Induction of Vascular Endothelial Growth Factor Protein by *Mycoplasma pneumoniae*

Kyung Eun Lee, Ph.D., Jung Yeon Hong, MSc, Myung Hyun Sohn, M.D., Ph.D.
Kyung Won Kim, M.D., Ph.D, In Dal Park, Ph.D.*
Myung Woong Chang, Ph.D.* and Kyu Earn Kim, M.D., Ph.D.

*Department of Pediatrics and Institute of Allergy, Brain Korea 21 Project for Medical Science, Severance Medical Research Institute, Yonsei University College of Medicine, Seoul, Korea, Department of Microbiology, Kosin University College of Medicine, Busan, Korea**

=Abstract=

Purpose : *Mycoplasma pneumoniae* is an extracellular pathogen that attaches to and destroys the ciliated epithelial cells of the respiratory tract. The vascular endothelial growth factor (VEGF) is a critical angiogenic factor that manages the formation and function of vascular networks. Thus, we examined whether *M. pneumoniae* lysate (MPL) induces VEGF and MPL-induced VEGF expression is regulated by the activation of mitogen-activated protein kinase (MAPK) pathways in airway epithelial cells.

Methods : Cells were treated with MPL in dose and time dependent manners or pretreated with chemical inhibitors of MAPK signaling molecules before the addition of MPL. The supernatants were measured by a specific human VEGF enzyme-linked immunosorbent assay (ELISA). The RNAs were extracted and synthesized into cDNAs for VEGF gene expression by polymerase chain reaction.

Results : MPL considerably increased VEGF mRNA 2 hours after treatment, which was gradually reduced thereafter. On the other hand, VEGF protein was continuously amplified for 12 hours after both 5 and 10 μg/mL MPL treatment. Pretreatment with U0126 (a specific extracellular signal-regulated kinase inhibitor) and SB202190 (a specific p38 inhibitor) abolished MPL-stimulated VEGF protein close to basal level (-85%), whereas JNK inhibitor II (a specific c-Jun N-terminal kinase inhibitor) partially decreased VEGF protein (57%).

Conclusion : We concluded that MPL induces VEGF expression through the activation of MAPK signaling molecules (ERK, p38 and JNK) in airway epithelial cells. **[Pediatr Allergy Respir Dis (Korea) 2010;20:100-106]**

Key Words : *Mycoplasma pneumoniae*, Vascular endothelial growth factor, Mitogen-activated protein kinase, Airway epithelial cell

Introduction

Mycoplasma pnuemoniae, one of the main causes of community-acquired pneumonia, has been recognized as a contributing factor in both stable asthma and asthma exacerbation. Infection of the lower respiratory tract with the atypical bacteria *M. pneumoniae*

This work was supported by Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (grant code: E00322)
접수 : 2009년 12월 21일, 승인 : 2010년 4월 15일
책임저자 : 김규언, 서울시 강남구 언주로 712
연세대학교 강남세브란스병원 소아청소년과
Tel : 02)2019-3353 Fax : 02)3461-9473
E-mail : kekim@yuhs.ac

has emerged as an important clinical issue in stable patients with chronic asthma.[1, 2] These organisms have been clinically observed in adults with inflammation and bronchial hyperreactivity,[3] and its acute infection increased bronchial resistance and cytokine production in a mouse model.[4] Mycoplasmas are characterized by their small size (150–250 nm), absence of a cell wall and the presence of a trilayered cell membrane. There is evidence that indicates that the cell membrane components of *M. pneumoniae* are not only major inducers of the host protective immune response, but also significantly contribute to the pathogenic response.[5] The lysate of Mycoplasmas was known to contain the soluble cell membrane–associated proteins, consistent with innate immune system.[6] It has been demonstrated in other studies that these mycoplasmal proteins are caused by the induction of various cytokines, which mediated the augmentation of immune response.

Vascular endothelial growth factor (VEGF) as a mediator of blood vessel formation and permeability becomes essential in allergic lung responses.[7] Recent studies have measured VEGF protein levels in bronchoalveolar lavage fluid from patients with asthma or examined VEGF expression in bronchial biopsy specimens.[8] These suggest that increased VEGF may be correlated with airway vascularity and edema in individuals with asthma. Furthermore, it is reported that proinflammatory actions of VEGF induce eosinophilic migration and cooperate with T_H2 cells in the development of allergic lung responses, at least in an experimental model of asthma in mice.[7] Transgenic mouse models have also demonstrated that overexpression of VEGF leads to increased vascularity in the airway epithelium, airway inflammation and airway hyperresponsiveness.[9] Although the interaction of *M. pneumoniae* with respiratory epithelial cells is a critical early phase of pathogenesis, little is known about the cascade initiated by *M. pneumoniae* in respiratory epithelial cells. Thus, in this study, we investigated whether *M. pneumoniae* lysate (MPL) induces VEGF and the MPL–induced VEGF is regulated via mitogen–activated protein kinase (MAPK) pathways in airway epithelial cells.

