치의학석사 학위논문

Inhibition of Inflammatory Responses of *Porphyromonas gingivalis by a* Quorum Sensing Inhibitor

Quorum Sensing Inhibitor를 이용한 *Porphyromonas gingivalis*의 염증반응 억제

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서울대학교 대학원
치 의 학 과
박 경 리

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지도교수 최 봉 규

이 논문을 치의학석사 학위논문으로 제출함

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치 의 학 과

박 경 리

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위 원 장 구 기 태 (인)

부 위 원 장 최 봉 규 (인)

위 원 최 영 님 (인)

Abstract

1. Background

Quorum sensing (QS) is a control system of genetic expression according to changes in cell population density. QS molecules play an important role in biofilm formation in oral environment as well. Recent studies have shown that QS inhibitors (QSIs) can inhibit biofilm formation and consequently, contribute to the attenuation of chronic inflammatory periodontitis. Therefore, the purpose of the study was to investigate whether D-galactose as a QSI can reduce inflammatory responses in human gingival epithelial cells by *P. gingivalis*.

2. Method

Human gingival epithelial cell line (HOK-16B) was infected with *P. gingivalis* (ATCC 33277) in the presence of D-galactose at 2, 20, 200 mM for 24 h. For the evaluation of inflammatory responses, the gene expression level of IL-6, IL-8, MMP-2, and MMP-9 was measured using real time RT-PCR.

3. Results and Discussion

P. gingivalis induced expression of IL-6, IL-8, MMP-2, and MMP-9 mRNA. D-Galactose at 2 and 20 mM significantly reduced the expression of IL-6, MMP-2, and MMP-9 induced by *P. gingivalis*. The gene expression was also reduced by D-galactose at 200 mM, but it was not statistically significant.

On the contrary, we observed that D-galactose treatment enhanced IL-8 expression induced by *P. gingivalis*.

This result indicates D-galactose may also play a role of promoting host defense mechanism against gingipain secreted by *P. gingivalis*.

4. Conclusion

In conclusion, these results indicate that D-galactose is capable of inhibiting proinflammatory responses and promoting host defense system in human epithelial cells infected with *P. gingivalis*. Although the exact mechanism behind

the effect of D-galactose still remains elusive, the results provide an evidence of D-galactose as a potential QSI.

Keywords : Periodontitis; *Porphyromonas gingivalis*; Quorom sensing inhibitors; D-Galactose

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I. Introduction

Periodontitis, a chronic inflammation of the tissues surrounding and supporting the teeth, is a highly prevalent oral disease, affecting millions of people each year (Darveau *et al.*,). Globally, the costs associated with conventional treatment of periodontal diseases are enormous (Listl *et al.*,).

Periodontitis is associated with a biofilm adhering to the surface of the tooth. During biofilm formation, microorganisms colonize the tooth in a coordinated sequential manner (Frias *et al.*,). Bacterial aggregation and virulence depend on gene expression, which can be stimulated by a cell-to-cell signaling called quorum sensing (QS) (Cho *et al.*,). QS bacteria produce, detect, and respond to chemical signal molecules called autoinducers (AIs) (Miller *et al.*,). When the concentration of an AI reaches a threshold level, an alteration in gene expression occurs, resulting in various physiologic activities including bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Rutherford *et al.*,). QS provides an effective and efficient means for the bacterial community to interact and survive.

Autoinducer-2 (AI-2) is a universal QS molecule that mediates interspecies communication among bacteria (Kolenbrander *et al.*,). Specifically, Frias *et al.* has shown that periodontal pathogens important in the development of periodontitis such as *Prevotellaintermedia*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* also produced extracellular signaling molecule related to AI-2 (Frias *et al.*,). Therefore, AI-2 seems to be a suitable target for the control of periodontopathogens. QS inhibitors (QSIs) can disrupt or prevent the formation of biofilm and reduce virulence while exerting less selective pressure on the bacteria, suggesting that QSIs are potential alternatives for antibiotics (Chen *et al.*,).

