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# The Effects of *Porphyromonas endodontalis* Lipopolysaccharide on Production of Several CC Chemokines by Periodontal Ligament Fibroblast

Department of Conservative Dentistry, College of Dentistry, Seoul National University

Hyun Jung Ko, D.D.S., M.S.D., Ph. D. (postdoctoral research fellow), Sang Yup Lee, D.D.S., M.S.D., (graduate student)
Woo Chul Lee, D.D.S., M.S.D., Ph. D. (Full-time Instructor), Seung Ho Baek, D.D.S., M.S.D., Ph. D. (assistant professor)
Kwang Sik Bae, D.D.S., M.S.D., Ph. D. (professor), Sung Sam Lim, D.D.S., M.S.D., Ph. D (professor)

## ABSTRACT

### *Porphyromonas endodontalis*의 lipopolysaccharide가 치주인대 섬유아세포의 CC Chemokines 생성에 미치는 영향

고현정 D.D.S., M.S.D., Ph.D - 연구원, 이상엽 D.D.S., M.S.D. - 박사과정 대학원생
이우철 D.D.S., M.S.D., Ph.D - 전임강사, 백승호 D.D.S., M.S.D., Ph.D - 조교수
배광식 D.D.S., M.S.D., Ph.D - 교수, 임성삼 D.D.S., M.S.D., Ph.D - 교수

이 연구의 목적은 치수 및 치근단 조직의 감염의 주요 원인균인 *Porphyromonas endodontalis*의 LPS가 치주인대 섬유아세포의 MCP-1, MIP-1$\alpha$ 그리고 RANTES의 생성에 미치는 영향을 관찰함으로써 치근단 염증의 발병에서 섬유아세포의 역할을 알아보는 것이다.

혐기성 조건에서 배양한 *P. endodontalis*에서 LPS를 추출, 정제한 후, 치주인대 섬유아세포에 0.01, 0.1, 1, 10$\mu g/m\ell$ 농도의 *P. endodontalis*와 E. coli의 LPS로 12, 24, 48시간 동안 자극하였다. MCP-1, MIP-1$\alpha$ 그리고 RANTES의 단백질 농도는 ELISA를 이용하여 분석하였다.

이 연구의 결과는 아래와 같다.

1. *P. endodontalis*의 LPS로 자극시킨 치주인대 섬유아세포에서 분비된 MCP-1의 수준은 시간과 농도에 비례하여 증가하였다.
2. *E. coli*의 LPS로 자극시킨 치주인대 섬유아세포에서 분비된 MCP-1의 양도 시간에 비례하여 증가함을 보였다. 이는 *P. endodontalis* LPS보다 통계적으로 유의성 있게 많은 양을 나타냈다 ($p<0.05$).
3. 치주인대 섬유아세포에서 MIP-1$\alpha$과 RANTES는 거의 분비되지 않았다.

이 연구에서 세균의 LPS로 자극 시 치주인대 섬유아세포에서 MCP-1의 강한 발현은 만성 치근단 염증의 초기에 단핵세포와 대식세포의 recruitment를 증가시키는 것과 관련되어지는 것으로 보인다.

*Keywords ; Lipopolysaccharide, MCP-1, MIP-1$\alpha$, *Porphyromonas endodontalis*, RANTES

# I. INTRODUCTION

Periapical bone resorption is the hallmark of periradicular disease initiated by the invasion of bacteria and their byproduct from the infected root canal system[1]. Many recent studies have demonstrated that interleukin-1 (IL-1) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) are potent inflammatory cytokines having bone-resorbing activity[2-4]. Since these cytokines are produced by monocytes/macrophages, the recruitment of inflammatory cells is of particular importance in the root canal system and periapical tissue because it is likely that cells will be challenged with bacteria during acute infection and once localized, these cells in periapical tissue may play a functional role in the pathogenic mechanism(s) of periapical disease through production of these cytokines[5]. Periapical infections may result in the exposure of periodontal ligament (PDL) cells to microbial products as well as to proinflammatory cytokines liberated from neighboring tissue. These cytokines affect the functions of PDL cells.

