3.5 cm dishes in 2 ml of DMEM containing 10% FBS. When the cells reached 80% confluence, the medium was changed to DMEM containing 0.1% bovine serum albumin (BSA, GibcoBRL, USA). After incubation for 12 h, the cells were exposed to

E.coli LPS (Sigma, USA) or *A. actinomycetemcomitans* LPS (0.1, 1, and 10 $\mu\text{g/ml}$). The mRNA was isolated from RAW cells by using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The expression of MMP-9 mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The levels of MMP-9 in culture supernatants were also measured using an enzyme linked immunosorbent assay (ELISA, R & D Systems, USA).

3. Culture of osteoblasts

The osteoblastic cells were isolated from the calvaria of 1 day ICR mice as previously described¹³. The calvaria were digested in 40 ml of α -minimum essential medium (α -MEM) containing 0.2% collagenase (Charlottesville Upstate, USA) and 0.1% dispase (GibcoBRL, CA, USA) for 20 min at 37°C with vigorous shaking and were then centrifuged at 1500 \times g for 5 min. The first supernatant was discarded, another 10 ml of the collagenase-dispase enzyme solution was added, and the preparation was incubated for 20 min. The digestion procedure was repeated four times, and the cells isolated by the last three digestions were combined. They were cultured in α -MEM containing 10% FBS and 1% antibiotic/antimycotic solution in 10 cm tissue culture dishes and grown to confluence. The cells were then detached from the culture dishes with trypsin-EDTA. The cells (1.5×10^5) were seeded into 3.5 cm dishes in 2 ml of α -MEM containing 10% FBS. When the cells had grown to 80% confluence, the medium was changed to DMEM containing 0.1% BSA. After culture for 12 h, the cells were exposed to

E.coli or *A. actinomycetemcomitans* LPS (0.1, 1, and 10 $\mu\text{g/ml}$). The mRNA was isolated from cells by using the TRIzol reagent. Expression of SDF-1 α mRNA was determined by RT-PCR. The levels of SDF-1 α in culture supernatants were also measured using an ELISA kit (R & D Systems, USA). The culture supernatants of osteoblasts treated with each bacterial LPS were used for the transmigration assay of the pre-OCs.

4. RT-PCR

The expression of MMP-9, SDF-1 α and β -actin mRNAs was determined by RT-PCR. Total RNA (1 μg) from non-treated or treated cells was used as a template for cDNA synthesis. cDNA synthesis was performed using an RT premix (Bioneer, Korea) according to the manufacturer's instruction. The RNA (1 μg) and oligo (dT) primer (1 mM) were denatured at 70°C for 5 min and incubated for 1 to 2 min on ice. The denatured RNA and oligo (dT) primer were added to RT premix and incubated at 42°C for 60 min, followed by 94°C for 5 min.

The cDNA (4 μl) was amplified by PCR in a 20 μl PCR premix (Bioneer, Korea). The following primer pairs were used: forward, 5'-CTGTCCAGACCAAGGGTACAGCCT-3'; reverse, 5'-GTGGTATAGTGGGACACATAGTGG-3' for mouse MMP-9; forward, 5'-CTGCATCAGTGACGGTAAACC-3'; reverse, 5'-CAGCCGTGCAACAATCT-GAAG-3' for mouse SDF-1 α ; forward, 5'-GGACTCCTATGGTGGGTGACGAGG-3'; reverse, 5'-GGGAGAGCATAGCCCTCGTAGAT-3' for mouse β -actin. For MMP-9, amplification was performed at 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min. For SDF-1 α , amplification was performed

