Lipoteichoic Acid from *Staphylococcus aureus* Induced Expression of MMP-9 in Human Middle Ear Epithelial Cells

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Major in Medicine

Department of Medical Sciences

The Graduate School, Ajou University

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This certifies that the dissertation of Jung Hwan Song is approved.

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June, 22nd, 2012

감사의 글

아무것도 모르고 아련한 동경에서 시작되었던 의학도의 길, 선배 의학도들이 일구어 놓은 길을 힘겹게 달려왔던 지난 날들을 되돌아보게 됩니다. 수 많은 의학서적에 담겨있던 한 구절 한 구절들이 생명의 소중함을 아끼는 여러 의학자들의 피땀 어린 연구들이었다는 것에 의학 공부를 시작한지 20년이 넘게 지난 지금에서야 고개를 숙이게 됩니다. 진료실에서 환자들에게 “제가 친구에게 할 수 있는 이야기와 환자분에게 할 수 있는 이야기는 다릅니다. 제가 환자분에게 할 수 있는 이야기는 제 생각이 아닌 제가 공부한 교과서에 나온 이야기만 드릴 수 있습니다”라는 이야기를 이따금 하게 됩니다. 선배 의학도들의 힘으로 지금까지 올 수 있었고, 또 새로운 의학의 발전에 작은 나사라도 될 수 있다는 기쁨이 미숙한 저를 웃음짓게 합니다. 배움의 끝은 없는 것이겠지만 이제 또 새로운 시작을 할 수 있도록 지금까지 끊임없는 격려와 질책으로 저를 이끌어 주신 많은 분들께 이 글을 통해 감사의 마음을 전하고자 합니다.

지도교수님이신 김현준 교수님께 감사 드립니다. 전공의 시절부터 연구강사 시절까지 저를 항상 큰형의 모습으로 따뜻하게 감싸주신 모습 지금도 아른 합니다. 코 수술을 전공으로 사는 저에게 힘든 역경이 있었던 시절 선생님의 진심 어린 충고와 보살핌이 없었다면 지금의 저의 모습은 없었을 것입니다. 항상 열린 아이디어로 새로운 것을 탐구하시는 선생님의 모습에 존경과 찬사를 보내며, 본 논문이 완성될 수 있도록 지도 편달해 주신 것 진심으로 감사 드립니다.

박기현 교수님께 감사 드립니다. 이비인후과라는 전공을 선택한 저에게 많은 학문적 가르침과 인생에 대한 말씀들은 오늘의 제가 있게 해주신 큰 힘이었습니다. 때로는 아버지 같은 엄격함으로 때로는 어머니 같은 자상함으로 항상 보살펴 주신 선생님의 은혜에 진심으로 감사의 말씀 드립니다. 예전과 같은 건강한 모습을

곧 뵐 수 있기를 기도합니다.

항상 바쁜 임상의 나날들에도 끊임 없는 연구의 자세로 많은 지도 편달해 주신 정연훈 교수님께도 감사의 말씀 드립니다. 따뜻함과 온화함 속에서도 언제나 냉철함으로 현명한 조언을 주시는 김철호 교수님 감사 드립니다. 바쁘신 와중에도 논문 심사에 고생해 주신 세브란스 병원 김창훈 교수님께 감사 드립니다. 함께 보낸 시간이 많지 않아 아쉽지만, 이번 논문에 크나큰 힘이 되어준 박헌이 교수님 감사 드립니다. 이번 실험에 큰 도움을 주신 연구원 선생님들께 감사 드립니다.

힘든 시간을 형제처럼 동거동락하며 지금의 제가 될 수 있게 해준 또 한 분의 스승인 최윤석 원장님께 감사의 말씀을 전하고 싶습니다.
새롭게 태어나는 저희에게 항상 평온함과 현명함을 가르쳐주시는 배준호 선생님께도 감사의 말씀 드립니다. 이번 논문에 마무리에 큰 도움을 주고 항상 고마운 동생인 이진석 선생님. 감사합니다.

제 인생의 가장 큰 스승이자 그 이름만 불러도 가슴이 찡해지는 어머니, 감사합니다. 지금은 먼 곳에서 지켜봐 주시고 계실 아버지, 감사합니다. 보고 싶습니다. 저를 위한 기도로 매일 새벽잠을 설치시는 할머니, 감사합니다. 건강하세요.

마지막으로 언제나 든든한 기둥이 되어주는 아내와 친구 같은 딸 민주, 어릴 적 저를 보는 듯한 아들 민준에게 고마움을 전합니다.

2012년 5월

저자 씀

-ABSTRACT –

Lipoteichoic acid from *Staphylococcus aureus* induced expression of MMP-9 in human middle ear epithelial cells

Change in matrix metalloproteinases (MMPs) and regulation of their tissue inhibitors of metalloproteinases (TIMPs) could play certain role in the pathogenesis of otitis media. This study was designed to evaluate the modulation of MMPs and TIMPs in middle ear epithelium by lipoteichoic acid (LTA) isolated from *Staphylococcus aureus*.Human middle ear epithelial cells (HMEE) were treated with LTA. Changes of MMP and TIMP and signal transduction were examined by PCR, ELISA, zymography and western blot. LTA isolated from *S. aureus* increased MMP-9 mRNA expression and secretion in HMEEC, whereas no effect on the expressions of MMP-1, 2, 3, 7 and TIMP-1, 2 was observed. Only the extracellular signal-regulated kinases (ERK) 1/2 of mitogen-activated protein kinase (MAPK) pathway was activated by LTA. And the MEK1/2 inhibitor prevented the expression of MMP-9 by LTA.LTA increased the activity of MMP-9, not TIMPs in middle ear epithelia, suggesting that disturbed balance between MMP-9 and TIMPs could play an active role in LTA-induced otitis media.

Key words : matrix metalloproteinase; middle ear; otitis media

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I. INTRODUCTION

Otitis media (OM) is the most common diagnosis in children who visit physicians for illness in the United States (Bluestone and Klein, 1996). The children in the United States have 9.3 million episodes of acute otitis media.The annual cost of the medical and surgical treatment of otitis in the United States is over $3 billion (Berman, 1995) .Although microbial infection and eustachian tube dysfunction are well known to be involved in the pathogenesis of OM(Iwano et al., 1993; Revai et al., 2008), more thorough understanding of the pathophysiology and the inflammatory events that occur during this disease process is needed to provide more effective treatment. In particular, evidences suggest that chronic inflammatory responses to middle ear pathogens may be important in the development of OM and raise the possibility that control of this response may reduce OM-related morbidity(Hall-Stoodley et al., 2006; Lieberthal, 2006).