Periodontal ligament resides between the cementum of the roots of teeth and the alveolar bone. In this location, PDL cells are uniquely situated to maintain the overall integrity of the periodontal ligament. Among the PDL cells, fibroblasts are the predominant cell type and play a central role in the maintenance of the periodontium since they are responsible for the synthesis and degradation of collagen and are involved in the regulation of alveolar bone remodeling. It is now clear that the fibroblasts are a key sentinel cells that have important functions in fibrosis, wound healing, maintenance of tissue integrity, as well as the regulation of immune responses in various tissues. In order to execute these functions, fibroblasts should synthesize and release a wide array of cytokines including chemokines[6].

Chemokines are chemotactic cytokines that stimulate recruitment of leukocytes to the site of inflammatory reactions[7-8]. Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$) and RANTES (regulated on activation, normal T cell expressed and secreted) are 3 well-characterized CC-chemokines that regulate mononuclear cell recruitment.

MCP-1 is expressed by mononuclear phagocytes and endothelial cells, MIP-1$\alpha$ is produced by activated inflammatory cells, such as macrophages and eosinophils, and noninflammatory cells including fibroblasts, myofibroblasts, endothelial cells and epithelial cells. The role of RANTES in allergic inflammation has been well defined and one of the major eosinophil chemoattractants in allergic inflammatory diseases[8]. Despite of the fact that some of these chemokines are secreted by various normal cell types, including fibroblasts, epithelial cells, and leukocytes[9], there are few studies on the expression of chemokines from the PDL fibroblasts.

In this regard, Safronova et al.[10] have demonstrated that MCP-1 was expressed in human synovial fibroblasts and Hanazawa et al.[11] showed that gingival fibroblasts also produced MCP-1.

Bacterial lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria. It functions as a virulence factor and has the ability to induce a number of

inflammatory and immunopathological reactions by stimulating inflammatory cells to release a variety of cytokines, which in turn leads to the destruction of host tissue. In last few years, although many studies have investigated the role of anaerobic bacterial LPS, the role of endodontopathic bacterial LPS and the interaction between these bacterial LPS and inflammatory cells have received less attention[12-14].

*P. endodontalis*, an asaccharolytic, black-pigmented bacteria, is found in approximately 50% of chronic endodontic lesions and exclusively found in infections of endodontic origin, suggesting that there is a specific association between *P. endodontalis* and pulpal and periapical diseases[15-16]. Recently, Ko et al.[17] demonstrated that human peripheral polymorphonuclear neutrophils (PMNs) have the ability to release MIP-1$\alpha$ and MIP-1$\beta$ after stimulation of *P.endodontalis* LPS.

It is postulated as a possibility that chemokines, MCP-1, MIP-1$\alpha$ and RANTES expressions in the PDL fibroblasts may be induced by the action of cell components of *P. endodontalis*. Therefore, the purpose of present study was to evaluate the protein level of these chemokines expression in PDL fibroblasts stimulated with *P. endodontalis* LPS.

## II. Materials and Methods

### 1. Culture of Bacteria and Purification of LPS

The bacterial strains used was *P. endodontalis* ATCC 35406 and was cultured in brain heart infusion (BHI) broth supplemented with Hemin (10㎍/㎖), vitamin K1 (1㎍/㎖), and yeast. Bacterial cells were grown anaerobically at 37℃. After sufficiently growing, the bacterial cell pellet was obtained by centrifugation at 10,000×g for 10min.

This pellet was washed with distilled water and then lyophilized. LPS was extracted and purified by the method as described by the rapid method[18].The lyophilized bacteria was mixed with 1㎖ of distilled water and then boiled for 15min, with 5min of inverted mixing period. Following centrifugation at 12,000×g for 5min, cellular debris was removed, and the supernatant was collected. Then proteinase K (1mg dissolved in 50 liter of water) was added and incubated for 1h at 60℃. After the tube was boiled for 5min to precipitate any residual proteinase K, the supernatant was dialyzed against distilled water. After lyophilize the supernatant, the final crude LPS was redissolved to a concentration of 1㎎/㎖ of distilled water.

