대한치주과학회지 : Vol. 36, No. 3, 2006

The role of CD14 and Toll-like receptors on the release of MMP-8 in the LPS recognition pathway

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I. INTRODUCTION

Periodontal diseases are infections that are caused by microorganisms which are colonized on the tooth surface at or below the gingival margin, and these initiate the breakdown of connective tissue attachment to the tooth and alveolar bone loss. For a periodontal pathogenesis, it is essential that the pathogen should be able to colonize the subgingival area and produce factors that either directly damage the host tissue or lead to the host tissue damaging itself. It has been designated that A. actinomycetemcomitans, P. gingivalis and B. forsythus were major periodontal pathogens among oral microflora[1]. Among them, P. gingivalis species produce collagenase, an array of proteases, hemolysins, endotoxin etc. and can inhibit migration of polymorphonuclear leukocytes (PMNs) across an epithelial barrier[2].

The pathogenicity of microorganisms relates as much to the particular host□s innate and/or inflammatory and/or immune capabilities, as to the virulence of the bacteria themselves. Some of substances produced by the biofilm can be directly injure host cells and tissues. Other microbial constituents may activate inflammatory or cellular and humoral immune systems which secondarily damage the periodontium. Among the enzymes released by bacteria, lipopolysaccharides (LPS) of Gram-negative microorganisms are capable of invoking both the inflammatory and immune responses as they interact with host cells. LPS binding protein(LBP), CD14, and Toll-like receptors(TLRs) are parts of major innate host defense inflammatory activation pathway that recognize and facilitate the host inflammatory response to LPS[3-5]. One role of

This study was supported by grant #03-2004-0033 from the SNUDH researchfund

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CD14 is to concentrate microbial component such as LPS on the host cell surface for further recognition by the innate host response system[6]. But two studies suggested that host cells recognize LPS antagonists after CD14 binding by an uncharacterized LPS recognition protein[7,8]. Recently TLR family of proteins has been identified as a crucial component of the mammalian innate host defense microbial recognition pathway that is able to functionally recognize different microbial ligands[9,10].

The neutrophil is the predominant leukocyte within the gingival crevice and pocket in both health and disease[11,12]. Neutrophils from the circulation are attached to the area via chemotactic stimuli elicited from microorganisms in the biofilm and histologically neutrophils can be seen transversing the gingival connective tissue in inflammation[13]. The periodontium is structurally comprised of fibrous elements including collagen, elastin and glycoproteins, minerals, lipids, water and tissue-bound growth factors. Matrix metalloproteinases(MMP) are responsible for remodeling and degradation of matrix components[14]. One of the MMPs receiving much attention is the neutrophil collagenase(MMP-8) which is found in higher concentration in inflamed gingival specimens than in clinically healthy gingival. The increased presence of MMP-8 in diseased over healthy sites[15], their increase during experimental gingivitis[16] and decrease after periodontal treatment[17,18] suggest that MMPs are involved in periodontal tissue breakdown. Among the MMPs both neutrophil and fibroblast collagenase have the unique ability of cleaving the triple extracellular matrix degradation which is not shared by the other members of the family.

Thus, the purposes of this study were to investigate the effects of LPS on the release of MMP-8 of neutrophils in vitro and to find how this release can be occurred through what kind of pattern recognition factor and finally to find whether transcriptional events can be involved in the MMP-8 signaling pathway.

II. MATERIALS AND METHODS

1. Subjects selection and isolation of neutrophils

Peripheral blood was collected from 16 healthy donors(13 males and 3 females, age 23-28 years), after the donors gave their informed consent to participation, and incubated in glass tubes containing EDTA. Neutrophils were isolated from EDTA blood by centrifugation using Ficoll-Hypaque®(Amersham Biosciences, Uppsala, Sweden) according to the manufacturer□s instructions and suspended in RPMI 1640 containing 2% human serum within 2 h after blood collection.

2. Preparation of A-LPS

The bacterial cells(50-ml cultures for A. *actinomycetemcomitans*[ATCC33384]) were harvested by centrifugation at 5,000 × g for 10 min at 4℃. The cells were then washed with phosphate-buffered saline(PBS). LPS-like materials were isolated by phenol-water extraction using an LPS extraction kit(iNtRON Biotechnology, Korea) according to the manufacturer's protocol. The first preparation was incubated with endonucleases(100 ㎍/㎖ of DNaseI and 100㎍/㎖ of RNaseA, 1 h at 37℃)

and subsequently proteinase K(250 μg/mℓ, 1 h at 50℃), and was then reextracted with phenol-water as described above. LPS was quantified by lyophilization and dry weight measurement. The LPS pellets were dissolved in endotox-in-free dH_2O at the concentration of 1mg/mℓ, and solubility was ensured by sonication.