During bacterial infections, bacterial constituents can induce the production of various host factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and platelet-activating factor (PAF) which can cause inflammation(Vogels et al., 1994; Cavaillon et al., 2003). Lipoteichoic acid (LTA), component of the Gram-positive bacterial envelope, is analogous to lipopolysaccharide (LPS) in structure and function(Finney et al., 2012). The glycolipid anchor of LTA, which is analogous to the lipid A of LPS, activates the immune response and induces the proinflammatory cytokines (Morath et al., 2005). Moreover, LTA can activate matrix metalloproteinases (MMPs)(Souza et al., 2009; Hsieh et al., 2010). There was several reports of problems for commercial LTA, endotoxin contamination could alter the result of the study and structural damage of LTA was another problem (Gao et al., 2001; Morath et al., 2002; Morath et al., 2005). Highly purified and structurally intact LTAcan minimize the distortionof result of the study.MMPs are zinc-binding proteolytic enzymes that participate in degrading and remodeling of the extracellular matrix in various physiological and pathological conditions(De et al., 2005). MMPs have three distinct domains. Firtst domain is Zinc-containing cayalytic domain. Second domain is N-terminal pro-peptide domain, chelates zinc ion with cystein residue, it holds the enzyme in a latern pro-form. Third doma is C-terminal haemopexin-like domain for substrate recognition (Kelly and Jarjour, 2003).

Several cytokines are known to increase release of MMPs and cell migration(Brown et al., 2007). These endopeptidases participate in the migration and infiltration of immune cells, and they also regulate inflammation and immunity by acting on proinflammatory cytokines and chemokines, and altering the circulatory concentration of these mediators(Chakraborti et al., 2003; Parks et al., 2004).Tissue inhibitor of Metalloproteinase (TIMPs) are low-molecular-weight endogenous inhibitor of MMPs(Woessner, 2002). Four mammalian TIMPs are identified (Moore and Crocker, 2012). TIMPs neutralize MMPs activity via interaction of their N-terminal domain with the catalytic site of MMPs(Edwards et al., 1996). It is reported that changes in MMPs and their tissue inhibitor of metalloproteinases (TIMPs) regulations could play certain role in the pathogenesis of OM (Jang et al., 2006; Moon et al., 2008) .In acute otitis media, characterized by rapid bacterial proliferation with pus accumulation inside middle ear cleft, bacterial endotoxin has been demonstrated to cause a rise in MMP-9 but not MMP-2 (Albert et al., 2003). In otitis media with effusion, MMP-2 and MMP-9 activity was higher in mucoid effusion than serous effusion (Jennings et al., 2001). Cholesteatoma demonstrates proteolytic activity with destructive process. In cholesteatoma specimen MMP-9 is increased, but not MMP-2(Schmidt et al., 2001).

To our best knowledge, however, there is no study so far on the relationship between LTA and MMPs in the middle ear epithelium. This study was, therefore, designed to evaluate the modulation of MMPs and TIMPs in middle ear epithelium by highly purified LTA from Staphylococcus aureus.

Mitogen-activated proteinkinase(MAPK) signaling pathway known to have certain role in controlling MMP regulation. There are three well-characterized subfamilies of MAPKs. Extracelluar signal-regulated kinases, ERK1 and ERK2 ; the c-Jun NH2-terminal kinase, JNK; and P38 enzymes (Johnson and Lapadat, 2002).We also try to find out which signal transduction play a role in MMP regulation.

II. MATERIALS ANDMETHODS

A. Cell Culture and LTA Treatment

Cells used in the present study were human middle ear epithelial (HMEE) cells from a transformed cell line stored in liquid nitrogen (These cells were generously provided by Dr D. Lim, House Ear Institute). From the stock supply of HMEE cells, aliquots were thawed and grown in a 50/50 mixture of Bronchial Epithelial Cell Basal Medium (Gibco, Carlsbad, CA, USA) and Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA). Each 500 ml portion of media was supplemented with antibiotic/antimycotic solution (1000 U/ml of penicillin G sulfate, 1000 μg/ml of streptomycin sulfate, and 250 ng/ml of amphotericin B; Invitrogen, Carlsbad, CA, USA) and BEGM aliquots (Clonetics, San Diego, CA, USA) which contain 2 ml of 13 mg/ml bovine pituitary extract, 0.5 ml of 5 mg/ml insulin, 0.5 ml of 0.5 mg/ml hydrocortisone, 0.5 ml of 0.1 mg/ml retinoic acid, 0.5 ml of 10 mg/ml transferrin, 0.5 ml of 6.5 mg/ml triiodothyronine, 0.5 ml of 0.5 mg/ml epinephrine, and 0.5 ml of 0.5 mg/ml human epidermal growth factor. When the cells reached approximately 70% confluency, they were removed from the flask using Trypsin/EDTA passaging. The cells were counted by hemocytometer and plated in 12 well plates at approximately 1 x 105 cells per cm2. The cells were grown in a humidified atmosphere at 37°C containing 95% air and 5% carbon dioxide. Cells were grown to confluency and used for experimentation.

We had used the highly purified LTA that was provided by Dr. Han, Department of Oral Microbiology, Dental College, Seoul National University. The LTA was extracted from S. aureus by organic solvent and purified through various chromatographies(Han et al., 2006). The LTA preparation used in this study was proved to contain less than 5 pg of endotoxin/mg LTA (Han et al., 2006).

B. RT-PCR

RNA was extracted from the HMEE cells treated with LTA by Trizol reagent (Invitrogen, Carlabad, CA, USA). After quantitative analysis of the RNA extracted, reverse transcription polymerase chain reaction (RT-PCR; Superscript Ⅲ First Strand kit; Invitrogen, Carlabad, CA, USA) was carried out to synthesize cDNA. The synthesized cDNA was used

as template of PCR for analysis of gene expression of MMP-1, -2, -3, -7, -9 and TIMP-1, -2. The primer sequences and PCR conditions of MMPs and TIMPs are summarized in Table 1.