The purified LPS was confirmed in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 1).

Commercial preparations of *E. coli* LPS (Sigma chemical company, St Louis, MO, USA) were obtained as lyophilized powder and used as positive control.

### 2. Culture of periodontal ligament fibroblasts and stimulation by LPS

PDL cells were obtained from PDL explants dissected from the mid-root of premolars extracted for orthodontic reasons, according to the method described by Oates et al[19]. PDL tissue was scraped from the middle third of the root surface with sharp blade under sterile conditions.

The tissue explants were placed in 60㎜ tissue

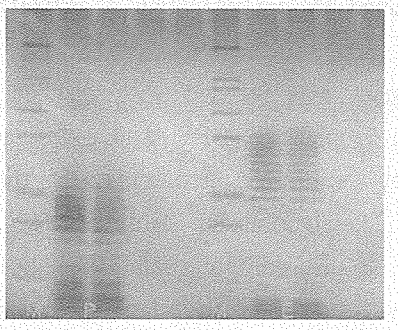

Fig 1. *P. endodontalis* (left) and *E. Coli* (Right) LPS confirmed by SDS-PAGE.

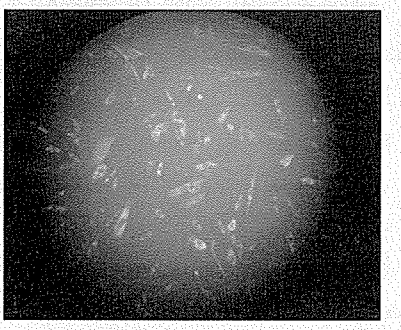

Fig 2. Immunohistochemical staining of human PDL cell cultures for the confirmation of cytoplasmic marker of fibroblasts.

culture plates containing Dulbecco's modified eagle's medium (DMEM) with high glucose (4,500㎎/L), 10,000U/L penicillin, 10,000㎍/L streptomycin, 50㎍/L fungizone and 20% heat inactivated fetal bovine serum (FBS). The cells were incubated in humidified air at 37℃ with 5% $CO_2$ for 2 to 4 weeks. The medium was replaced every 2 or 3 days until sufficient cell proliferation was evident.

Brief incubation with 0.25% trypsin was employed to remove the cells, which were then transferred into 100㎜ dish in alpha-MEM supplemented with 10% FBS, 1mM sodium pyruvate, 1mM non-essential amino acid and antibiotics for continued growth, and then cultured at 37℃ in 5% $CO_2$. Cells were grown in 35mm dish or on glass coverslips for immunohistochemical staining with 5B5 (Dako) as cytoplasmic marker of human fibroblasts (Fig 2). After confluency, the cells were passaged on 6 well plate and stimulated by LPS (0.01, 0.1, 1, 10㎍/㎖) of *P. endodontalis* and *E. coli* (positive control) for various periods of time (12, 24, 48hrs).

### 3. ELISA assay of chemokines

The concentrations of MCP-1, MIP-1$\alpha$ and RANTES were analyzed using enzyme-linked immunosorbent assay kits from Quantikine R&D system(Quantikine colorimetric sandwich ELISA kits; R&D System, Inc., Minneapolis, MN, USA). Briefly, monoclonal antibodies specific for MCP-1, MIP-1$\alpha$ and RANTES were precoated onto enzyme-linked immunosorbent assay plate. Dilutions of standards and culture supernatants were then added to individual wells in duplicate. After any unbound substances were washed away, an enzyme-linked polyclonal antibody specific for MCP-1, MIP-1$\alpha$ and RANTES was added to the wells.

After a further wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and the color was developed.

The optical density was read at an absorbance of 450nm, and the cytokine concentration was quantified from the standard curve. Three samples in each group were analyzed in duplicate.

4. Statistical Analysis

All statistical analyses were performed according to Student's t-test and Mann-Whitney sum test. The statistical significance risk rate was set at $p<0.05$.