To check the activity of the A-LPS, the expression of ICAM-1 of the THP-1 cells unstimulated or stimulated as described below was analyzed by flow cytometry. THP-1 cells cultured in RPMI 1640 medium containing 2% human serum were collected by centrifuging and washed with PBS. Cells(1×10^6 cells/100 μl) were reacted with anti-human ICAM-1 mAb(BD Bioscience, SanJose, CA, USA) at 4oC for 20 min. After washing, the cells were stained with fluorescein isothiocyanate(FITC)-labeled goat anti-mouse immunoglobulin G(IgG)(BD Biosciences) at 4℃ for 20 min. The cells were washed with PBS, and the protein expressions were analyzed using a fluorescence-activated cell sorter(FACSCalibur, Becton Dickinson, San Jose, CA, USA). The data were obtained by counting 15,000 cells. As a control for nonspecific binding, the cells were stained with isotype-matched IgG, and no nonspecific reactivity was observed.

3. Release, activation, and activity of MMP-8

Collected neutrophils were incubated at 37℃ under aerobic condition. The total content of MMP-8 was extracted from the neutrophils by treatment with 0.2% Triton X-100(Riedel-deHaen AG, Seelze, Germany) in the presence of 2% human serum. To detect MMP-8 activity, neutrophils(2.0×10^6 cells/mℓ) were incubated with 1 μg/ml A-LPS for 24hours. The amount of MMP-8 released extracellulary is expressed in relation to the total MMP-8 amounts of the neutrophils.

According to the time kinetics for the release of MMP-8, the neutrophils(2.0×10^6 cells/mℓ) were pre-incubation with 2μg/mℓ anti-TLR2 antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), 20μg/mℓ anti-TLR4 monoclonal antibody(HTA125, eBioscience, USA) for 1hour at 37℃, and then incubated with 1μg/mℓ A-LPS for 9 hour at 37℃.

In some experiment, neutrophils were pre-incubated with anti-CD14 monoclonal antibody(Coulter Clone MY4, 1/200 dilution, Coulter Corporation, Miami, FL, USA) for 30 min at 4oC and incubated with 1 μg/mℓ A-LPS for 9 hours at 37℃, or incubated with microtubule polymerization inhibitors(25μm colchicines, 10μm nocodazole, and 2μm demecolcine) and actin polymerization inhibitor(2μm cytochalasin B) in the absence or presence of A-LPS(1μg/mℓ) for 9 hours at 37℃. These inhibitors were purchased from Sigma-Aldrich Co.(St. Louis, MO, USA).

In the other experiment, the cells were pretreated with an NF-κB activation inhibitor, L-1-tosylamido-2-phenylethyl chlormethyl ketone(TPCK)(10μm)(Sigma-Aldrich Co. St. Louis, MO, USA) for 1 hour at 37℃ before A-LPS and then incubated for 9 hours at 37℃.

The cell viability was examined using the trypan blue exclusion test and was greater than 98% after isolation and maintained at greater than 92% after cell incubation with or without A-LPS, several antibodies and inhibitors. After the incubation of neutrophils, the conditioned medium was collected and mixed with protease inhibitor(cØmplete, Mini, Roche Applied Science, Penzberg, Germany) and used for the determi-