Materials and Methods

1. Cell culture

The human epithelial carcinoma cell line H292 from the American type culture collection (ATCC, MD, USA) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin (GibcoBRL, NY, USA). Cells were grown in a sterile 75T culture flask and maintained at 37 ℃ in an incubator with 5% CO_2.

2. *M. pneumoniae* lysate (MPL) preparation

MPL was provided from Kosin University. Briefly, *M. pneumoniae* (strain 15531 from ATCC, MD, USA) was grown in a Chanock modified medium. The colonies in the exponential growth phase were collected by centrifugation at 20,000 g for 30 minute (min). The pellet was suspended with phosphate buffered saline (PBS), sonicated and then the lysate was centrifuged for clearance.[10] As evaluated with a Bradford assay reagent (Bio–Rad, CA, USA), the total protein in the lysate was 329 μg/mL.

3. VEGF protein and mRNA expression

Cells were treated with MPL and their supernatants were measured by a specific human VEGF enzyme–linked immunosorbent assay (ELISA) (R&D system, MN, USA). In some experiments, cells were pretreated with inhibitors of MAPK signaling molecules, U0126, SB202190 or JNK inhibitor II for 1 hour (hr) before the addition of MPL. (Calbiochem, CA, USA)

For the synthesis of VEGF mRNA, the total RNA (2 μg) was suspended in 12 μL diethylpyrocarbonate–treated water with random hexamers. The mix was incubated at 65℃ for 10 min and cooled on ice. The first strand buffer (100 mM Tris–HCl, pH 8.4, 250 mM KCl), 10 mM dNTP mix, 0.1 M DTT and 25 U

superscript II RT (GibcoBRL, NY, USA) were all added to the samples. The samples were incubated at 42℃ for 60 min, and the reverse transcriptase was heat-inactivated at 70℃ for 10 min. PCR conditions for VEGF included an initial denaturation step at 94℃ for 5 min, 30 cycles of 94℃ for 30 second (sec), 55℃ for 30 sec, 72℃ for 30 sec and a final extension step at 72℃ for 7 min. Primers were designed as human VEGF: 5'-CGA AAC CAT GAA CTT TCT GC-3' (sense) and 5'-CCT CAG TGG GCA CAC ACT CC-3' (antisense); GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). The final PCR products were run on ethidium bromide-stained 1.5% agarose gels in 0.5 X TAE buffer.

4. Statistical analysis

Data were expressed as mean±SD from three independent experiments. Statistical analysis comparing the treatment and control groups was assessed by Student's t test, and a $P<0.05$ was considered significant.

Results

1. MPL induces VEGF in H292 cell line

To investigate whether MPL induces VEGF expression, we treated cells with MPL in various times and doses. Based on a 10 μg/mL MPL treatment, the VEGF protein was increased by 8.4±0.8, 51.1±1.7, 782.4±18.1, and 1565.0±49.5 pg/mL according to 1, 2, 6, and 12 hr, respectively (Fig. 1A). Although the stimulation with 5 μg/mL MPL also elevated the VEGF protein expression associated with incubation time, the treatment with 10 μg/mL MPL showed more dramatic effect of VEGF amplification. The mRNA levels of VEGF sharply rose by 2 hr after MPL treatment and then was gradually reduced (Fig. 1B). These results demonstrated that MPL effectively induced the VEGF protein and mRNA in human airway epithelial cells.

2. MPL regulates VEGF via MAPK pathways in H292 cell line

We next tested the requirement of the MAPK pathway activation on MPL-induced VEGF expression. MAPK signaling molecules comprise of three main family members - p42/44 ERK, p38, and JNK - and mediate cellular responses against extracellular stimuli such as bacteria, cytokines, and osmotic shock.[11] Initially, cells were pretreated with 30 μM of U0126 (a specific MEK 1/2 inhibitor), SB202190 (a specific p38 inhibitor) or JNK inhibitor II (a competitor of ATP for JNK) for 1 hr, followed by a stimulation with 10 μg/mL MPL for an additional 6 hr. Then, each supernatant was measured by a specific VEGF ELISA. As shown in Fig. 2, the inhibition of each MAPK

