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博士學位論文

Activation of Toll-like receptors by a secretory bacterial protease to induce an inflammatory response

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指導敎授 李 正 燮

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ABSTRACT

Activation of Toll-like receptors by a secretory bacterial protease to induce an inflammatory response

By Jung Eun Park
Advisor: Prof. Jung Sup Lee, Ph. D
Department of Bio-materials Engineering
Graduate School of Chosun University

Vibrio vulnificus (V. vulnificus) is a pathogenic bacterium that is associated with septicemia and serious wound infection in susceptible individuals. Most of the fatal cases are caused by septic shock, which is resulted from various virulence factors including capsular polysaccharide, lipopolysaccharide and haemolysin secreted by the bacterium. A metalloprotease secreted by *V. vulnificus* has many biological activities and seems to induce vascular permeability and hemorrhagic reactions in animal models. In this laboratory, a broad-specificity extracellular metalloprotease designated to as vEP has been purified and characterized from *V. vulnificus* ATCC29307. vEP is capable of cleaving a variety of plasma proteins which are associated with blood clotting and also proteolytically activating prothrombin to active thrombin (Chang *et al.*, 2005). The protease can be inhibited by its own N-terminal propeptide

(Chang *et al.*, 2007). In this study, the entire vEP-coding region was obtained by polymerase chain reaction (PCR), based on the sequence of vEP gene, cloned into a periplasmic-directed expression vector (pFLAG-ATS), and expressed in *E. coli*. The active recombinant vEP was purified from the periplasmic proteins of the transformant *E. coli* cells. The purified vEP enzyme appeared both as 45- and 35-kDa forms on SDS-polyacryamide gel. It has been shown that the intact 45-kDa protease could be autoprocessed to make 35-kDa in size by the removal of its C-terminal region. Two mutant proteases designated as ΔC99 and G202D were also expressed. In addition, the properties of C-terminal region were studied by comparing with those of the wild type vEP enzyme. One mutant protease ΔC99, which has a deletion of the C-terminal 99 amino acids from vEP showed a similar level of specific activity compared with that of wild type vEP. Another mutant protease G202D that has a substitution of glycine^{202} to aspartate^{200} exhibited approximately 58% level of specific activity, compared that of wild type vEP. The vEP-derived peptides (C-ter100 and N-ter139) were also purified and characterized. In this study, an additional role of vEP was also examined in terms of an inflammation-associated cytokine production in macrophage Raw 264.7 cells. vEP could mediate the production of pro-inflammatory cytokines such as TNF-α and IL-1β, in which NF-κB signaling pathway was activated through the degradation of IκB, as judged by ELISA, RT-PCR, real-time PCR, Western blotting, EMSA, and supershift assay. Among Toll-like receptors (TLRs), TLR2 and TLR4 could be activated by vEP, as judged by RT-PCR, immunoprecipitation, western blotting, and confocal imaging analysis.

The results showed that vEP could induce an inflammatory response by activating TLR2 and TLR4, leading the translocation of NF-κB proteins into nucleus to activate the target genes including TNF-α gene. Increase in NO production and Cox-2 expression by vEP further supported the fact that vEP can act as an inflammatory inducer. In addition, a deletion mutant enzyme (called ΔC99) of vEP deficient in C-terminal 99 amino acids failed to show those kinds of responses at all. However, C-ter100 only was sufficient to produce TNF-α, suggesting that the C-domain of vEP may play a critical role in the induction of inflammatory response by activating the Toll-like receptors. It was also observed by conforcal imaging analysis that vEP could bind directly to TLR2 and TLR4 receptors through by its C-domain region. Taken together, the results obtained by the present study suggest that the production of extracellular protease(s) from the bacterium Vibrio can disturb blood homeostasis as well as induce an inflammation during the bacterial infection.

I. INTRODUCTION

Pathogenic bacteria produce various kinds of molecules including lipopolysaccharide, capsular polysaccharide, cytolysin, and metalloprotease (Fig. 1) that may have a role in bacterial virulence and pathogenesis (Hoogerwerf *et al.*, 2002; Gulig *et al.*, 2005; Kawase *et al.,* 2004; Kwon *et al.*, 2007). Among these molecules, metalloprotease may have an important role in many regulatory processes, such as acquisition of nutrients, escape from host defense system, disturbance of host's blood homeostasis, and induction of inflammatory response.