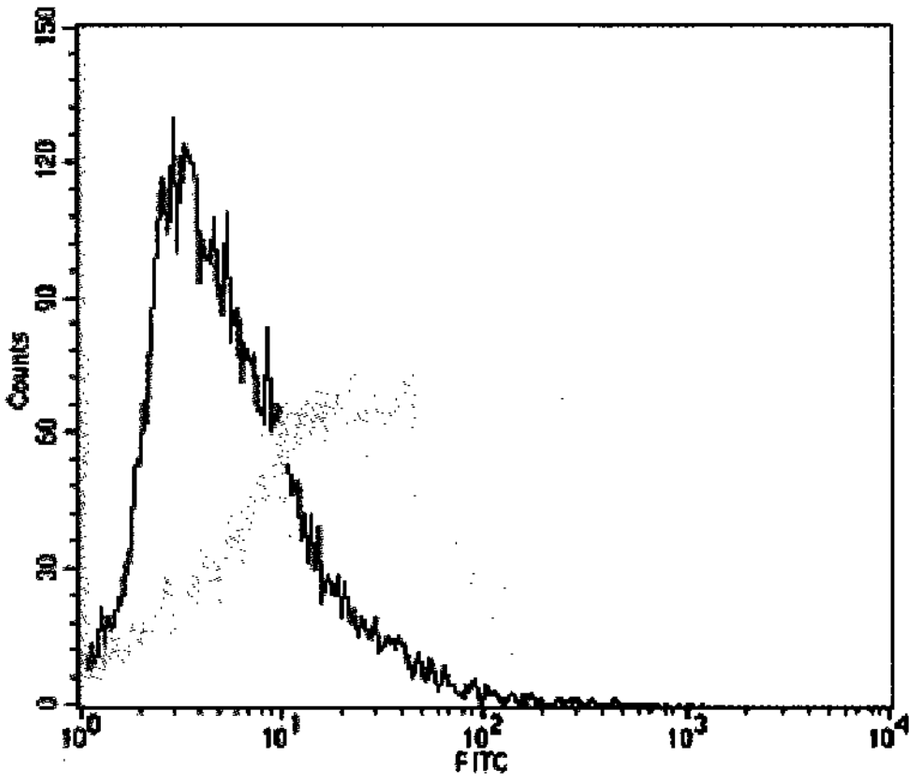

Figure 1. Flow cytometric analysis of the activity of A-LPS on ICAM-1 expression in THP-1 cells. ICAM-1 expression was analysed by fluorescence-activated cell sorting by using anti-human ICAM-1 monoclonal antibody and FITC-labeled IgG.

nation of the released amount of MMP-8 by using ELISA kits obtained from Amersham Biosciences (Amersham Biosciences, Uppsala, Sweden).

4. Statistical analysis

The statistical significance of the differences in the release of MMP-8 between untreated and A-LPS treated cells was evaluated by ANOVA test ($p<0.05$).

III. RESULTS

1. Time kinetics for the release of MMP-8

The activity of A-LPS on neutrophils were examined via expression of ICAM-1 by THP-1 cells. A-LPS strongly induced ICAM-1 expression (Figure 1). Pure A-LPS induced a time-dependent releases of MMP-8 from the neutrophils. When the cells were incubated for determination of kinetics of the release of MMP-8 from

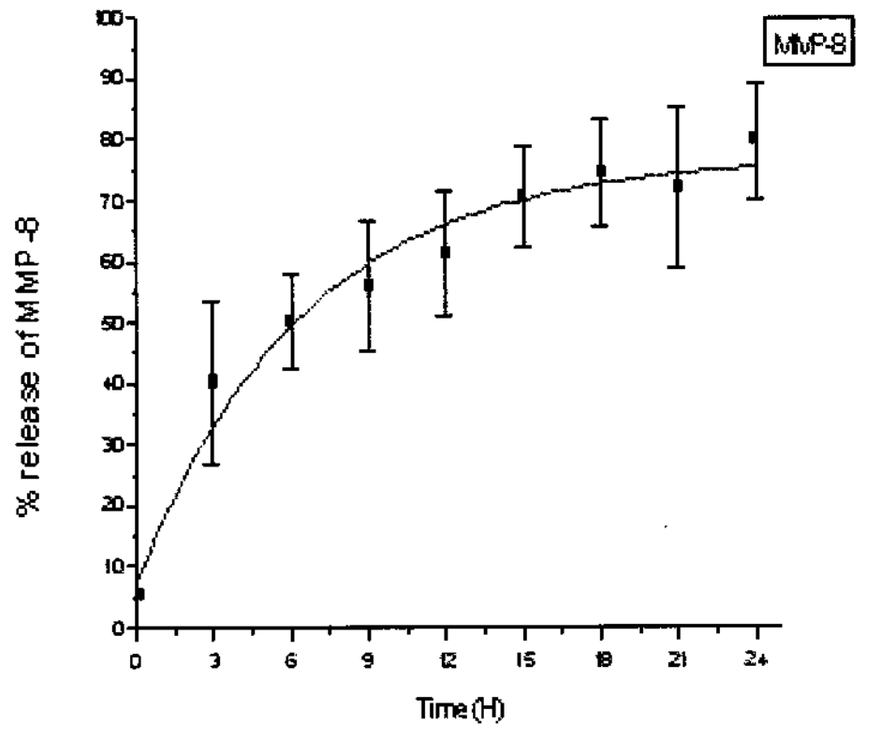

Figure 2. The kinetics of the release of MMP-8. Neutrophils(2.0 x 106 cells/ml) were incubated with Triton X-100 and they also were incubated with A-LPS(1㎍/㎖) for 24h. The release is expressed in percent of the total MMP-8 amount extracted from the neutrophils by treatment with Triton X-100. The released amount of MMP-8 was determined by ELISA. Values are means ± SD for 8 donors'samples

human neutrophils, they showed time-dependent but stag after 12 hours(Figure 2).