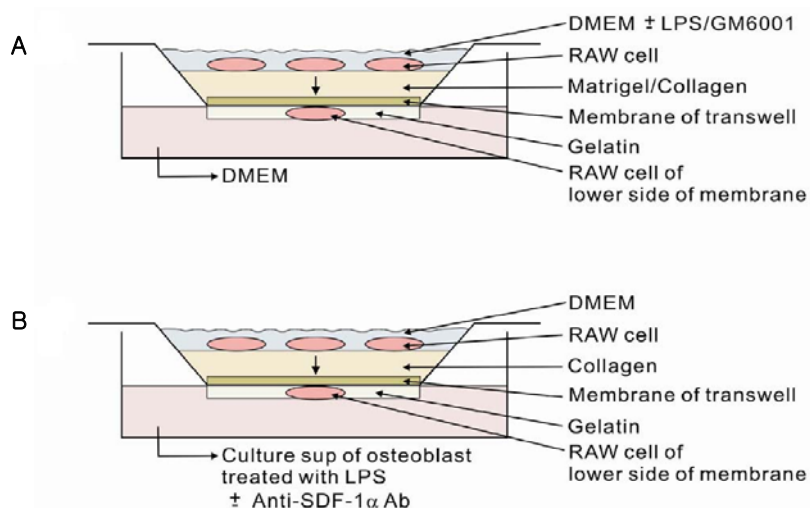


Figure 1. The method for migration of RAW cells treated with LPS through matrigel or collagen. For analyzing the involvement of MMPs in LPS-induced transmigration of pre-OCs, RAW cells were allowed to transmigrate for 16 h through porous transwell membrane pre-coated with matrigel or collagen, in the presence or absence of LPS and/or the general MMP inhibitor (GM6001) which was added to the upper chamber of the transwell (A). For analyzing the involvement of SDF-1 α in LPS-induced transmigration of pre-OC, RAW cells were allowed to transmigrate for 16 h through porous transwell membrane pre-coated with collagen in the presence or absence of culture supernatant of osteoblasts treated with LPS and/or anti-SDF-1 α antibody added to the lower chamber of the transwell (B).

at 95°C for 30 sec, 60°C for 5 sec, and 72°C for 27 sec. For β -actin, amplification was performed at 94°C for 1 min, 58°C for 30 sec, and 72°C for 1 min. Products were separated on a 1.5% agarose gel electrophoresis and were visualized by ethidium bromide staining. The relative intensities of gel bands were measured using Gel Doc (Alpha Innotech, USA) and MMP-9 and SDF-1 α signals were normalized to β -actin signals determined in parallel for each sample.

5. Transmigration assay of RAW cells

Transmigration assay using matrigel or type I collagen were performed using a transwell with 5- μ m-pore membrane (Nalgene, USA;

Figure 1). The upper side of the membrane was pre-coated with 10 μ l of 3 mg/ml matrigel (BD Bioscience, USA) or 150 μ l of 0.35 mg/ml type I collagen (Upstate, USA) and the lower side of the membrane was pre-coated with 20 μ l 1 mg/ml gelatin. For analyzing the involvement of MMPs in LPS-induced transmigration of pre-OCs, RAW cells (5×10^4) were resuspended in 100 μ l DMEM containing 0.1% BSA, added to the upper chamber, and each bacterial LPS (1 μ g/ml) and/or the general MMP inhibitor GM6001 (30 μ M) were added simultaneously to the upper chamber. DMEM (600 μ l) containing 0.1% BSA was added to the lower chamber. After 16 h of culture, cells on the membrane were methanol fixed and stained with hematoxylin. The cells on the upper side of the

membrane were removed. The number of cells that had transmigrated through the matrigel or collagen matrix to the lower side of the membrane was counted in a microscope in 4 fields. For analyzing the involvement of SDF-1 α in LPS-induced transmigration of pre-OCs, RAW

cells (5×10^4) were resuspended in DMEM containing 0.1% BSA, added to the upper chamber, and the culture supernatants of osteoblasts treated with LPS (1 $\mu\text{g}/\text{ml}$) and/or anti-SDF-1 α antibody (Ab, 20 $\mu\text{g}/\text{ml}$, R & D Systems, USA) were added to the lower chamber.