Among different periodontopathogens, *P. gingivalis*is is a keystone

pathogen that is capable of producing several virulence factors. Recently, D-galactose has been demonstrated to significantly inhibit the biofilm formation of *F. nucelatum*, *P. gingivalis*, and *Tannerella forsythia* induced by the AI-2 of *F. nucelatum* (Ryu *et al.*,). However, the role of D-galactose as a QSI on *P.gingivalis*-induced inflammation has yet been assessed.

This study aims to investigate whether D-galactose as a QSI can reduce expression of virulence by *P. gingivalis in vitro*. We hypothesized D-galactose treatment would inhibit the action of *P. gingivalis* AI-2 and thus reduce inflammatory cytokine levels in human oral epithelial cell induced by *P. gingivalis*. To test this hypothesis, we evaluated the effect of D-galactose on the expression of IL-6, IL-8, MMP-2, and MMP-9 mRNA.

II. Materials and Methods

1. Bacteria Culture

P. gingivalis (ATCC 33277) was cultured in a brain heart infusion medium (BD Bioscience, San Jose, CA, USA) supplemented with hemin (20 mg/ml) and vitamin K (0.2 mg/ml). The bacteria were incubated under anaerobic conditions (10% H_2, 10% CO_2,and 80% N_2) at 37° C for 2 days.

2. Cell Culture

Human epithelial cell line (HOK-16B cells) was grown in keratinocyte growth medium containing a supplementary growth factor bullet kit (PromoCell cat.no. c-20011). HOK-16B cells (5x10^5/well) were cultured in six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for 24 h and washed with antibiotic-free medium prior to bacterial infection.

3. QSI and Cell Treatment

D-Galactose compound (Sigma-Aldrich, St. Louis, Mo., USA) was used as a quorum sensing inhibitor. HOK-16B cells were infected with *P. gingivalis* at concentration of D-galactose of 2, 20, 200 mM for 24 h. HOK-16B cells infected only with *P. gingivalis* without D-galactose treatment served as a positive control, and cells treated only with D-galactose at 200 mM served as a negative control. The cells were harvested for RNA isolation.

4. Real-Time RT-PCR

Total RNA was extracted from the cells using the easy-BLUE total RNA extraction kit. The cDNA synthesis was performed from 1 ㎍ of RNA using an M-MLV reverse transcriptase kit (Promega, Madison, WI, USA). For PCR of IL-6, IL-8, MMP-2, and MMP-9, cDNA (1 ㎕) was mixed with 10 ㎕ of Power SYBR Green Master mix (Applied Biosystem, Warrington, UK) and primer pairs (0.2 ㎕ each) in a 20 ㎕ reaction volume. PCR was repeated for 40 cycles of the following protocol: denaturation step at 95° C for 15 s and annealing/extension step at 60° C for 1 min. GAPDH expression was used to normalize gene expression. The sequences of the primers were as follows: (5′-GTC GCC AGC CGA GCC-3′ and 5′-TGA AGG GGT CAT TGA TGG CA-3′ for GAPDH; 5′-GAT TCA ATG AGG AGA CTT GCC TGG-3′ and 5′-GCA GGA ACT GGA TCA GGA CTT T-3′ for IL-6; 5′-CTG TGT GAA GGT GCA GTT TTG C-3′ and 5′-AAC TTC TCC ACA ACC CTC TGC-3′ for IL-8; 5′-GGG CAG CCA TAG AAG TT-3′ and 5′-GCT ACG ATG GAG GCG GA TA-3′ for MMP-2; 5′-CTC TGG AGG TTC GAC GT GA-3′ and 5′-CTG CAG GAT GTC ATA GGT CA-3′ for MMP-9.)

5. Statistical Analysis

Statistical analyses were performed using Student's *t*-test. Statistically significant differences between the control and D-galactose groups were analyzed. A *p*-value less than 0.05 was considered statistically significant.