2. Effect of CD14 inhibitor and Toll-like receptor antagonists

When the cells were preincubated with anti-CD14 monoclonal antibody, A-LPS induced the release of MMP-8 was significantly inhibited and its level decreased almost to the level of the culture treated with anti-CD14 monoclonal antibody alone(Figure 3). The anti-CD14 monoclonal antibody alone did not significantly affect the MMP-8 release. HTA125, the TLR4 monoclonal antibody didn□t affect the release of MMP-8 and anti-TLR2 antibody also didn't affect the release of MMP-8 (Figure 4). These results suggest that MMP-8 release is induced by A-LPS via CD14, not TLR2 and

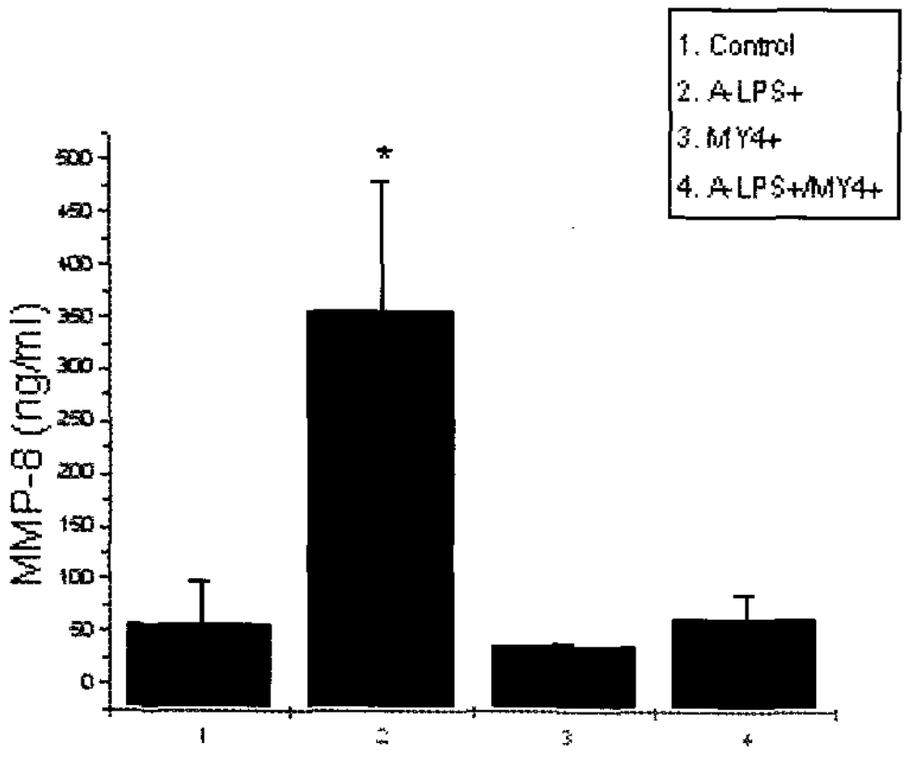

Figure 3. Effect of anti-CD14 monoclonal antibody on A-LPS induced MMP-8 release. Neutrophils(2.0 x 106 cells/ml) were pre-incubated with anti-CD14 monoclonal antibody(1/200 dilution) for 30min, and then incubated with A-LPS(1㎍/㎖) for 9h. The released amount of MMP-8 was determined by ELISA. Values are means ± SD for 8 donors' samples. * There was significant difference (P<0.05).