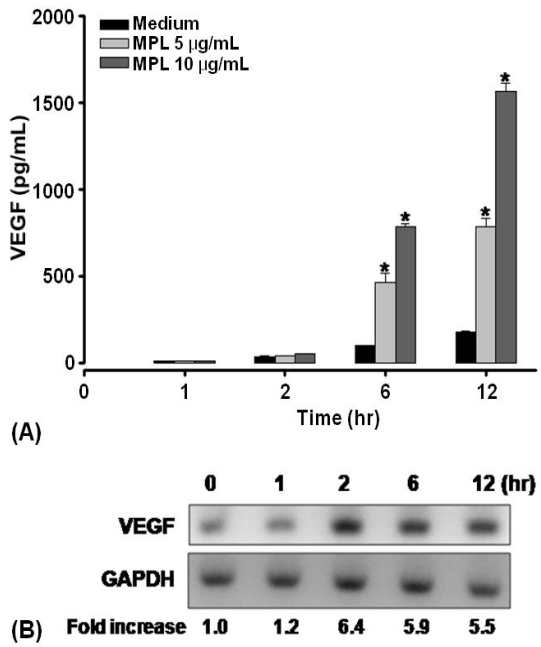

Fig. 1. MPL induces VEGF protein and mRNA. (A) H292 cell line was stimulated with MPL depending on time and dose. The VEGF concentrations of supernatants were measured by ELISA (*$P<0.05$ vs. 0 hr). All data represent the mean SD of at least three independent experiments. (B) The RNAs from cells treated with MPL for the indicated times were harvested and used for RT-PCR. Similar results were obtained in two other independent experiments.

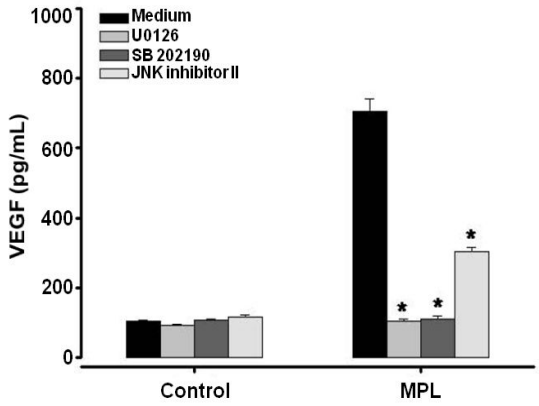

Fig. 2. Requirements of ERK, p38 and JNK activation for MPL-induced VEGF expression. Cells were pretreated with inhibitors of ERK, p38, and JNK before the addition of MPL. Then the supernatants were measured by ELISA (*P<0.05 vs. MPL alone). All data represent the mean±SD of at least three independent experiments.

affected the MPL-induced VEGF production. For instance, the inhibition of ERK and p38 decreased the amount of MPL-induced VEGF protein by −85% and JNK inhibitor II also blocked MPL- induced JNK activity by 57%. Together, these suggested that both ERK and p38 activity more mainly regulate MPL-induced VEGF expression than JNK activity in airway epithelial cells.

Discussion

M. pneumoniae, a common cause of atypical pneumonia and tracheobronchitis,[12] attaches to ciliated airway epithelial cells, infecting the cells and causing epithelial damage and ciliary dysfunction.[13] Both new-onset wheezing exacerbations of prevalent asthma and long-term decrements in lung function by *M. pneumoniae* suggest that this organism can play an important role in asthma.[14] Lipoproteins, glycoproteins, glycolipids, and lipoglycans among Mycoplasmal membrane molecules are known to be essential antigens that induce host pathogenic response.[5, 15] In this study, to determine whether *M. pneumoniae* induces VEGF in airway epithelial cells, we specifically used MPL and also investigated the MAPK activity related to MPL-stimulated VEGF expression in epithelial carcinoma cell line.

VEGF as one of the critical angiogenic factors is a heparin-binding glycoprotein that is secreted by most types of cells. Potential cellular sources of VEGF in the airways include airway epithelial cells, mast cells, and airway smooth muscle cells.[16] Several studies have proven that VEGF contributes to the development of asthma, airway responsiveness and airway remodeling. Expression of VEGF and its receptors is increased in asthma, and VEGF is the major proangiogenic activity in asthmatic airways. Increases in the size and number of airway wall blood vessels are characteristic of airway remodeling, even in mild asthma, and moreover, overexpression of VEGF in the airways of mice has been shown to result in both vascular and airway remodeling.[17, 18] Also, VEGF has been clearly implicated in the destruction of alveolar wall components including microvasculature.[19, 20] Wang et al.[21] reported the correlation of airway responsiveness with bronchial epithelial cell VEGF and VEGFR mRNA expression in asthmatics. The current study showed a dose and time dependent increase in VEGF secretion when cultured with 5 and 10 μg/mL of MPL relative to control for 1, 2, 6, and 12 hr, respectively. Also, VEGF mRNA maximally increased at 2 hr after MPL treatment and then slowly decreased.