## III. Results

The levels of MCP-1, MIP-1α and RANTES released from freshly isolated human PDL fibroblasts in response to *P. endodontalis* and *E. coli* LPS were examined. Table 1 shows that the levels of MCP-1 released from cells stimulated with either *P. endodontalis* and *E. coli* LPS were significantly higher than unstimulated control group by ELISA technique ($p<0.05$).

The levels of MCP-1 produced a significant dose-dependent increase when PDL fibroblasts were treated with 0.01, 0.1, 1, 10㎍/㎖ of *P. endodontalis* and *E. coli* LPS compared with the control ($p<0.05$), but the levels of MCP-1 released from those cells stimulated with *E. coli* LPS were higher than those released from cells stimulated with *P. endodontalis* LPS except 10㎍

Table 1. Mean concentration and standard deviation of MCP-1

| | conc.of LPS (㎍/㎖) | ELISA (pg/ml) 12h | 24h | 48h |
|---|---|---|---|---|
| control | 0 | 68.0±12.0 | 129.8±18.3 | 459.6±52.3 |
| MCP-1(*E. coli*) | 0.01 | 496.7±23.5+ | 670.4±30.0+ | 2098.4±33.2+ |
| | 0.1 | 503.0±32.9+ | 629.4±32.2+ | 3098.4±323.4+ |
| | 1.0 | 329.3±92.2 | 890.3±58.8+ | 5098.4±152.4+ |
| | 10 | 381.4±221.1 | 1047.4±50.3 | 1639.3±133.2 |
| MCP-1(*P. endo*) | 0.01 | 140.3±65.6 | 192.6±24.5 | 566.5±22.1 |
| | 0.1 | 192.0±112.8 | 234.3±33.4 | 725.4±24.3 |
| | 1.0 | 269.0±31.4 | 417.3±32.5 | 974.9±375.6 |
| | 10 | 322.7±22.4 | 1487.1±19.5+ | 2093.4±123.2 |

+ indicates significant differences ($P<0.05$) between cells stimulated with *P. endodontalis* LPS and cells stimulated with *E. coli* LPS (n=10 for each group).

Table 2. Mean concentration and standard deviation of MIP-1α

| | conc. of LPS (㎍/㎖) | ELISA (pg/ml) 12h | 24h | 48h |
|---|---|---|---|---|
| control | 0 | 0.0 | 1.4 | 1.4 |
| MIP-1α (*E.coli*) | 0.01 | 0.0 | 0.0 | 1.4 |
| | 0.1 | 1.1 | 0.0 | 0.8 |
| | 1.0 | 1.7 | 0.0 | 1.1 |
| | 10 | 2.0 | 0.2 | 0.5 |
| MIP-1α (*P.endo*) | 0.01 | 0.8 | 0.8 | 0.8 |
| | 0.1 | 0.0 | 1.4 | 2.3 |
| | 1.0 | 0.0 | 0.0 | 1.4 |
| | 10 | 0.0 | 1.1 | 1.7 |

Table 3. Mean concentration and standard deviation of RANTES

| | conc. of LPS (㎍/㎖) | ELISA (pg/ml) 48h |
|---|---|---|
| control | 0 | 0.0 |
| RANTES (*E.coli*) | 0.01 | 424.2 |
| | 0.1 | 398.4 |
| | 1.0 | 1137.5 |
| | 10 | 20.7 |
| RANTES (*P.endo*) | 0.01 | 0.0 |
| | 0.1 | 0.0 |
| | 1.0 | 0.0 |
| | 10 | 76.7 |

/㎖ concentration.

Table 1 shows that the levels of MCP-1 released from PDL fibroblasts stimulated with *P. endodontalis* and *E. coli* LPS were time-dependent increase at 1㎍/㎖ of each LPS. Production of MCP-1 by PDL fibroblasts stimulated with either *P. endodontalis* and *E. coli* LPS continued to increase and reached a peak at 48h. However, MIP-1α and RANTES in PDL fibroblasts were undetectable by ELISA (Table 2 and 3).