Vibrio vulnificus is a Gram-negative marine bacterium that is often found in warm coastal water. It is an opportunistic human pathogen that primarily causes serious and often fatal systemic infection and septicemia (Janda *et al.*, 1988; Powell *et al.*, 2003). *V. vulnificus* infection is usually acquired from contact of the wound with sea water or through consumption of contaminated raw seafood. *V. vulnificus* causes a rapid and severe disease process resulting in extensive tissue damage. In fact, death can occur within 24 h after contact with the bacterium. Typically, individuals infected by *V. vulnificus* exhibit fever, chills, hypotension, and characteristic bullous skin lesions. It has been proposed that several factors such as metalloprotease, haemolysin, cytolysin, and capsular polysaccharide may be involved in the pathogenesis by *V. vulnificus.*

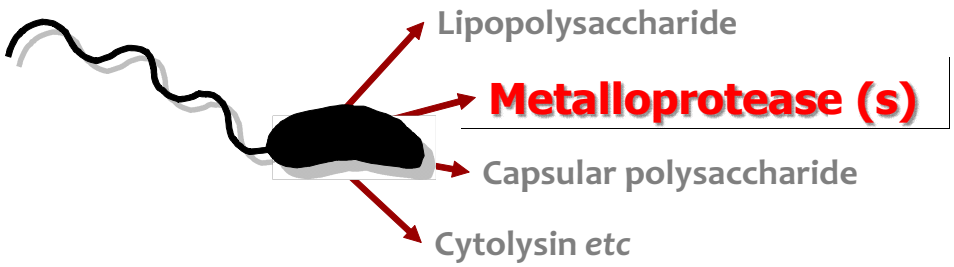
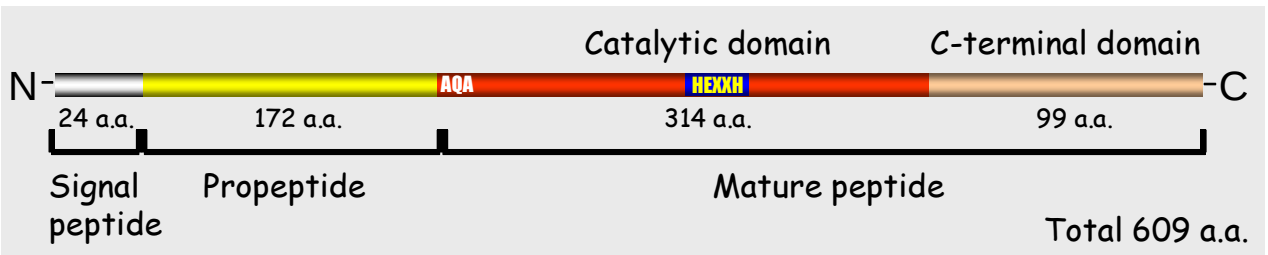


Fig. 1. Bacterial-secreted molecules. Pathogenic bacteria secrete a variety of molecules that may have a role in bacterial virulence and pathogenesis.

V. vulnificus ATCC29307 secretes a 45 kDa metalloprotease. The enzyme is a broad-specificity protease that has many biological activities and is associated with skin lesions and serious hemorrhagic complications (Miyoshi, 2006). The enzyme is synthesized as a zymogen consisting of a signal peptide, an N-terminal propeptide and a mature peptide that contains a catalytic site and a C-terminal propeptide region (Fig. 2). The signal sequence targets the enzyme for secretion, while the propeptide function as intra-molecular chaperone required for the folding of the polypeptide and as an inhibitor preventing premature activation of the enzyme. Tang and coworkers (2003) have studied the general function of the N-terminal propeptide of an extracellular thermolysin-like metalloprotease produced by *Aeromonas caviae* T-64. They showed that it did act as an intra-molecular chaperone to assist the folding of thermolysin-like protease and also had an inhibitory activity toward its cognate mature enzyme. The molecular weight of mature vEP enzyme that has been purified and characterized in this laboratory is 45 kDa and the enzyme can undergo auto-processing with the loss of the C-terminal propeptide yielding a smaller 35 kDa form. This protease shows a broad substrate specificity and can activate prothrombin to have an actual thrombin activity (Fig. 3) (Chang *et al.*, 2005).