Ⅲ. Results

1. Effect of D-galactose on inflammatory factors induced by P.gingivalis

To examine whether D-galactose reduces inflammatory responses induced by *P. gingivalis*, we evaluated the inhibitory effect of D-galactose (2, 20, and 200 mM) on the gene expression of IL-6, IL-8, MMP-2, and MMP-9 in HOK-16B cells. As shown in Fig. 1A, D-galactose significantly reduced the expression of IL-6 mRNA in a dose-dependent manner, except for D-galactose at 200 mM. A similar trend was shown in MMP-9, where the level of MMP-9 mRNA expression was decreased in a dose-dependent manner except for D-galactose at 200 mM (Fig. 1B). In both cases, D-galactose showed an inhibitory effect at 200 mM compared with the control, but the results were not statistically significant. These results were within the scope of our hypothesis. For MMP-2, we saw a decrease in the expression in a dose-dependent manner, yet the standard deviation values for positive control and D-galactose at 2 mM were too high for any analysis to be made (Fig. 1C).

For IL-8, however, we saw an opposite trend, with the expression of IL-8 increasing in a dose-dependent manner (Fig. 2). This suggests that the inhibitory effect of D-galactose on QS of *P. gingivalis* could also result in an increased host defense responses by producing more chemokines such as IL-8.

IV. Discussion

In the study, we have shown the potential of D-galactose as a QSI to attenuate virulence by *P.gingivalis*. To the best of our knowledge, this study is the first to investigate the effect of D-galactose in reducing the expression of specific inflammatory factors induced by *P. gingivalis*.

Both gram-negative and gram-positive bacteria use QS to regulate a variety of physiological functions enabling bacteria to survive even in a hostile environment (Bassler *et al.*,). QS molecules modulate both intra- and inter-species bacterial communication. Especially, AI-2 encoded by *luxS* plays an important role in the biofilm formation of periodontopathogens. The universality of AI-2 has emerged as a great potential target against bacterial infection. *P. gingivalis* also utilizes AI-2/luxS system for its physiological actions. As *P. gingivalis* is the major etiologic bacteria of periodontitis and produces numerous virulence factors by modulating the host inflammatory responses (How *et al.*,), the inhibition of AI-2 in *P. gingivalis* may help attenuate its virulence.

Although the *P. gingivalis* receptor for AI-2 has not been identified (Shao *et al.*,), LuxS gene in *P.gingivalis* shows to control hemin acquisition and the stress responses (Chung *et al.*,). While the exact role of AI-2 in *P. gingivalis* virulence is not clear yet, AI-2 certainly plays a vital role for the survival of *P. gingivalis* (Shao *et al.*,). Accordingly, quorum sensing inhibitors (5Z)-4-bromo-5-(bromomethylene)-2(5 H)-furanone(2 mM) and D-ribose (50 mM) have been shown to reduce both *P.gingivalis* monospecies and *F. nucleatum* and *P.gingivalis* mixed-species biofilm development (Gerits *et al.*,). Ryu *et al.* have shown the potential of D-galactose as a QSI in inhibiting the biofilm formation of *F.nucelatum*, *P. gingivalis*, and *T. forsythia* induced by the AI-2 of *F. nucelatum* (Ryu *et al.*,).

From the RT-PCR results, we could see that *P. gingivalis* infection

resulted in an increase in the inflammatory cytokine expressions, suggesting the activation of bacteria's virulence. Subsequently, D-galactose treatment showed an expected inhibitory trend in IL-6, MMP-2, and MMP-9 in HOK-16B cells. Whether the suppression of *P. gingivalis* virulence by D-galactose could be attributed exclusively to AI-2 inhibition is not clear, but the consistent inhibitory trend indicates D-galactose plays an important role in decreasing inflammatory responses to *P. gingivalis*. Although MMP-2 also showed the inhibitory trend, the results were not statistically significant, leaving room for further investigation in order to verify and confirm our hypothesis on MMP-2.

Interestingly, IL-8 showed an opposite tendency. D-galactose treatment increased the expression of IL-8 in a dose-dependent manner. Previous studies have shown that *P. gingivalis* upregulates IL-6 and IL-8 at the transcriptional level, but *P. gingivalis*' protease, lysine gingipain, directly degrade cytokine extracellularly (Stathopoulou *et al.*,). Gingipain is known to subvert the host defense system and sustain chronic inflammation by suppressing IL-8 (Uehara *et al.*,). In our study, we have shown a consistent result where *P. gingivalis* infection increased the IL-8 gene expression. Yet, the gene expression was increased even more with the treatment of D-galactose. Our result indicates that D-galactose may be capable of enhancing the protective host proinflammatory responses by increasing the level of IL-8 expression. Increased expression of IL-8 may antagonize the effect of gingipain and promote the host defense system. However, this hypothesis remains to be investigated.