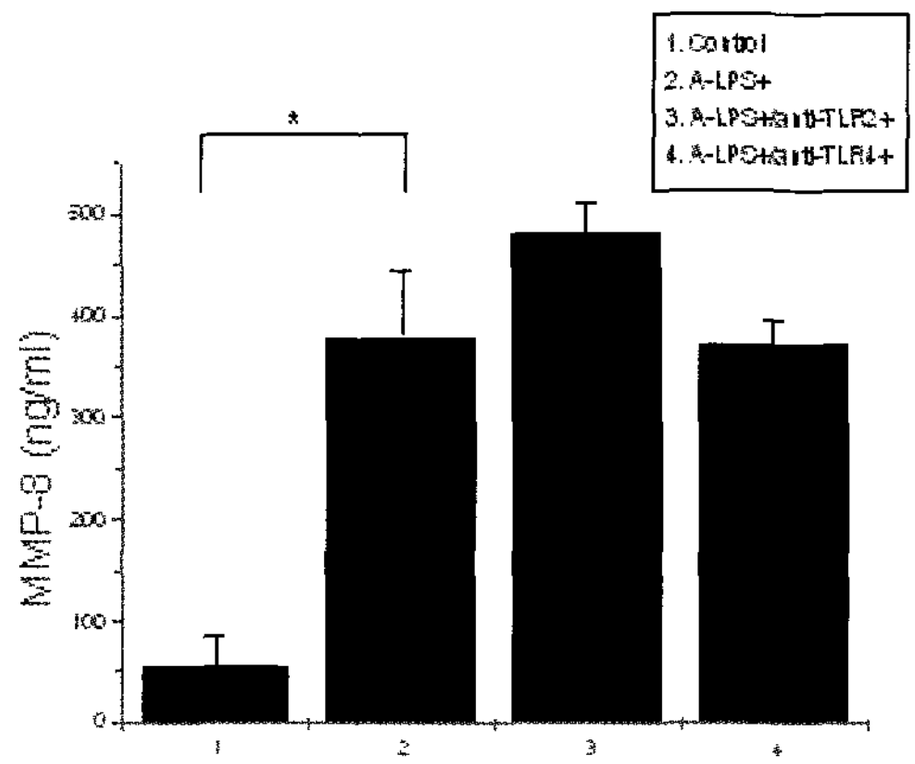

Figure 4. Effect of anti-TLR antibodies on A-LPS induced the release of MMP-8. Neutrophils(2.0 X 106 cells/ml) were pre-incubated with HTA 125(20㎍/㎖) or anti-TLR 2 antibody(2㎍/㎖) for 9h. The release of MMP-8 was determined by ELISA. Values are means ± SD for 15 donors'samples. * There was significant difference (P<0.05).

TLR4.

3. Effect of an NF-κ B activation inhibitor

TPCK(an Iκ B protease inhibitor) was used to inhibit the effects of the A-LPS in order to analyze the involvement of NF-κ B activation in the signal transduction pathway leading to release of MMP-8 from human neutrophils by the A-LPS. A-LPS-stimulated NF-kB activation was completely blocked by the pre-treatment with TPCK. TPCK clearly suppressed LPS-induced the release of MMP-8 from neutrophils(Figure 5). These results suggest that A-LPS induced the release of MMP-8 is regulated via the CD14 and NF-κ B pathway.

4. Effect of cytosol inhibitors

In the first step to elucidate the mechanism of the release of MMP-8, the effects of tubulin polymerization inhibitors(colchicine, nocodazole and demecolcine) and actin polymerization inhibitor(cytochalasin B) on the release of MMP-8 were investigated. Colchicine, nocodazole and demecolcine didn□t suppressed A-LPS induced the release of MMP-8 but cytochalasin B inhibited MMP-8 release greater than three tubulin polymerization inhibitors to 52% of the A-LPS induced level(Figure 6). However, each inhibitor alone did not demonstrate a significant effect on the release of MMP-8(data not shown).