Table 1.Primers and cycling conditions for detection of MMPs and TIMPs transcripts by PCR.

MMPs/TIMPs	Oligonucleotides	Temp./time (s)	Cycles
MMP-1	Up: 5'-GGTGATGAAGCAGCCCAG-3' Down: 5'-CAGTAGAATGGGAGAGTC-3'	60 °C/30	35
MMP-2	Up: 5'-ACCTGGATGCCGTCGTGGAC-3' Down: 5'-TGTGGCAGCACCAGGGCAGC-3'	66 °C/30	30
MMP-3	Up: 5'-GATTACAGACATGGGTCACG-3' Down: 5'-GAATTCACATCACTGCCACC-3'	60 °C/30	35
MMP-7	Up: 5'-TGGCCTACCTATAACTGGAA-3' Down: 5'-TCCCTAGACTGCTACCATCC-3'	55 °C/30	28
MMP-9	Up: 5'-GGGGAAGATGCTGTTCA-3' Down: 5'-GGTCCCAGTGGGGATTTACA-3'	55 °C/30	28
TIMP-1	Up: 5'-GCTGGGTGGTAACTCTTTAT-3' Down: 5'-CACCTTGCCTGCCTGCCTC-3'	61 °C/55	45
TIMP-2	Up: 5'-GGTCCTCGATGTCGAGAAAC-3' Down: 5'-CTGGATGGACTGGGTCACAG-3'	61 °C/55	45
β-Actin	Up: 5'-GACCTGACTGACTACCTCAT-3' Down: 5'-TCGTCATACTCCTGCTTGCT-3'	61 °C/20	25

C. Real-Time PCR

Real-time PCR was carried out according to the standard protocol provided with the SYBR ® Green protocol (SensiMixTMLowRef; Quantace, USA). The upstream and

downstream primers for β-actin were 5'-GGCACCCAGCACAATGAAG-3' and 5'-CCGATCCACACGGAGTACTTG-3', respectively. The upstream and downstream primers for MMP-9 were 5'-TGGGCTACGTGACCTATGACAT-3' and 5'-GCCCAGCCCACTCCACTCC TC-3', respectively. The PCR conditions for β-actin and MMP-9 were 95℃ for 10min, followed by 45 cycles of 95℃ for 15sec, 60℃ for 20sec, and 72℃ for 40sec. The SYBR® Green Ⅰ fluorescence was measured after extension step. Expression of MMP-9 mRNA was quantified using the threshold cycle (Ct) method and was standardized to the value of intrinsic β-actin mRNA expression.

D. Enzyme-Linked ImmunosorbentAssay (ELISA)

To determine relative quantity of MMP-9 released in the supernatants of HMEE cells which had been treated with 0, 0.1, 1, 5, 10, 20 μg/ml LTA for 24h, an enzyme-linked immunosorbent assay (ELISA) was performed. The levels of MMP-9 were measured using commercially available ELISA kits (R&D systems, Minneapolis, MN, USA). Parallel measurements of standards and samples were performed by applying them to an antibody-precoatedmicroplate. The entire procedure was carried out according to the manufacture's protocol. For total MMP-9 measurement, the samples were acidified before assay. Colorimetric results were read at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). MMP-9 concentration was determined from the best linear curve drawn with the absorbance of standards versus their concentrations.

E. Gelatin Zymography

To determine the activity of MMP-9 in the supernatants of the HMEE cells which had been treated with 0, 0.1, 1, 5, 10, 20 μg/ml LTA for 24h, gelatin-zymography was performed. For the activation of zymography sample (MMP-9), 1mM APMA (4-Aminophenylmercuric acetate; Sigma, St. Louis, MO, USA) was added to the cell supernatants, and the mixture was incubated at 37℃ for 1h. The zymography sample was mixed with 5X zymography sample buffer [2.5 ml of 150 mMTris-HCL (pH 6.8), 7.5 ml of glycerol, 1.5 ml of 20% SDS, 0.45 mg of bromophenol blue], and the volume was adjusted with 20 ml of distilled water, however, the samples were not boiled. The whole sample was loaded onto an 8% polyacrylamide gel containing 0.1% gelatin and electrophoresed with running buffer (0.03%

Tris-HCl, 0.14% glycine, 0.01% SDS, pH 8.3) at 130 V until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was equilibrated in renaturation solution (2.7% Triton X-100) at room temperature for 1 h with gentle agitation. The zymogram was developed in developing buffer (Novex, Carlsbad, CA, USA) at room temperature for 30 min and then at 37°C overnight. Following the incubation, the gels were stained with the stain solution (0.5% Coomassie brilliant blue R-250; Sigma, St. Louis, MO, USA) at room temperature for 2h, and destained with the destain solution (MeOH : H_2O : acetic acid = 45 : 45 : 10) until the activated MMP-9 band was visible. The area of the activated MMP-9 appeared as a clear band. The gel was washed with distilled water to fix the activated MMP-9 band, dried with drying solution (20% MeOH, 2% glycerol), and fixed on cellophane paper. The dried gel was scanned, and the area of the band was measured using the image analysis program (FUJIFILM Multi Gauge V3.0).

F. Western Blot Analysis

After treating HMEE cells with 10 μg/ml LTA for 0, 5, 15, 30, 60, and 180 minutes, cellular protein was extracted using cell lysis buffer (RIPA buffer; Sigma) that contained a protease inhibitor cocktail (1 tablet/10 ml RIPA buffer; Roche Diagnostics, Mannheim, Germany). 20 μg of protein were loaded in each lane. The protein was denaturized using buffer (2.5 ml Tris-HCL [150mM], pH 6.8, 7.5 ml glycerol, 1.5 ml 20% SDS, 3.75 ml β-mercaptoethanol, 0.45 mg bromophenol blue) and electroblotted onto nitrocellulose membrane. The membrane was incubated in blocking solution (5% skim milk in PBST; PBS+0.1% Tween 20) at room temperature for 2 hours and then incubated using rabbit polyclonal antibodies specific for phosphylated and total extracellular signal-regulated kinases (ERK)1/2, Jun-N terminal protein kinase(JNK) and p38 (Cell Signaling Technology, Beverly, MA, USA) diluted in 5% blocking solution at 4°C overnight. The membrane was then washed with PBST, incubated in secondary antibody (horse radish peroxidase [HRP]-conjugated anti-rabbit IgG, Cell Signaling Technology) diluted in 5% blocking solution at room temperature for 1 hour, and washed again with PBST. After treating HMEE cells with 10 μg/ml LTA for 0, 5, 15, 30, 60, and 180 minutes, cellular protein was extracted using cell lysis buffer (RIPA buffer; Sigma) that contained a protease inhibitor cocktail (1 tablet/10 ml RIPA buffer; Roche Diagnostics, Mannheim, Germany).The