MAPK signaling pathways have been shown to regulate VEGF expression in different cell types, including smooth muscle cells, human umbilical vein endothelial cells, macrophages and renal glomerular epithelial cells by various stimuli.[22-24] For example, childhood human rhinovirus (HRV)-induced generation of VEGF was regulated by p38 and ERK 1/2 pathways in airway epithelial cells.[25] Kim et al reported that *Chlamydophila pneumoniae*-infected bronchial epithelial cells increased the ratio of phosphorylated to non-phosphorylated p38.[26] In addition, members of the IL-6 cytokine family (which includes IL-6 itself and IL-31 etc) have been shown to induce

VEGF. The activation of MAPKs can be crucial for IL-31-mediated activation of bronchial epithelial cells, thereby providing an immunological role for IL-31 in bronchial inflammation, at least partly, via epithelial VEGF.[27] In this study, we examined the role of MAPK pathways including ERK, p38 and JNK in relation with regulating MPL-mediated VEGF secretion in airway epithelial cells. It was found that both ERK and p38 inhibition showed 85% and less decrease of MPL-stimulated VEGF and JNK inhibition reduced VEGF secretion to 57%. The data suggest that MAPK pathways contribute to regulating VEGF expression by MPL in airway epithelial cells.

In this study, we concluded that MPL directly induces VEGF secretion in airway epithelial cells and the regulation might be mainly mediated by MAPK pathways. In addition, we showed that MPL-induced VEGF is more dependent on activation of the ERK and p38 than JNK pathway.

Acknowledgement

We thank Dr. Myung Woong Chang and Dr. In Dal Park at Kosin university in Korea for *M. pneumoniae* lysate.

한 글 요 약

호흡기상피세포 내 *Mycoplasma pneumoniae*에 의한 혈관내피세포성장인자의 유도

연세대학교 의과대학 소아과학교실, 알레르기 연구소,
BK21 의과학사업단, 의생명과학부,
고신대학교 의과대학 미생물학교실*

이경은 · 홍정연 · 손명현 · 김경원
박인달* · 장명웅* · 김규언

목 적 : *Mycoplasma pneumoniae*는 세포 외 병원균으로 기도점막에 부착하여 기관지상피세포들을 공격한다고 알려져 있다. 혈관내피세포성장인자(VEGF)는 혈관계의 형성 및 기능을 담당하는 매우 중요한 혈관형성인자이다. 본 연구에서는 기관지 상피세포에 *M. pneumoniae* 항원을 자극하여 VEGF가 유도되는지를 확인하였고 이 과정에서 MAPK 신호전달경로가 관여하는지를 살펴보았다.

방 법 : 기관지 상피세포주인 H292 세포에 *M. pneumoniae* 항원을 농도별 시간별로 변화를 주어 자극한 후 그 상층액 및 RNA를 분리하여 각각 VEGF 단백생성 및 mRNA 발현정도를 측정하였다. 그리고 MAPK 신호전달물질의 활성을 특이적으로 억제할 수 있는 화학물질들을 사용하여 그 연관성을 확인하였다.

결 과 : 기관지 상피세포에 *M. pneumoniae* 항원을 노출시켰을 때 항원의 농도 및 반응시간을 증가시킴에 따라 VEGF 단백 및 mRNA 합성이 증가하는 것을 관찰할 수 있었다. 또한, MAPK 신호전달물질 중 ERK와 p38의 억제제인 U0126과 SB202190을 각각 전처치한 후 항원과 반응시켰을 때 -85%까지 VEGF 단백생성이 감소하는 것을 확인할 수 있었으며, JNK의 억제제인 JNK inhibitor II를 처리한 경우에는 VEGF 단백합성이 57% 줄어드는 것을 관찰할 수 있었다.

결 론 : *M. pneumoniae* 항원은 기관지 상피세포에서 VEGF 발현을 유도하며 그 과정에서 MAPK 신호전달경로가 중요한 역할을 담당하고 있는 것으로 생각된다.