V. Conclusion

Our results indicate that D-galactose is capable of inhibiting proinflammatory responses and promoting host defense system in human epithelial cells infected with *P. gingivalis*. Although the exact mechanism behind the effect of D-galactose still remains elusive, the results provide an evidence of D-galactose as a potential QSI.

In the future, construction of *P.gingivalis* mutant strain defective in the AI-2 receptor may allow us to validate the role of D-galactose. In conclusion, D-galactose is a good candidate to attenuate virulence of *P. gingivalis* effectively, and its application in treating periodontitis seems promising.

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Tables and Figures

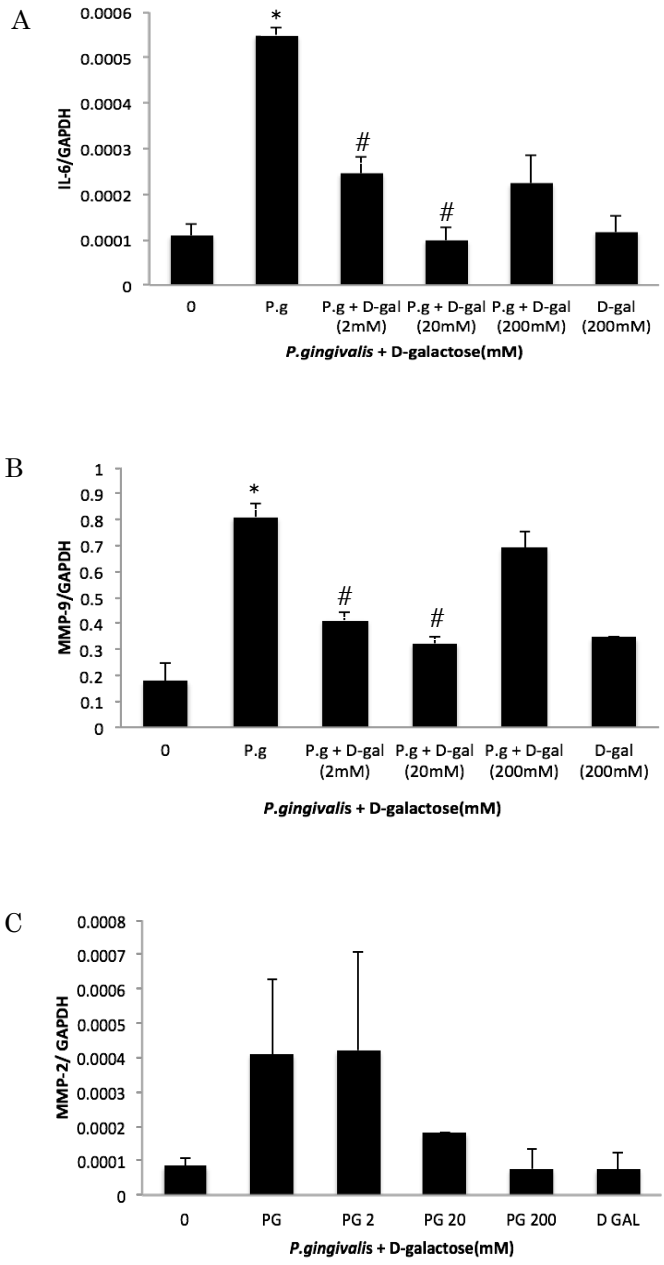

Fig.1 Inhibitory effect of D-galactose on (A)IL-6, (B)MMP-9, (C)MMP-2 expression

HOK-16B cells were cultured in six-well plates for 24 h. Then, the cells were infected with *P. gingivalis* at concentration of D-galactose of 2, 20, 200 mM for 24 h. HOK-16B cells infected only with *P. gingivalis* served as a positive

control, and cells treated only with D-galactose at 200 mM served as a negative control. The cells were harvested for RNA isolation.