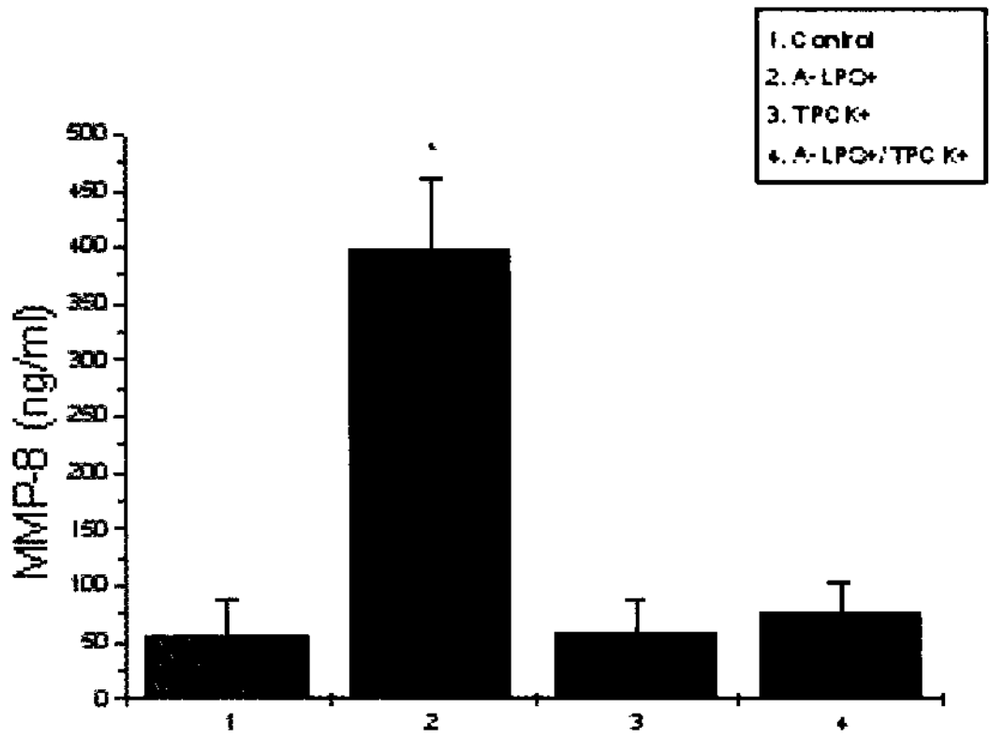

Figure 5. Effect of NF-κ B inhibitors on A-LPS induced NF-κ B activation and MMP-8 release. Neutrophils(2.0 X 106 cells/ml) were pre-incubated with TPCK(10μ M) for 1h and then incubated with A-LPS for 9h. The release of MMP-8 was determined by ELISA. Values are means ± SD for 9 donors□samples. * There was significant difference in the presence of A-LPS without TPCK (P<0.05).

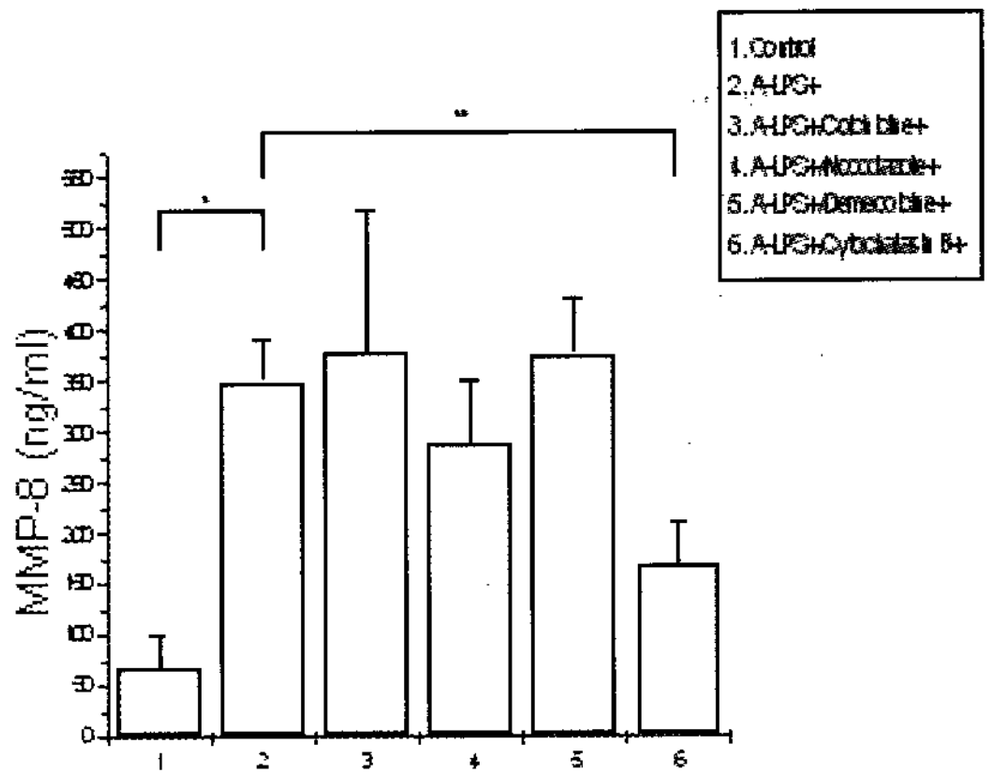

Figure 6. Effect of cytoskeleton-related inhibitors on A-LPS induced MMP-8 release. Neutrophils(2.0 X 106 cells/ml) were incubated with colchicine(25μ M), nocodazole(10μ M), demecolcine(2μ M) or cytochalasin B(2μ M) in the presence of A-LPS(1μg/㎖) for 9h. The release of MMP-8 was determined by ELISA. Values are means ± SD for 15 donors'samples. * There was significant difference between control and A-LPS presence (P<0.05). ** There was significant difference between A-LPS presence and A-LPS with cytochalasin B (P<0.05).