참 고 문 헌

1) Martin RJ, Kraft M, Chu HW, Berns EA, Cassell GH. A link between chronic asthma and chronic infection. J Allergy Clin Immunol 2001;107:595-601.
2) Brunetti L, Colazzo D, Francavilla R, Tesse R, De Sario V, Lore M, et al. The role of pulmonary infection in pediatric asthma. Allergy Asthma Proc 2007;28:190-3.
3) Musikacharoen T, Matsuguchi T, Kikuchi T, Yoshikai Y. NF-kappa B and STAT5 play important roles in the regulation of mouse Toll-like receptor 2 gene expression. J Immunol 2001;166:4516-24.
4) Hardy RD, Jafri HS, Olsen K, Wordemann M, Hatfield J, Rogers BB, et al. Elevated cytokine and chemokine levels and prolonged pulmonary airflow resistance in a murine Mycoplasma pneumoniae pneumonia model: a microbiologic, histologic, immunologic and respiratory plethysmographic profile. Infect Immun 2001;69:3869-76.
5) Chmura K, Bai X, Nakamura M, Kandasamy P,

McGibney M, Kuronuma K, et al. Induction of L-8 by Mycoplasma pneumoniae membrane in BEAS-2B cells. Am J Physiol Lung Cell Mol Physiol 2008;295:L220-L30.
6) Brightbill HD, Libraty DH, Yang RB, Belisle JT, Bleharski JR, Maitland M, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 1999;285:732-6.
7) Rothenberg ME. VEGF obstructs the lungs. Nat Med 2004;10:1041-2.
8) Demoly P, Maly FE, Mautino G, Grad S, Gougat C, Sahla H, et al. VEGF levels in asthmatic airways do not correlate with plasma extravasation. Clin Exp allergy 1999;29:1390-4.
9) Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, et al. Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. Am J Pathol 2004;165:1071-85.
10) Sohn MH, Lee KE, Choi SY, Kwon BC, Chang MW, Kim K. Effect of Mycoplasma pneumoniae lysate on interleukin-8 gene expression in human respiratory epithelial cells. Chest 2005; 128:322-6.
11) Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298:1911-2.
12) Baseman JB, Tully JG. Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. Emerg Infect dis 1997;3:21-32.
13) Andersen P. Pathogenesis of lower respiratory tract infections due to Chlamydia, Mycoplasma, Legionella and viruses. Thorax 1998;53: 302-7.
14) Sutherland ER, Martin RJ. Asthma and atypical bacterial infection. Chest 2007;132:1962-6.
15) Rawadi G, Roman-Roman S. Mycoplasma membrane lipoproteins induced proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharide. Infect and Immun 1996;64:637-43.
16) Koyama S, Sato E, Tsukadaira A, Haniuda M, Numanami H, Kurai M, et al. Vascular endothelial growth factor mRNA and protein expression in airway epithelial cell lines in vitro. Eur Respir J 2002;20:1449-56.
17) Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. Am J Respir Crit Care Med 1997;156:229-33.
18) Lee CG, Link H, Blauk P, Homer RJ, Chapoval S, Bhandari V, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. Nat Med 2004;10: 1095-103.
19) Kanazawa H, Yoshikawa J. Elevated oxidative stress and reciprocal reduction of vascular endothelial growth factor levels with severity of COPD. Chest 2005;128:3191-7.
20) Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. Am J Respir Crit Care Med 2001;163:737-44.
21) Wang K, Liu C, Wu Y, Feng Y, Bai H. Budesonide/formoterol decreases expression of vascular endothelial growth factor (VEGF) and VEGF receptor 1 within airway remodelling in asthma. Adv ther 2008;25:342-54.
22) Chang HJ, Park JS, Kim MH, Hong MH, Kim KM, Kim SM, et al. Extracellular signal-regulated kinases and AP-1 mediate the up-regulation of vascular endothelial growth factor by PDGF in human vascular smooth muscle cells. Int J Oncol 2006;28:135-41.
23) Bian Z, Elner SG, Elner VM. Thrombin-induced VEGF expression in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2007;48:2738-46.
24) Jung YD, Liu W, Reinmuth N, Ahmad SA, Fan F, Gallick GE, et al. Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway. Angiogenesis 2001;4:155-62.
25) Leigh R, Oyelusi W, Wiehler S, Koetzler R, Zaheer RS, Newton R, et al. Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling. J Allergy Clin Immunol 2008;121: 1238-45.
26) Kim TB, Moon KA, Lee KY, Park CS, Bae YJ, Moon HB, Cho YS. Chlamydophila pneumoniae triggers release of CCL20 and vascular endothelial growth factor from human bronchial

epithelial cells through enhanced intracellular oxidative stress and MAPK activation. J Clin Immunol 2009;29:629-36.
27) Ip WK, Wong CK, Li ML, Li PW, Cheung PF, Lam CW. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signalling pathways: implications for the allergic response. Immunology 2007;122:532-41.