*P < 0.05,compared with non-treated HOK-16B cells

#P < 0.05,compared with *P.g*-treated HOK-16B cell

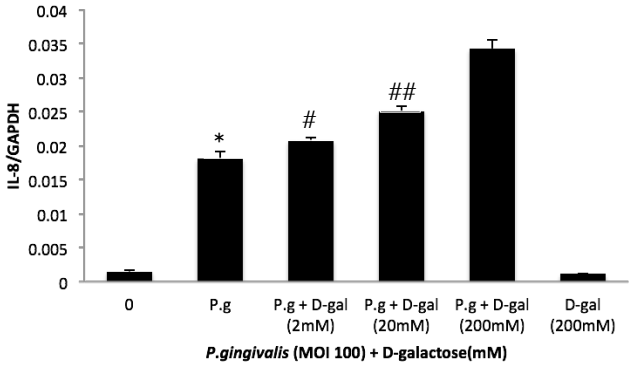

Fig.2 Positive effect of D-galactose on IL-8 expression

HOK-16B cells were cultured in six-well plates for 24 h. Then, the cells were infected with *P. gingivalis* at concentration of D-galactose of 2, 20, 200 mM for 24 h. HOK-16B cells infected only with *P. gingivalis* served as a positive control, and cells treated only with D-galactose at 200 mM served as a negative control. The cells were harvested for RNA isolation.

*P < 0.05,compared with non-treated HOK-16B cells

#P < 0.05,compared with *P.g*-treated HOK-16B cell

국문초록

1. 목 적

Quorum sensing (QS)이란 세포 군의 밀도 변화에 대한 신호 전달로서 유전자 발현의 조절에 관여한다. 구강 내에서도 QS 분자들이 바이오 필름 형성에 중요한 역할을 한다. 최근 연구들에서 QS 억제제 (QSIs)의 사용이 바이오 필름 형성을 억제하여 치주염 완화에 기여할 수 있음을 보였다. 따라서 본 논문의 목적은 in vitro 환경에서 QSI와 D-galactose 처치를 통해 *Porphyromonas gingivalis*에 의한 염증성 인자의 발현을 억제할 수 있는지 연구하는 것이다.

2. 방 법

P. gingivalis (ATCC 33277)를 감염시킨 human oral epithelial cell line (HOK-16B)에 QSI인 D-galactose를 2, 20, 200 mM의 농도에 24시간동안 처리하였다. 염증반응 평가를 위해서 real time RT-PCR을 통하여 IL-6, IL-8, MMP-2, MMP-9의 유전자 발현량을 측정하였다.

3. 결 과

IL-6, MMP-2, MMP-9에서 D-galactose 처리를 통한 유전자 발현의 억제를 확인할 수 있었다. D-galactose를 2 mM, 20 mM 농도로 처리하자 발현이 점점 더 유의미하게 억제되었으나, 최대 농도인 200 mM에서는 발현이 증가함을 보였다. 이는 통계적으로 유의하지 않은 범위로 추후 실험에서 D-galactose 농도의 범위 조절이 필요한 것으로 보인다. 이로써 D-galactose가 IL-6와 MMP-2의 발현을 억제하여 *P. gingivalis*에서의 QSI 작용을 한다는 점을 알 수 있었다.

반대로, IL-8에서는 D-galactose를 처리하였을 때 발현의 증가를 관찰할 수 있었다. D-Galactose 2, 20, 200 mM를 처리하자 발현이 점점 더 유의미하게 증가하였다. 이는 D-galactose가 IL-8의 발현을 증가시켜 숙주의 방어 체계를 향상시키는 것으로 보인다.

4. 결 론

본 실험을 통해 *P. gingivalis*에 감염된 human epithelial cell에서 D-galactose가 염증성 인자의 발현을 억제하고 숙주의 방어 체계를 향상시킬 수 있

음을 확인할 수 있었다. *P. gingivalis*의 병독성을 억제할 수 있는 D-galactose 역할은 장차 D-galactose가 QSI로서 치주염 치료에 적용될 수 있는 유망한 가능성을 가지고 있음을 시사한다.

주요어 : 치주염; *Porphyromonas gingivalis*; 쿼럼 센싱 억제제 (Quorom sensing inhibitors); D-Galactose

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