IV. DISSCUSSION

One of the conclusions supported by the present findings is that a secretion of extracellular activation of MMP-8 can be stimulated by the periodontopathogen A. actinomycetemcomitans in human neutrophils. The results are in line with previous observations showing that P-LPS[19] and leukotoxin[20] of A. actinomycetemcomitans induce human neutrophil degranulation. The degranulation is an active but slow process that differs from the leukotoxin-induced granulation[20]. In the present study, the results showed the degranulation reaches 50% of total amount of MMP-8 release after 6h of the interaction. It is known that LPS stimulates the release of inflammatory cytokines including IL-1β , TNF-α and IL-6 via CD14-TLR-NF-κ B pathway in polymorphonuclear leukocytes and monocytes[21]. Sugita et al.[22] demonstrated anti-CD14 monoclonal antibody (MY4) partially blocked P-LPS-induced NF-κ B activation in human neutrophils and CD14 was closely related to P-LPS signal transduction. From those findings and our results that A-LPS-induced the release of MMP-8 was suppressed by anti-CD14 monoclonal antibody(MY4) and NF-κ B inhibitor, it is suggested that A-LPS stimulates the release of MMP-8 from neutrophils via the LPS signal pathway including CD14 and NF-κ B.

TLR2 and TLR4 have been reported to function as LPS-signaling receptors[23,24]. TLR2 is

associated with the recognition of lipoproteins and peptidoglycan and TLR4 is related to the recognition of LPS[25]. Tabeta et al. and Wang et al. reported that anti-TLR4 monoclonal antibody inhibited P-LPS-induced IL-6 gene expression[26] and IL-1 production[27] in human gingival fibroblast. However, we found that anti-TLR2 antibody and anti-TLR4 monoclonal antibody did not show significantly inhibition of A-LPS-induced the release of MMP-8 from human neutrophils. The innate host response to lipopolysaccharide(LPS) obtained from P. gingivalis is unusual in that different studies have reported that it can be an agonist for TLR2 as well as an antagonist or agonist for TLR4[28]. Hirschfeld et al. showed that P-LPS induced IL-6 production in the human astrocytoma cell line that overexpressed TLR2, but not TLR4, and suggested that P-LPSs do not signal through TLR4[29]. The signals of most LPSs are transmitted via TLR4, but the P-LPS signal is recognized by TLR2 in macrophages because this LPS has a different structure from many Gram-negative LPSs[29]. The present findings suggest that A-LPS couldn□t act not only via TLR4 but also via TLR2 or other signal transducers in neutrophils. Therefore the A-LPS signal for the release of MMP-8 from human neutrophils may not be mediated through TLR4 and TLR2.

Degranulation is preceded by the fusion of the granule membranes with plasma membrane of the neutrophil, which involves rearrangement of the cytoskeleton via specific intracellular reactions. Thus, degranulation is not a passive leakage of intracellular components, but the effect of activation of an intracellular signaling triggered by the interaction of A-LPS with the neutrophils.

Ryder et al. showed microtubules may provide a tracking mechanism whereby lysosomes are specifically parceled out to phagocytic vacuoles[30]. In this study, we used 3 types of microtubule polymerization inhibitors, colchicine, nocodazole, and demecolcine. Colchicine can cause the mitotic arrest of dividing cells at metaphase by interfering with microtubule organization, in particular, those of the mitotic spindle[31]. Nocodazole showed biochemical actions that disrupt microtubules by binding to β-tubulin and preventing formation of one of the two interchain disulfide linkages, thus inhibiting microtubule dynamics, disruption of mitotic spindle function, and fragmentation of the Golgi complex[32]. Demecolcine depolymerizes microtubules[33]. But, none of the microtubule polymerization inhibitors showed inhibition of the release of MMP-8. Previous other studies reported that ammonium can inhibit degranulation, decrease cytoskeletal actin, and increase actin depolymerization rates[34] and the amounts of actin and myosin in rabbit neutrophils were decreased by cytochalasin B which caused a shift in the amount of actin and myosin from the cytoskeleton to the cytoplasm[35]. In the present study, actin polymerization inhibitor significantly suppressed A-LPS-induced the release of MMP-8 from neutrophils, but tubulin polymerization inhibitors did not. These results could be explained that first, the A-LPS signal to the release of MMP-8 was dominantly affected by actin polymerization which determine the shape of the cell□s surface and second, the nucleation of tubulin needs more time than that of actin. Third, culture time is too long to eval-

uate the polymerization inhibitors. To evaluate thses assumptions, we need further study. These results supported the existence of the release of MMP-8 mechanism in addition to that of cell death. It is suggested that several NF-κ B inhibitors suppressed A-LPS-induced the release of MMP-8 and there is an associa-tion of NF-κ B activation and the release of MMP-8, but the exact mechanism between these two responses caused by A-LPS is not elucidated. It is known that E.coli LPS (E-LPS) regulates protein production and secretion by its effects on microtubules and microfilaments depolymerization, and that NF-κ B activation is modulated by Iκ B degradation with ubiquitin, a component of the microtubule organization in-duces NF-κ B activation[36]. The present find-ings and those of other studies suggest that MMP-8 may be released by LPS stimulation via microfilament-dependent pathway with NF-κ B activation or a microfilament-dependent pathway.