## IV. Discussion

The attraction of leukocytes to tissues is essential for inflammation and the host response to infection. The regulation of leukocytes migration into and through the tissues is determined by the expression of adhesion molecules on firstly endothelial cells and on other cells such as keratinocytes which are induced by pro-inflammatory cytokines as well as a group of cytokines with chemotactic properties, the chemokines[20]. Chemokines are responsible for the recruitment and subsequent activation of particular leukocytes into inflamed tissues and therefore play a central role in the final outcome of the immune response by determining which subsets of leukocytes form the inflammatory infiltrate[21-23].

Chemokines are a group of structurally related cytokines whose importance in the inflammatory process is best illustrated by their ability to specifically recruit discrete leukocytes subpopulation. Chemokines are 8 to 10 kDa proteins with 20 to 50 percent homology in amino acid sequences. They have been subdivided into families on the basis of the relative position of their cysteine residues. There are at least four families of chemokines. The α- and β- chemokines are the largest and most well characterized families[9].

MCP-1 is expressed by monocytes, vascular endothelial cells, smooth muscle cells, glomerular meningeal cells, osteoblastic cells, and human pulmonary type-2-like epithelial cells in culture[24]. MCP-1 is chemotactic for monocytes but not neutrophils. MCP-1 is a potent activator of human basophils, inducing the degranulation and the release of histamines. It is now assumed that the combinatorial effects of multiple chemokines and other mediators are responsible for the cellular composition at inflammatory sites. In addition, many chemokines also directly cause cell activation. Some of them activate granulocytes and/or monocytes and cause respiratory bursts, degranulation, and the release of lysosomal enzymes. Studies[25-27] have suggested that MCP-1 may play an important role in producing the pathogenic state of inflammatory disease. Therefore, in the present study, we examined MCP-1 expression in PDL fibroblasts with ELISA technique, and as expected, MCP-1 was produced by PDL fibroblasts and demonstrated time- and dose-dependent increase. In this study the levels of MCP-1 expression were significantly higher than MIP-1α and RANTES. This result is in sharp contrast to the Gemmell's report[25] that *P. gingivalis* induces lower percentages of MCP-1-positive monocytes, B cells and *P. gingivalis*-specific T cells in comparison with the other chemokines, MIP-1α and RANTES. Furthermore, *P. gingivalis* has been shown to inhibit the production of MCP-1

by *P. gingivalis*-specific T cells, B cells and monocytes to inhibit neutrophils migration into *P. gingivalis*-induced lesions in the mouse model[26]. On the contrary, Yu et al.[27] reported the expression of MCP-1 on endothelial cells as well as monocytes/macrophages in inflamed gingival tissues correlated with the degree of inflammation. We can infer from this study that the strong expression of MCP-1 in PDL fibroblasts may be related to increased recruitment of monocytes/macrophages at the early stage of chronic inflammation when stimulated with bacterial LPS. Since these results of other studies[25-28] are at variance with the present study, further studies are required to determine the significance of MCP-1 in periapical disease. Collectively, these results imply that MCP-1 produced by *P. endodontalis*-stimulated PDL fibroblasts may be involved predominantly in recruitment of monocytes from the circulating pool into periapical tissue.

MIP-1$\alpha$ , like MCP-1, is a potent chemoattractant for monocytes and macrophages and MIP-1$\alpha$ is the major factor produced by macrophages following their stimulation with bacterial endotoxin[29,30]. MIP-1$\alpha$ is also involved in the cell activation of human granulocytes such as neutrophils, eosinophils, and basophils, and appeared to be involved in acute neutrophilic inflammation.

Circulating T-cells produces RANTES and T-cell clones in culture but not by any T-cell lines tested so far. RANTES is chemotactic for T-cells, human eosinophils and basophils and plays an active role in recruiting leukocytes into inflammatory sites. RANTES increases the adherence of monocytes to endothelial cells[31]. It selectively supports the migration of monocytes and T-lymphocytes expressing the cell surface. RANTES activates human basophils from some select basophils donors and causes the release of histamines. On the other hand RANTES can also inhibit the release of histamines from basophils induced by several cytokines. RANTES is also reported that it is expressed by human synovial fibroblasts[32-33].