immunoreactivitywas detected using an enhanced chemiluminescence (ECL) kit (ECL advanced western blotting detection kit, Lumigen^{TM} TMA-6; Amersham, Little Chalfont, Buckinghamshire, UK) and a Luminescent Image Analyzer (LAS-3000, FUJIFILM) .

G. MEK1/2 Inhibitor Treatment

To determine the relationship between the expressed intracellular phosphokinase and MMP-9 gene expression, HMEEC were treated with 10 μM MEK1/2 inhibitor U0126 (Calbiochem, San Diego, CA, USA) for 1 hour prior to treatment with 10 μg/ml LTA for 24 hours. The extracted total RNA fromhuman middle ear epithelial cells treated with U0126 and LTA was used to synthesize cDNA, and MMP-9 mRNA expression was determined by PCR. The activation of MMP-9 protein was determined by zymography.

H. Statistical Analysis

Results are expressed as means ± SD for at least triplicates and are representative of three or more independent experiments. Differences between means were analyzed by Student's t-test. Statistical significance was defined as $p < 0.05$.

III. RESULTS

A. Expression of MMPs and TIMPs mRNAs in HMEE Cells After Treatment with LTA

To determine the effect of LTA on HMEE cells, we also examined the molecular expression of MMPs and TIMPs after treatment of HMEE cells with LTA for 24h. Although LTA failed to enhance the expression of MMP-1, -2, -3, -7, it increased MMP-9 expression (Fig. 1). Based on increased MMP-9 mRNA expression, we next examined mRNA expression of the TIMPs. There was no difference in mRNA expression of TIMP-1 and TIMP-2 after treatment with LTA (Fig. 1)

B. Real-time PCR of MMP-9

To quantitatively measure the expression of MMP-9 mRNA in HMEE cells after LTA treatment, real-time PCRwas performed. To find optimal time for LTA treatment, we measured the expression of MMP-9 after treatment of 10 μg/ml LTA over time. The expression of MMP-9 increased in a time-dependent manner until 24h, and then declined (Fig. 2A). To find optimal amount for LTA treatment, the mRNA expression of MMP-9 was analyzed after treatment with LTA (0 - 20μg/ml). The mRNA expression of MMP-9 increased approximately 2.3-fold after treatment with 10 μg/ml LTA compared to the control (Fig. 2B).

C. Secretion of MMP-9

HMEE cells constitutively secreted MMP-9 to the incubation medium, as assessed by ELISA. In ELISA, the level of MMP-9 significantly increased after treatment of 10 μg/ml LTA for 24h, whereas the levels of TIMP-1 and TIMP-2 were not changed (Fig. 3).

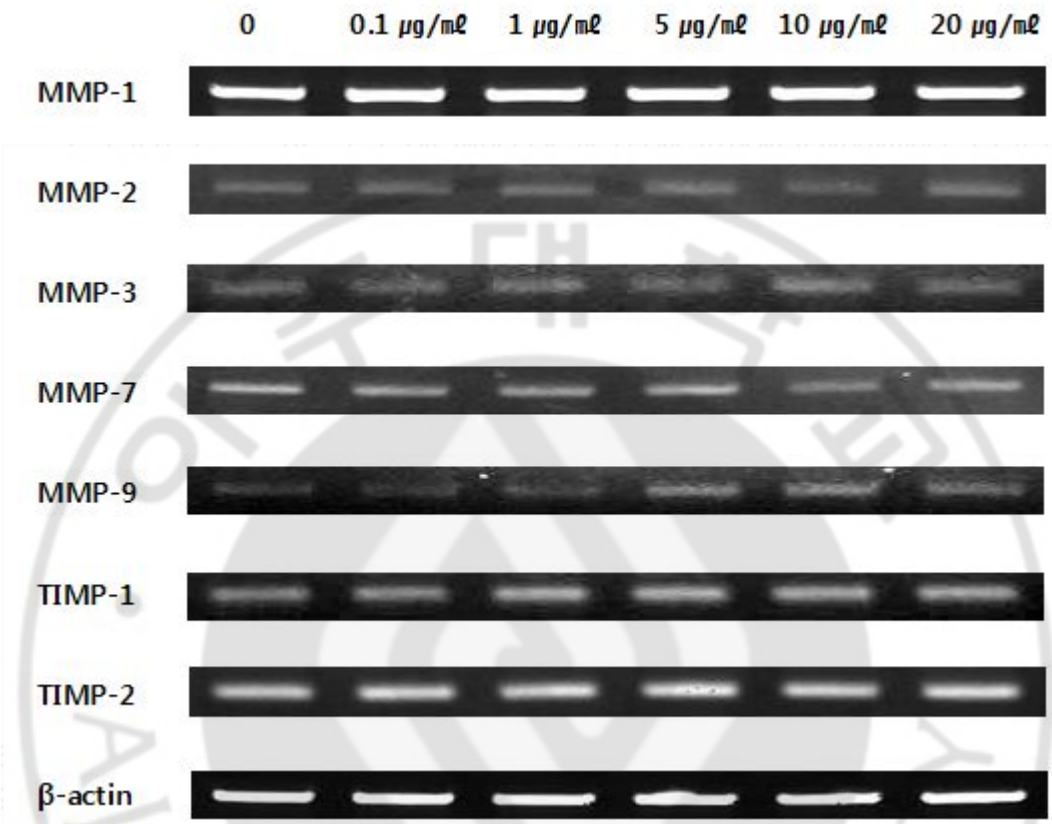


Fig. 1. Expression of MMPs and TIMPs mRNAs in HMEE cells after treatment with LTA. To investigate the effect of LTA on the expression of MMPs and TIMPs, we examined the mRNA expression of MMPs -1, -2, -3, -7, and -9 by PCR. MMPs -1, -2, -3, and -7 did not show differential expression, whereas MMP-9 expression was increased after LTA treatment. Also, there was no difference in mRNA expression of TIMP-1 and TIMP-2 after treatment with LTA.