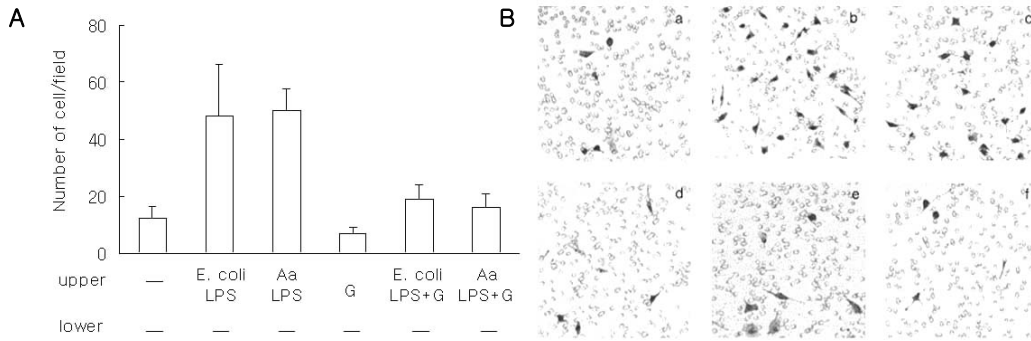


Figure 2. Migration of RAW cells treated with LPS through matrigel. RAW cells were allowed to transmigrate for 16 h through porous transwell membrane pre-coated with matrigel, in the presence or absence of *E. coli* LPS (1 $\mu\text{g}/\text{ml}$), *A. actinomycetemcomitans* LPS (Aa, 1 $\mu\text{g}/\text{ml}$), and/or the general MMP inhibitor GM6001 (G, 30 μM) added to the upper chamber of the transwells. After hematoxylin staining, the number of RAW cells that transmigrated through the transwell was counted (A). The RAW cells on lower side of membrane (x 200, B); non-treated culture (a), *E. coli* LPS (b), *Aa* LPS (c), GM6001 (d), *E. coli* LPS and GM6001 (e), *Aa* LPS and GM6001 (f).

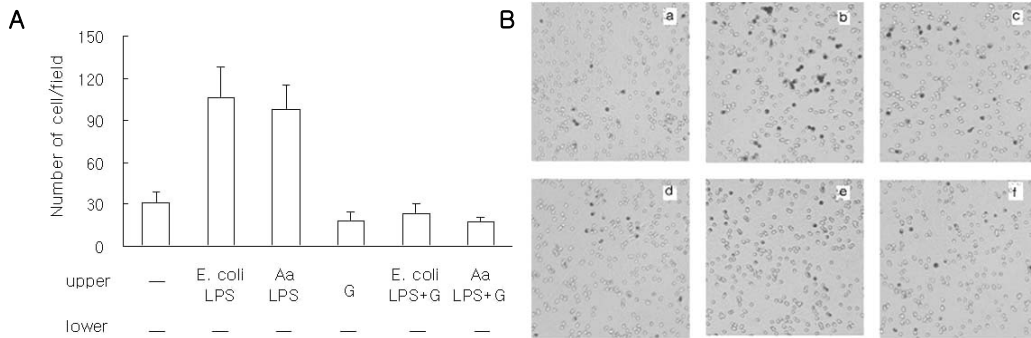


Figure 3. Migration of RAW cells treated with LPS through collagen. RAW cells were allowed to transmigrate for 16 h through the porous transwell membranes pre-coated with collagen, in the presence or absence of *E. coli* LPS (1 $\mu\text{g}/\text{ml}$), *A. actinomycetemcomitans* LPS (Aa, 1 $\mu\text{g}/\text{ml}$), and/or the general MMP inhibitor GM6001 (G, 30 μM) added to the upper chamber of the transwell. After hematoxylin staining, the number of RAW cells that transmigrated through transwells was counted (A). The RAW cells on lower side of membrane (x 200, B); non-treated cultures (a), *E. coli* LPS (b), *Aa* LPS (c), GM6001 (d), *E. coli* LPS and GM6001 (e), *Aa* LPS and GM6001 (f).

III. RESULTS

1. Transmigration of RAW cells treated with LPS

After the addition of each bacterial LPS and/or the general MMP inhibitor GM6001 to the upper chamber of transwell, RAW cells were allowed to transmigrate through the porous membrane of the transwell pre-coated with matrigel. The number of cells on the lower side of membrane, that had transmigrated through the matrigel, increased in the presence of *E. coli* or *A. actinomycetemcomitans* LPS (Figure 2). In the presence of each bacterial LPS and MMP inhibitor, the number of cells on the lower side decreased. When collagen coated-membranes were utilized, similar results were observed (Figure 3).