Inflammation is the first response of the immune system against a certain infection. The symptoms of inflammation are redness and swelling, which are caused by increased blood flow into a tissue. Inflammation is produced by ecosanoids and cytokines, which are released by injured or infected cells (Kawai *et al.*, 2006). Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They

A



B

Component		Function
Signal peptide		Targets the protein for secretion
Propeptide		May acts as inhibitor for the proteolytic activity
Mature peptide	Catalytic domain	Contains the catalytic machinery including the zinc-binding motif
	C-domain	May be important for binding to certain substrates such as collagen and elastin

Fig. 2. Overall structure of Vibrio extracellular protease vEP. (A) Schematic representation of the structure of vEP. (B) The functions of components of vEP protease.

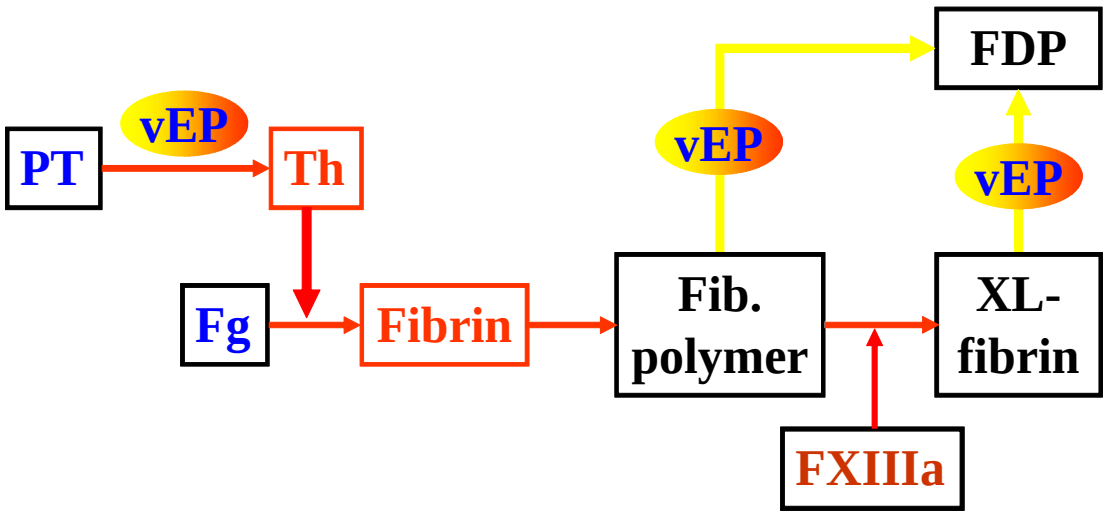


Fig. 3. vEP has prothrombin activation and fibrinolytic activities. Prothrombin is a very efficient substrate for vEP and the activated enzyme can catalyze the formation of fibrin polymer from fibrinogen. vEP can also cleave the cross-linked fibrin formed by factor XIIIa (Chang *et al*., 2005). PT, prothrombin; Th, thrombin; Fg, fibrinogen; FDP, fibrin degradation product.

act by binding to specific membrane receptors, which then signal the cell to make second messengers, often tyrosine kinases, that can alter the expression levels of target genes. The responses to cytokines include increasing or decreasing the expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules.

Common cytokines include; (1) interleukins that are responsible for communication between white blood cells; (2) chemokines that promote chemotaxis; and (3) interferons that have anti-viral effects such as shutting down protein synthesis in the host cell (Le *et al.*, 2004). These cytokines recruit immune cells to the site of infection and promote healing of any damaged tissue following the removal of pathogens (Martin *et al.*, 2005). Cytokines are also made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Major cytokines produced from macrophage are summarized in table 1.