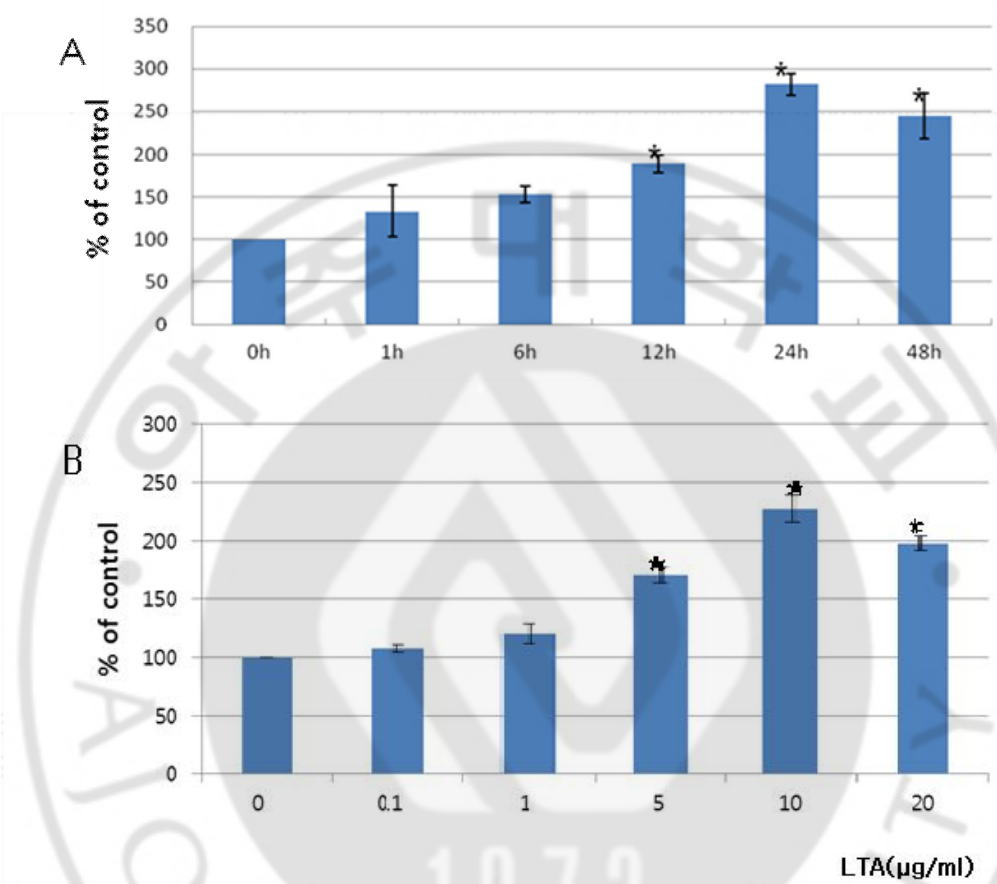
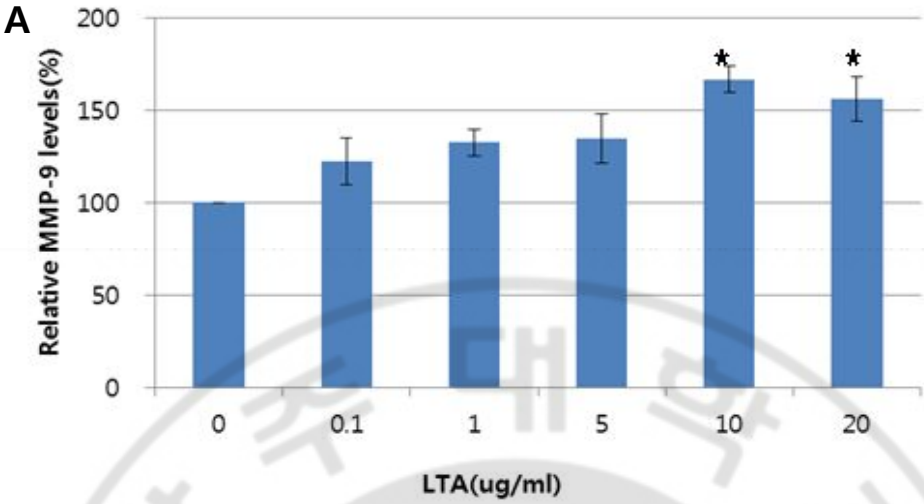
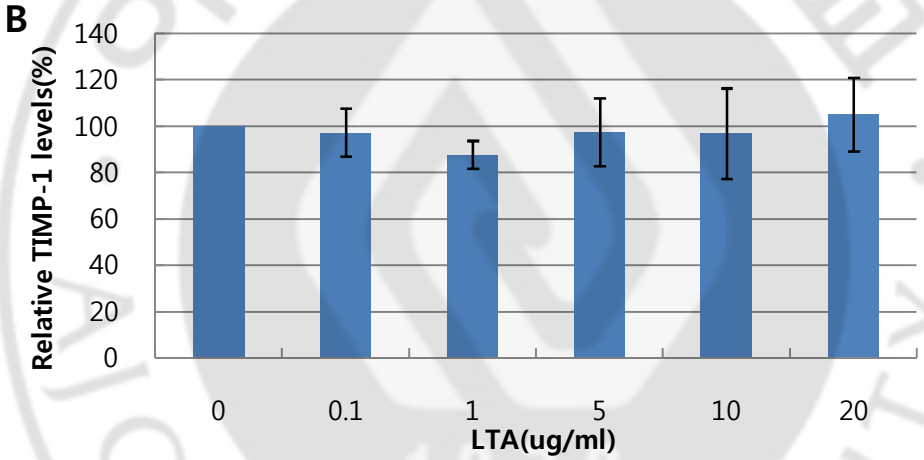


Fig. 2.Real-time PCR of MMP-9. After treatment of HMEE cells with 10μg/ml LTA , the mRNA expression of MMP-9 was measured according to treatment time. LTA increased significantly the expression of MMP-9 compared to the control. *Values* are the ratios compared to the control (A). After treatment of HMEE cells with LTA (0 - 20μg/ml), the mRNA expression of MMP-9 was analyzed by real-time PCR. LTA increased significantly the expression of MMP-9 compared to the control (B). *Values* are the ratios compared to the control. *$p<0.05$ versus control, n=3 experiments.