2. Expression of MMP-9 in RAW cells treated with LPS

In the RAW cells treated with either *E. coli* or *A. actinomycetemcomitans* LPS, MMP-9 mRNA expression was estimated. Treatment

with each bacterial LPS increased the expression of the MMP-9 mRNA and the expression of MMP-9 mRNA was markedly enhanced at a concentration of 1 $\mu\text{g/ml}$ (Figure 4A). The concentration of MMP-9 in the culture supernatant of cells was determined by ELISA. MMP-9 was detected in the absence and presence of LPS treatment (Figure 4B). When compared to non-treated cells, the concentration of MMP-9 in the LPS-treated cells was higher.

3. Expression of SDF-1 α in osteoblasts treated with LPS

In osteoblasts treated with either *E. coli* or *A. actinomycetemcomitans* LPS, SDF-1 α mRNA expression was estimated. Treatment with each bacterial LPS increased the expression of the SDF-1 α mRNA levels. Expression of SDF-1 α mRNA was markedly enhanced at a concentration of 10 $\mu\text{g/ml}$ (Figure 5A). The concentration of SDF-1 α in the culture supernatant of cells was determined by ELISA (Figure 5B). SDF-1 α was detected in the absence and pres-

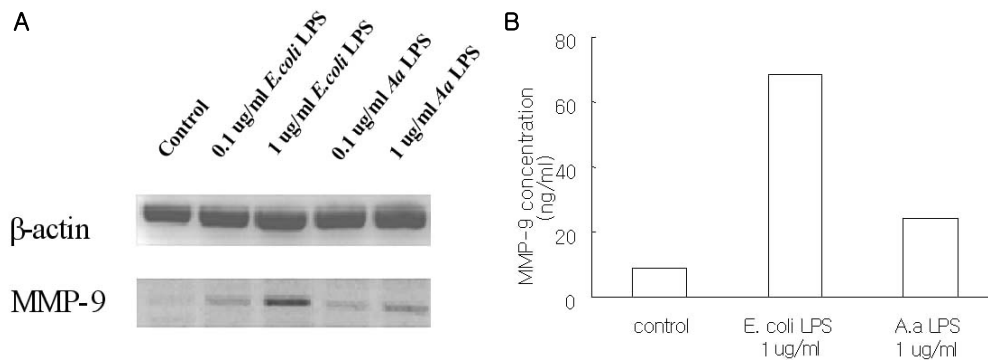


Figure 4. Expression of MMP-9 in RAW cells treated with LPS. RAW cells were treated with *E. coli* or *A. actinomycetemcomitans* LPS for 16 h (A). MMP-9 and β -actin mRNA levels were determined by RT-PCR. The concentration of the proteins in the culture supernatant was determined by ELISA for MMP-9 (B).

ence of LPS treatment. When compared to non-treated cells, the concentration of SDF-1 α in the LPS-treated cells was higher.

4. Transmigration of RAW cells treated with LPS through an SDF-1-dependent mechanism

Whether LPS could stimulate the migration

of RAW cells by increasing the expression of SDF-1 α in osteoblasts was examined. After the culture supernatant of osteoblasts treated with LPS and/or anti SDF-1 α antibody was added to the lower chamber of the transwell, the RAW cells of upper chamber were allowed to migrate through the membrane of the transwell pre-coated with collagen (Figure 6A, B). When the culture supernatant of non-treated cells