V. CONCLUSIONS

The purpose of present study was to inves-tigate whether the release of MMP-8 by A-LPS is induced via the CD14-TLR-NF-κ B pathway and the cellular mechanism of the release of MMP-8 in human neutrophils. According to our experiment, first, we could concluded that the release of MMP-8 was induced by A-LPS and that release occurred through CD14 and NF-κ B signal pathway. Second, the release of MMP-8 was not dependent on TLR2 or TLR4 signal pathway. Third, the release of MMP-8 was de-pendent on actin polymerization process not on microtubule polymerization process. Within the limit of our experiment, it is concluded that MMP-8 release is induced by A-LPS via the CD14 and NF-κ B signal pathway in human neutrophils and may be dependent on micro-filament systems.

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-국문초록-

치질 다당질 인지경로에서 기질금속단백분해효소-8 분비에 대한 CD14와 Toll-like receptors의 역할 연구

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2. 성균관대학교 의과대학 치과학교실

1. 연구배경

교원질 분해작용을 하는 호중구의 세포질 효소인 기질금속단백분해효소-8은 치주질환, 류마티스 관절염, 그리고 궤양결장염과 같은 염증성 질환에서 농도가 증가한다고 알려져 있다. 최근에는 A. actinomycetemcomitans의 leukotoxin이 사람호중구에서 기질금속단백분해효소-8의 분비를 유도하는 것이 보고되었다. 이 연구의 목적은 선천면역 체계에서 세포표면 항원무리14, Toll-like 수용기, 그리고 NF-κ B경로를 통하여 A. actinomycetemcomitans의 지질다당질로 유도된 기질금속단백분해효소-8의 분비 여부와 세포기전을 알아보고자 하였다.

2. 연구재료 및 방법

건강한 개인 제공자(남자 13명, 여자 3명)로부터 얻은 개개인의 20㎖ 말초혈액을 제조사의 지침에 따라 호중구를 추출한 후 항세포표면 항원무리14와 함께 4℃에서 30분간 전배양 한 후, 37℃에서 9시간 동안 배양시켰다. 추출한 호중구에 Toll-like 수용기 억제제 또는 NF-κ B 억제제인 TPCK를 첨가한 후 37℃에서 1시간 동안 전배양하고 37℃에서 9시간 동안 배양시켰다. 호중구에 세포뼈대 억제제인 cholchicine, nocodazole, demecolcine, 그리고 cytochalasin B를 A. actinomycetemcomitans의 지질다당질과 함께 37℃에서 9시간 동안 배양시켰다. 기질금속단백분해효소-8 분비량은 효소면역측정법을 통하여 결정하였다. 통계처리는 일원배치 분산분석법을 이용하였다($p<0.05$).

3. 결과

A. actinomycetemcomitans 지질다당질은 기질금속단백분해효소-8의 분비를 증가시켰다. 기질금속단백분해효소-8의 분비는 항세포표면 항원무리14에 의해서 억제되었지만, 항 Toll-like 수용기2, 항 Toll-like 수용기4 항체는 억제시키지 못했다. NF-κ B 억제제는 A. actinomycetemcomitans의 지질다당질로 유도된 NF-κ B 결합 활성도와 기질금속단백분해효소-8 분비를 억제하였다. 미세섬유 중합반응 억제제는 A. actinomycetemcomitans의 지질다당질로 유도된 기질금속단백분해효소-8의 분비를 억제시켰으나, 미세관 중합반응 억제제는 억제시키지 못했다.

4. 결론

위의 연구결과를 종합하여 볼 때, 기질금속단백분해효소-8은 A. actinomycetemcomitans의 지질다당질로 유도되며, 세포표면 항원무리-NF-κ B 경로를 통하여 분비되고, 이 분비 과정은 미세섬유 계통이 관여하는 것으로 보인다.

주요어 : 세포표면 항원무리14, Toll-like 수용기 억제제, 기질금속단백분해효소-8, A. actinomycetemcomitans지질다당질