A
Relative MMP-9 levels(%)
200
150
100
50
0
*
*
0
0.1
1
5
10
20
LTA(ug/ml)


B
Relative TIMP-1 levels(%)
140
120
100
80
60
40
20
0
0
0.1
1
5
10
20
LTA(ug/ml)

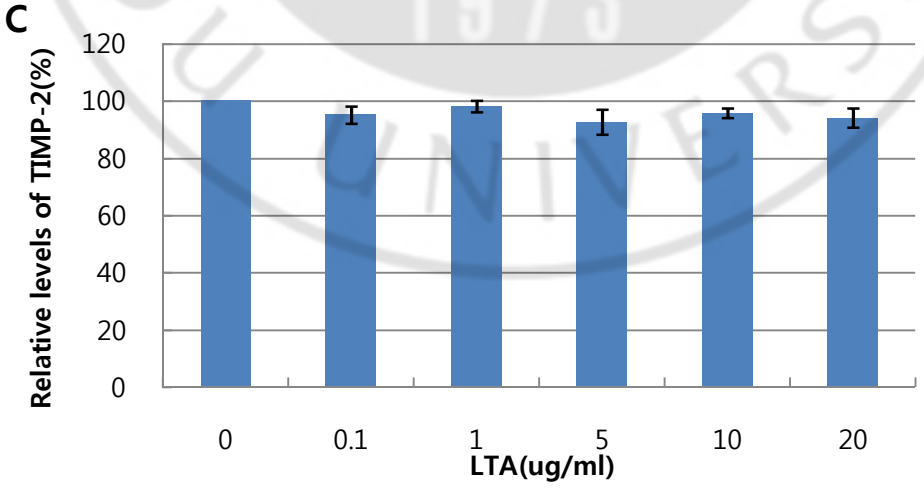

C
Relative levels of TIMP-2(%)
120
100
80
60
40
20
0
0
0.1
1
5
10
20
LTA(ug/ml)

Fig. 3. Secretion of MMP-9, TIMP-1 and TIMP-2. After treatment of HMEE cells with LTA (0−20 mg/ml), the secretions of MMP-9, TIMP-1 and TIMP-2 were analyzed by ELISA. The levels of MMP-9 were significantly elevated after treatment with 10 mg/ml LTA compared to the control(A). However, the levels of TIMP-1 and TIMP-2 were not changed after treatment of LTA(B and C). Values are the ratios compared to the control. *$p < 0.05$ versus control, n = 4 experiments.

D. Activation of MMP-9 Enzyme (gelatinase B)

To measure the enzymatic activity of MMP-9 (gelatinase B), we performed gelatin zymography using supernatants of HMEE cells treated with LTA (0–20 μg/ml). The activity of MMP-9 was increased by LTA treatment in a dose-dependent manner, and the data indicated that the greatest activation of the MMP-9 enzyme was achieved at 10 μg/ml LTA (Fig. 4).

E. Western blot analysis

In this study, we find that ERK1/2 (Thr202/Tyr204) of the mitogen-activated protein kinase (MAPK) pathway showed the greatest activation at 5 minutes. A protein downstream of ERK1/2 was seen with maximum phosphorylation at 15 minutes. JNK (the c-Jun NH2-terminal kinase) and p38 path showed no activation at various LTA concentrations (Fig. 5).

F. MEK1/2 Inhibitor Treatment

Our previous data confirmed that LTA induced activation of ERK1/2 in the HMEEC. To investigate whether the phosphorylated ERK1/2 influenced MMP-9 mRNA expression, HMHEE cells with a MEK1/2 inhibitor were pretreated, U0126, prior to treatment with LTA. We find the result that U0126 inhibited the release of MMP-9 enzyme (Gelatinase B) following co-treatment with 10 μM U0126 and 10 μg/ml LTA (Fig. 6)

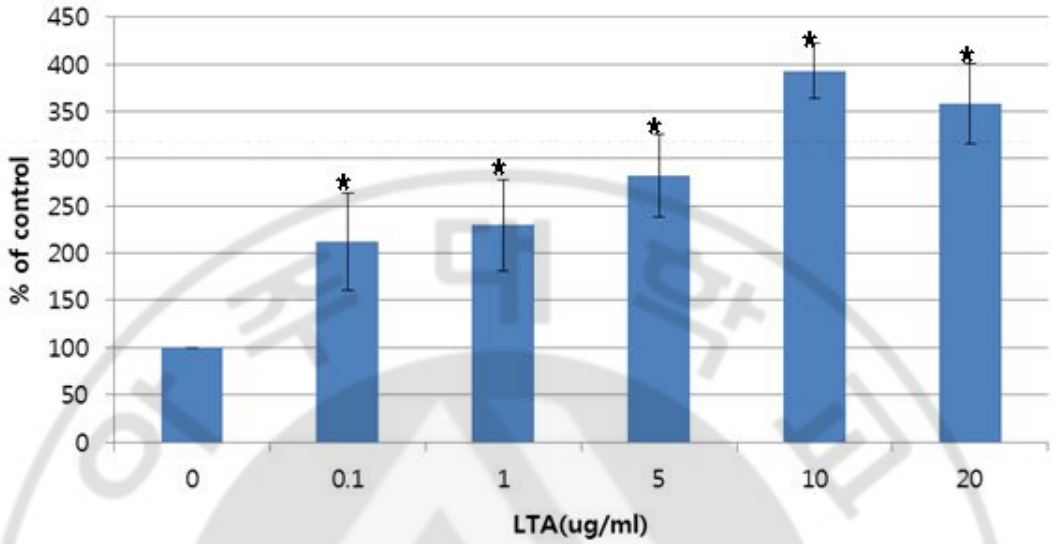


Fig. 4.Activation of MMP-9 enzyme. The HMEE cells were treated with LTA (0−20 mg/ml) and the cell supernatants were harvested for determination of the released MMP-9 enzyme (gelatinase B) activation by gelatin zymography. The activity of MMP-9 increased significantly after treatment of LTA. Values are the ratios compared to the control. *$p < 0.05$ versus control, n = 3 experiments.