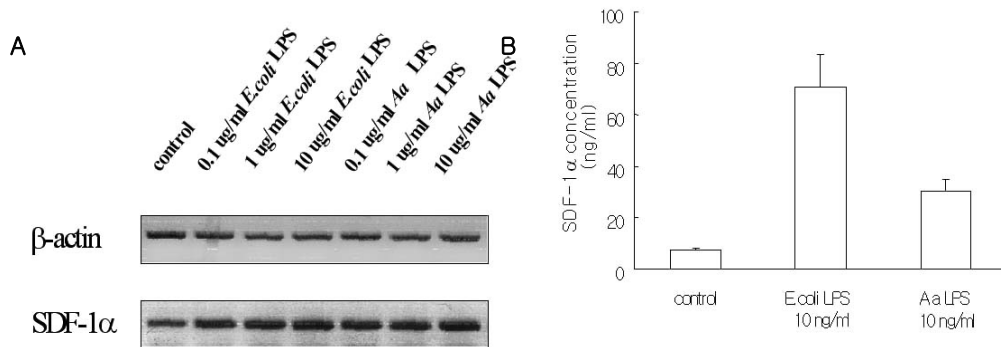


Figure 5. Expression of SDF-1 α in mouse calvaria-derived osteoblasts treated with LPS. Mouse calvaria-derived osteoblasts were treated with *E. coli* or *A. actinomycetemcomitans* LPS for 16 h. SDF-1 α and β -actin mRNA levels were determined by RT-PCR (A). The concentration of the proteins in the culture supernatant was determined by ELISA for SDF-1 α (B).

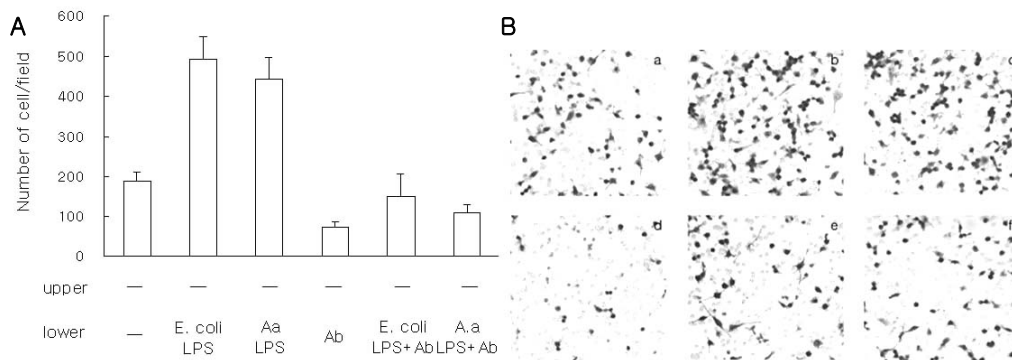


Figure 6. LPS stimulates RAW cell migration via a SDF-1 α -dependent mechanism. RAW cells were allowed to transmigrate for 16 h through porous transwell membrane pre-coated with collagen, in the presence or absence of culture supernatant of osteoblasts treated with *E. coli* LPS (10 μ g/ml), *A. actinomycetemcomitans* LPS (*Aa*, 10 μ g/ml), and/or anti-SDF-1 α antibody (*Ab*, 10 μ g/ml) added to the lower chamber of the transwell. After hematoxylin staining, the number of RAW cells that transmigrated through the transwell was counted (A). The RAW cells on lower side of membrane (x 200, B); non-treated cultures (a), *E. coli* LPS (b), *Aa* LPS (c), Anti-SDF-1 α *Ab* (d), *E. coli* LPS and Anti-SDF-1 α *Ab* (e), *Aa* LPS and Anti-SDF-1 α *Ab* (f).

was compared to the culture supernatants of osteoblasts treated with LPS, the number of cells on the lower side of membrane increased. When adding the anti SDF-1 α antibody to the LPS-treated culture supernatant, the number of cells on the lower side of membrane decreased.

IV. DISCUSSION

In this study, we examined the role of LPS in the migration of pre-OCs. *E. coli* and *A. actinomycetemcomitans* LPS increased the migration of pre-OCs through MMP induction in the cells. In addition, each bacterial LPS increased the migration of pre-OCs through the induction of SDF-1 α in osteoblasts.