However, under the same detection method as in the MCP-1 detection, we were unable to monitor the expression of MIP-1$\alpha$ and RANTES. The reason that MIP-1$\alpha$ and RANTES were not detected by ELISA technique is not clear, but it is plausible that this phenomenon may be due to the fact that PDL fibroblasts do not produce much of these two chemokines as other inflammatory cells or as we mentioned above, MIP-1$\alpha$ and RANTES are involved in later stage of inflammation, therefore, these chemokines were not produced enough to be detected by ELISA method up to 48 hours after bacterial LPS stimulation. Therefore, further studies such as northern blot or RT-PCR(reverse transcription-polymerase chain reaction) method which can detect mRNA of these proteins are needed.

These three chemokines are produced by virtually all somatic cells including monocytes, alveolar macrophages, neutrophils, platelets, eosinophils, mast cells, T and B lymphocytes, NK cells, keratinocytes, epithelial cells, hepatocytes, fibroblasts, smooth muscle cells, and endothelial cells. These cells can produce chemokines in response to variety of factors, including viruses, bacterial products, IL-1, TNF, complement C5a, Leukotriene B4 and Interferons[31-37]. The production of chemokines by

both immune and nonimmune cells supports the contention that these cytokines may play a pivotal role in orchestrating chronic inflammation. Among the chemokine-producing cells, fibroblasts are well known as a cell of connective tissues[33-41]. It is the predominating cell of the healthy gingiva and periodontal ligament, and is responsible for the synthesis and upkeep of the constituents of extracellular matrix. Fibroblasts release some specific mediators active on fibroblasts themselves (autocrine effect), or on other near cells (paracrine effect): osteoblasts, polymorphonuclear cells, mast cells, monocytes, endothelial and epithelial cells. In this way, fibroblasts appear as a true immune cell, able to control not only the extracellular matrix, but also the cells in its neighborhood.

The fact that PDL fibroblasts can synthesize and release chemokine should lead to a reconsideration of their role in immunoregulation and pathophysiology. Factors critical to understanding the homeostatic mechanisms regulating the regeneration of PDL during inflammation are poorly understood. The most important factors controlling tissue destruction and remodeling are proinflammatory cytokines and the potent immunostimulatory agent LPS. During pulpal and periradicular disease, due to its proximity to the infected root canal, the PDL is exposed to inflammatory exudates containing proinflammatory cytokines and bacterial products. However, studies on the expression of CC chemokines in PDL fibroblasts are lacking. To this end, we first determined if PDL fibroblasts could participate directly in the immune response through the action of chemokines elaborated in response to bacterial LPS.

In summary, Our finding that chemokine MCP-1 expression is unambiguously identified in PDL fibroblasts suggests that they can play a role in host defense by initiating the recruitment of monocytes in the early stages of chronic periapical inflammation. During the early stages of inflammation, large numbers of leukocytes are recruited from peripheral blood in response to changes in chemokine production and expression of inflammatory receptors and adhesion molecules on endothelial cells supplying the inflamed tissue.

## V. Conclusions

The production of MCP-1, MIP-1$\alpha$ and RANTES by human PDL fibroblasts stimulated with *P. endodontalis* LPS were monitored.

According to this study, the results were as follows:

1. The levels of MCP-1 from PDL fibroblasts stimulated with *Porphyromonas endodontalis* LPS were time- and dose-dependent increase at all concentrations of LPS treatment by ELISA.

2. MCP-1 production from PDL fibroblasts treated with *E. coli* LPS was also time dependent. This level was significantly higher than that of *P. endodontalis* LPS treated cells($p<0.05$).

3. MIP-1$\alpha$ and RANTES in periodontal ligament fibroblasts were undetectable by ELISA.

We can infer from this study that strong expression of MCP-1 in PDL fibroblasts may be related to increased recruitment of monocytes/macrophages at the early stage of chronic periapical inflammation when stimulated with bacterial LPS.

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