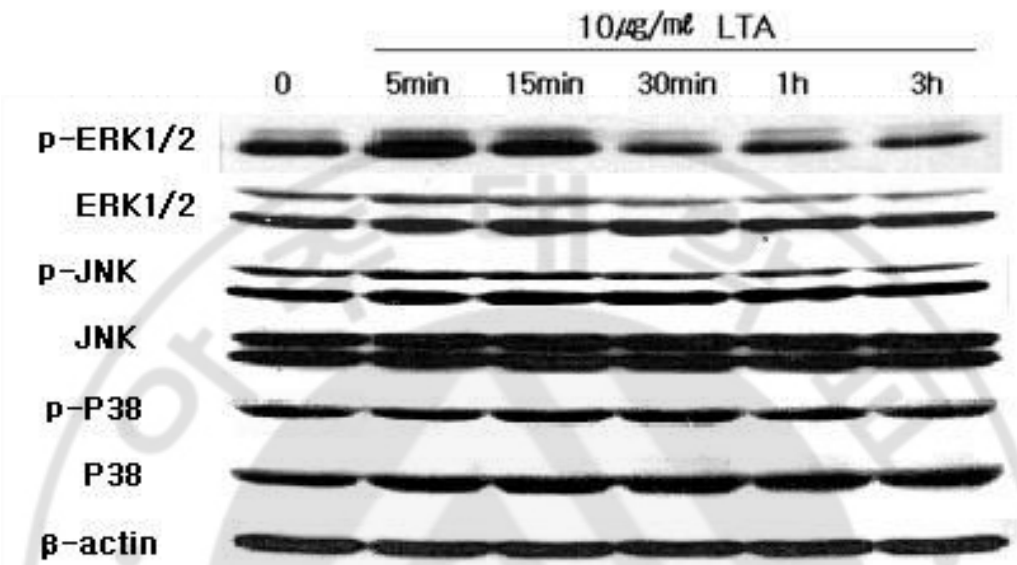


Fig. 5.The effect of LTA on phosphorylation of ERK1/2,p38 and JNKinHMEEC. After treating HMEE cells with 10 μg/ml LTA for 0, 5, 15, 30, 60, and 180 minutes. ERK1/2 (Thr202/Tyr204) of the mitogen-activated protein kinase (MAPK) pathway showed the greatest activation at 5 minutes. JNK (the c-Jun NH2-terminal kinase) and p38 path showed no activation at various LTA concentrations

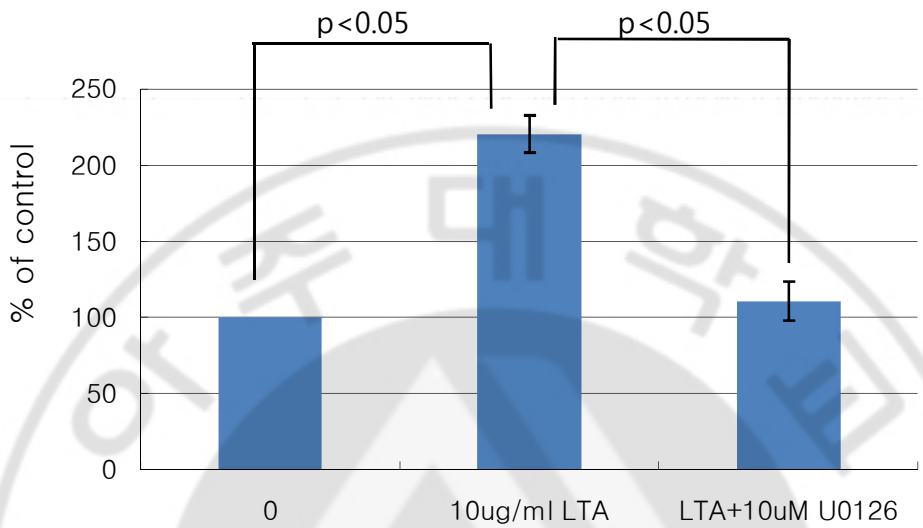


Fig. 6.Expression of MMP-9 after MEK1/2 inhibitor treatment.HMEE cells were treated with 10 μM U0126 (MEK1/2 inhibitor) for 1 hour prior to treatment with 10μg/ml LTA for 24 hours. MMP-9 enzyme activity in cell supernatants was analyzed by gelatin zymography.U0126 inhibited the release of MMP-9 enzyme (Gelatinase B) following co-treatment with 10 μM U0126 and 10 μg/ml LTA. Values are the ratioscompared to the control. *p < 0.05

IV. DISCUSSION

The respiratory epithelium and mucociliary clearance system play a key role in the primary defense system of the middle ear. Changes in the respiratory epithelium caused by infection and inflammation can lead to reduced mucociliary clearance due to loss of ciliated cells, squamous metaplasia, and secretory cell hyperplasia (Norlander et al., 1994). Furthermore, it is well known that *Staphylococcus aureus*, *Streptococcus pneumoniae*, *non-typableHaemophilusinfluenzae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* are involved in the pathogenesis of OM(Alter et al., 2011). Inflammation caused by these organisms is partly mediated by their cell membrane components LPS and LTA. LTA is the major glycolipid found in most Gram-positive bacteria and has most of the biochemical and physiologic properties of LPS, and has been shown to play a significant role in the initiation and progression of bacterial infection (Sriskandan and Cohen, 1999). In the past, LTA was prepared from bacteria and was contaminated with LPS. Furthermore, LTA preparations from *Bacilussubtilis* contained a significant amount of nucleic acid content (Morath et al., 2002).Several studies have indicated that commercial LTA has problems with purification and activity. Therefore, we used highly purified LTA from S. aureus, which was kindly provided by the Department of Oral Microbiology, Seoul National University. This highly purified LTA was prepared by organic solvent extraction, and Octyl-Sepharose and ion-exchange chromatography (Han et al., 2006).

Activation of the epithelial cells by LTA leads to a host innate immune response involving the recruitment of neutrophils and mucin production, and it can also be involved in the repair of epithelial cell damage during the process of airway inflammatory diseases including OM.