The receptor activator of NF- κ B (RANK) is a surface receptor on pre-OCs and all monocytes in peripheral blood have RANK on their surface¹⁾. This suggests that pre-OCs circulate in peripheral blood. To approach the bone surface, circulating pre-OCs must transmigrate through the endothelial cell layer of blood vessels, the underlying basal lamina, and the extracellular matrix. MMPs are involved in this process. The basal lamina consists of Type IV collagen, proteoglycan, and laminin¹⁴⁾. The extracellular matrix of periodontal tissue consists of type I collagen¹⁵⁾. The matrigel, which is used in this study, is a reconstituted matrix of basal lamina-laminin, type IV collagen, and proteoglycan. In this study, LPS increased the transmigration of pre-OCs through matrigel or type I collagen-coated transwell membrane. The general MMP inhibitor decreased the transmigration of pre-OCs induced by each bacterial LPS. These results suggest that LPS

stimulates the transmigration of pre-OCs in a MMP-dependent manner. MMP-9 expression in pre-OCs is obligatory for their migration to the developing marrow cavity¹⁰⁾. The receptor activator of NF- κ B ligand (RANKL) and SDF-1 has been shown to increase MMP-9 expression in pre-OCs and it suggests that MMP-9 is involved in the recruitment of pre-OCs via RANKL and SDF-1²⁾. In this study, LPS increased expression of MMP-9 in pre-OCs. The substrate of MMP-9 is type IV collagen and the degradation product of type I collagen⁸⁾. Type IV collagen and type I collagen are components of basal lamina and periodontal tissue, respectively^{14,15)}. Therefore, MMP-9 may be involved in LPS-induced transmigration of pre-OCs through the basal lamina and extracellular matrix of periodontal tissue.

SDF-1 stimulated the chemotactic recruitment of pre-OCs from human circulating monocytes and it was suggested that SDF-1 may play important role in eliciting the chemotactic recruitment of circulating human precursors capable of differentiating into bone resorbing osteoclast⁷⁾. SDF-1 is expressed in pre-osteoblastic stromal cells of bone and various factors affect the expression of SDF-1. Cytokines, known to be particularly important in bone physiology, control the expression of SDF-1 in osteoblasts. IL-1, tumor necrosis factor (TNF) and parathyroid hormone (PTH) were shown to increase SDF-1 synthesis in osteoblasts, while transforming growth factor (TGF) decreased SDF-1 secretion⁵⁾. SDF-1 α and SDF-1 β are derived by alternative splicing of the SDF-1 gene and share similar biological effects. In this study, we observed the effect of LPS on the expression of SDF-1 α . Each bacte-

rial LPS increased SDF-1 α secretion in osteoblasts. The migration of pre-OCs was higher in response to culture supernatants of LPS-treated osteoblasts as compared to culture supernatants of non-treated osteoblasts. The migration of pre-OCs induced by culture supernatants of LPS-treated osteoblasts was inhibited with an anti SDF-1 α antibody. These results indicate that LPS enhances the pre-OCs migration through an increase of SDF-1 α secretion in the osteoblast. SDF-1 increased expression of MMP-9 in pre-OCs and increased transcollagen migration of pre-OCs in an MMP-dependent manner²⁾. This indicates the possibility that LPS may enhance the pre-OCs migration through SDF-1-mediated MMP-9 expression in pre-OCs.

In addition to induction ability of pre-OCs chemotaxis, SDF-1 stimulates the early stages of osteoclast differentiation by promoting the rapid expression of tartrate resistant acid phosphatase and the fusion in pre-OCs and acts as a survival signal for mature osteoclasts⁷⁾. Therefore, in LPS-induced alveolar bone resorption, SDF-1 may play an important role through enhancing the chemotaxis of pre-OCs and the differentiation of osteoclasts. Alveolar bone is surrounded by gingival fibroblasts and periodontal ligament cells. SDF-1 is also expressed in human gingival fibroblasts¹⁶⁾. In contrast to osteoblasts, IL-1 and TNF inhibited the expression of SDF-1 in human gingival fibroblasts¹⁶⁾. These findings indicate that the production of SDF-1 may depend on the cell type. Therefore, it remains to be determined the effect of LPS on expression of SDF-1 in human gingival fibroblasts and periodontal ligament cells.

In summary, LPS enhanced the pre-OCs transmigration through the induction of MMP-9 and SDF-1 α secretion in pre-OCs and osteoblasts, respectively. These results suggest a mechanism for localizing pre-OC cells to the alveolar bone surface in periodontitis. LPS stimulates osteoclast differentiation through a decrease of osteoprotegerin (OPG) and an increase of RANKL¹²⁾. LPS may play an important role in bone destruction through the stimulation of osteoclast differentiation and pre-OCs migration on the alveolar bone surface.

V. CONCLUSIONS

To determine the role of LPS in migration of pre-OCs, the effect of LPS from *E. coli* or *Actinobacillus actinomycetemcomitans* on the migration of pre-OCs and the involvement

of MMP-9 and SDF-1 α in LPS-induced pre-OC migration was determined. The migration of RAW cells, a pre-OC cell line, was analyzed with transwells pre-coated with matrigel or collagen. The expression of MMP-9 and SDF-1 α was determined by RT-PCR or ELISA.

The transmigration of RAW cells through matrigel or collagen was increased in the presence of *E. coli* or *A. actinomycetemcomitans* LPS. The MMP inhibitor suppressed the migration of pre-OC induced by each bacterial LPS. LPS increased the expression of MMP-9 in RAW cells. The expression of SDF-1 α was also up-regulated in osteoblasts treated with each bacterial LPS. An anti SDF-1 α antibody down-regulated the migration of pre-OC which was increased by culture supernatant of osteoblasts treated with each bacterial LPS.

These results suggest that LPS increases the

migration of pre-OCs through up-regulation of MMP-9 and SDF-1 α expression in the pre-OCs and osteoblasts, respectively.

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Lipopolysaccharide가 파골세포 전구세포의 이동에 미치는 영향

이희영¹, 이대실³, 차정현², 유윤정^{2*}

1. 연세대학교 치과대학 구강생물학교실
2. 연세대학교 치과대학 구강생물학교실, 2단계 BK21 사업 및 구강과학연구소
3. 한국생명공학 연구원

파골세포에 의한 골흡수는 1) 혈관을 통한 파골세포 전구세포의 골표면 이동 및 2) 골표면에서 파골세포 전구세포로부터 파골세포 분화 두 단계를 거쳐 일어난다. Stromal cell derived factor (SDF)-1 α 는 파골세포 전구세포의 화학주성인자이며 matrix metalloproteinase (MMP)-9는 파골세포 전구세포의 이동에 관여하는 단백 분해효소이다. 파골세포 전구세포의 골표면 이동에 있어서 LPS의 역할을 규명하기 위하여 *E. coli* 및 *Actinobacillus actinomycetecomitans* LPS의 1) 파골세포 전구세포 유도능, 2) LPS에 의한 파골세포 전구세포의 이동에 있어서 MMP 및 SDF-1 α 의 관련성을 평가하였다.

LPS에 의한 파골세포 전구세포인 RAW 세포의 이동은 matrigel 또는 type I collagen을 도포한 transwell 을 이용하여 평가하였으며 MMP-9 및 SDF-1 α 의 발현은 RT-PCR 또는 ELISA로 평가하였다.

각 세균의 LPS는 matrigel 또는 type I collagen을 통한 파골세포 전구세포의 이동을 증가시켰다. MMP 억제제는 각 세균의 LPS에 의한 파골세포 전구세포의 이동을 억제하였다. LPS는 파골세포 전구세포의 MMP-9의 발현을 증가시켰다. 각 세균의 LPS는 마우스 두개골에서 분리한 조골세포의 SDF-1 α 의 발현을 증가시켰다. SDF-1 α 을 함유한 LPS 처리 조골세포 배양상층액은 파골세포 전구세포의 이동을 증가시켰으며 anti SDF-1 α Ab는 LPS처리 세포 배양상층액에 의한 파골세포 전구세포의 이동을 억제하였다.

이들 결과는 LPS가 파골세포 전구세포에서는 MMP-9을 조골세포에서는 SDF-1 α 의 발현을 증가시켜 파골세포 전구세포의 이동을 촉진 시킬 수 있음을 시사한다.