The extracellular matrix(ECM) is the extracellular part of animal tissue that usually provides structural support to the animal cells in addition to performing various other important functions such as a tissue remodeling, tumor invasion, wound healing, inflammatory diseases and fibrotic disorders. A collagen is a major component of the ECM that supports most tissues and gives cells structure from the outside. MMPs are involved in inflammatory response, participating in extracellular matrix remodeling and cellular migration and infiltration. A major role of TIMPs in tissue remodeling is to inhibit MMPs by

forming 1:1 enzyme-inhibitor complexes. In addition, previous studies have indicated that TIMPs inhibit the cellular invasion, tumorigenesis, metastasis and angiogenesis. MMP activity has been demonstrated to involve an interaction with TIMPs, and interruption of the balance between these 2 factors may result in pathologic processes (Wysocki et al., 1993; Mast and Schultz, 1996). Levels of MMP-9 protein are significantly increased in airway inflammatory diseases such as asthma, chronic sinusitis, and nasal polyps (Watelet et al., 2004). In addition, the plasma level of MMP-9 was found to be increased relative to the basal level in plasma from asthmatics, inducing collagen precipitation, smooth muscle hypertrophy, and an altered airway structure. This increase in MMP-9 production and activity can act as a non-invasive systemic marker of inflammation and airway remodeling in asthma patients (Belleguic et al., 2002).

It has been reported that LTA could be involved in OM (Kita and Himi, 1999; Vonk et al., 2008), suggesting that LTA stimulation of MMP in middle ear epithelia could be involved in OM associated with Gram-positive bacteria infections. On middle ear effusion, previous reports demonstrated increased activity of MMPs (Jennings et al., 2001; Moon et al., 2008) and significant inhibition of MMP activities by a protease inhibitor (Antonelli et al., 2003; Moon et al., 2008). However, there have been no reports on the relationship between LTA and activity of MMP in the middle ear epithelia. In this study, we report that LTA from *S. aureus* increased MMP-9 mRNA expression and secretion in HMEE cells, while no effect was observed in MMP-1, 2, 3, 7 and TIMP-1, 2. The activity of the MMPs and the expression levels of TIMPs are balanced in normal processes such as tissue remodeling and wound healing. However, in this study, MMP-9 expression was differentially increased by LTA, resulting in an imbalanced expression between MMP-9 and TIMP-1, 2. Such an imbalance may result in diseases, such as inflammation in the middle ear, after exposure to LTA. This result suggests that LTA mainly stimulates MMP-9 subtype in the middle ear and the extracellular matrix could be actively degraded due to the activation of MMP-9 by LTA.

The MAPK signaling pathways play an important role in MMP regulation. Specifically, previous studies have shown that LTA activates ERK1/2 and p38 MAPK pathways and induces immune response in various cells. You et al reported that LTA induced the ERK signaling pathway in the cornea(You et al., 2002), while Souza et al reported that LTA increased MMP-9 expression through the MEK/ERK pathway in RAW 264.7

macrophages(Souza et al., 2009). As LTA may induce production of several proteins, including cytokines, in the nasal epithelial cells, MMP-9 may be induced by activated ERK1/2 via various proteins. For example, whereas MAPK/JNK is activated by inflammatory cytokines, it is more active in programmed cell death and apoptosis than in inflammatory processes. p38, which is also activated by inflammatory cytokines in immune cells, participates in the activation of immune response associated with various diseases, such as asthma. Furthermore, auto-immune response is an important factor in the induction of inflammatory cytokines.

Several studies have indicated activation of the ERK1/2 and p38 MAPK pathways by LTA. Our study showed that LTA activates only the MEK1/2-ERK1/2 pathway. Souza et al reported that LTA increased MMP-9 expression through the MEK/ERK pathway in RAW 264.7 macrophages5 and You et al reported that LTA induced the ERK signaling pathway in the cornea(You et al., 2002). Only ERK1/2 among the MAPKs tested was expressed in response to LTA, and U0126 (a MEK1/2 inhibitor) inhibited MMP-9 activity. These results show that LTA-mediated MMP-9 expression is dependent on the MEK/ERK pathway in human middle ear epithelial cells.

V. CONCLUSION

We demonstrated increased activity of MMP-9 in the middle ear epithelial cells when treated with LTA, whereas it had no effect on the expression of TIMP-1 and TIMP-2. The disturbed balance between MMP-9 and TIMPs expressions after exposure to LTA may contribute to the destruction of middle ear epithelial cells, thus leading to OM.

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- 국문요약 -

인체중이점막세포 (human middle ear epithelial cells)에서포도상구균 (*Staphylococcus aureus*)의lipoteichoicacid에의한 MMP-9의발현

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송정환

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중이염의 병인에 있어 matrix metalloproteinases (MMPs)와 metalloproteinases의 tissue inhibitors (TIMPs)의 조절의 변화가 중요한 역할을 할 것으로 생각된다. 본 연구는 황색포도상구균(*Staphylococcus aureus*)에서 분리된 lipoteichoic acid (LTA)에 의한 중이 점막에서의 MMPs 와 TIMPs의 변화와 그 신호전달기전을 연구하고자 하였다. Human middle ear epithelial (HMEE) 세포들을 LTA로 처치하였다. MMPs와 TIMP의 변화와 신호전달을 PCR, ELISA, zymography, western blot으로 측정하였다. *S. aureus* 로부터 분리된 LTA는 HMEE 세포들에서 MMP-9 mRNA의 발현과 분비를 증가시킨 반면, MMP-1,2,3,7과 TIMP-1,2의 발현에는 영향을 미치지 않았다. Mitogen-activated protein kinase (MAPK) 중 오직 extracellular signal-regulated kinases (ERK) 1/2 만이 증가 하였으며, 이는 MEK1/2 inhibitor를 처치 하였을 경우 억제되었다. 중이 점막에서 LTA는 MMP-9의 활성도를 증가시킨 반면, TIMP의 활성도는 변화시키지 않았다. 이는 중이염의 병인에서 LTA에 의한 MMP-9과 TIMP의 불균형이 중요한 역할을 할 것으로 생각된다.

핵심어: matrix metalloproteinase; middle ear; otitis media