Many types of immune cells, synthesize nitric oxide (NO) and pro-inflammatory cytokines (Coleman, 2001). The pro-inflammatory cytokines (ex, TNF-α and IL-1β) have an important role in diverse cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation and apoptosis (Vandenabeele *et al.*, 1995; Johnson *et al.*, 1996). NO, prostaglandins E2 synthase (PGEs) and their associated enzymes such as NO synthase (NOs) and cyclooxygenases (specifically Cox-2) have been implicated in the development of inflammation (Nathan, 1997; Sautebin, 2000; Tsatsanis *et al.*, 2006). NO is a multifunctional biomolecule involved in a variety of physiological and pathological processes (Lala *et al.*, 2001). Besides, NO produced by iNOS is beneficial or even critical for host survival

Table 1. Major cytokines produced from macrophage.

Cytokine	Producing Cell	Target Cell	Function
IL-1α IL-1β	Monocytes Macrophages B cells DC	Th cells	Co-stimulation
		B cells	Maturation and proliferation
		NK cells	Activation
		Various	Inflammation, Acute phase response, Fever
IL-4	Th2 cells	Activated B cells	Proliferation and differentiation IgG_1 and IgE synthesis
		Macrophages	MHC Class II
		T cells	Proliferation
IL-6	Monocytes Macrophages Th2 cells Stromal cells	Aactivated B cells	Differentiation into plasma cells
		Plasma cells	Antibody secretion
		Stem cells	Differentiation
		Various	Acute phase response
IL-8	Macrophages Endothelial cells	Neutrophils	Chemotaxis
IL-10	Th2 cells	Macrophages	Cytokine production
		B cells	Activation
IL-12	Macrophages B cells	Activated Tc cells	Differentiation into CTL (with IL-2)
		NK cells	Activation
IFN-γ	Th1 cells Tc cells NK cells	Various	Viral replication
		Macrophages	MHC expression
		Activated B cells	Ig class switch to IgG_{2a}
		Th2 cells	Proliferation
		Macrophages	Pathogen elimination
MIP-1α	Macrophages	Monocytes, T cells	Chemotaxis
MIP-1β	Lymphocytes	Monocytes, T cells	Chemotaxis
TGF-β	T cells Monocytes	Monocytes, Macrophages	Chemotaxis
		Activated Macrophages	IL-1 synthesis
		Activated B cells	IgA synthesis
		Various	Proliferation
TNF-α	Macrophages Mast cells NK cells	Macrophages	CAM and cytokine expression
		Tumor cells	Cell death

* DC: dendritic cells; IL: interleukin; IFN: Interferon; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor.

in several infectious diseases, it is also known to be detrimental to the host. An enhanced formation of NO by iNOS resulting from an endotoxic shock can contribute to hypotension, vascular hyperactivity to vascular constrictors, and organ injury and dysfunction (Xie *et al.*, 1994; Moncada *et al.*, 1991). NO production has also been implicated in diseases such as septic shock, and autoimmune disease, in which NO-mediated apoptosis is often observed (Dimmeler *et al.*, 1997). Cox-2 is the key enzyme regulating the production of prostaglandins, central mediators of inflammation. Cox-2 expression is induced by several cellular signals, including TNF-α, IL-1β and IL-6 (Sautebin, 2000).

It is well known that LPS from bacteria can activate TLRs on macrophages to make a signal that can induce an inflammatory response (Xu *et al.*, 2007). In addition, several proteases such as thrombin can activate protease-activated receptors (PARs) (Macfarlane *et al.*, 2001). Therefore, it can be generally expected that vEP can also activate TLRs and/or PARs (Fig. 4).

TLRs are members of a larger superfamily of interleukin-1 receptors (IL-1Rs) that share a conserved stretch of ~200 amino acids in their cytoplasmic region known as the Toll/IL-1R (TIR) domain (Medzhitov *et al*., 2001; Jiang *et al.*, 2005). The region of homology in the TIR motifs is confined to three conserved boxes that contain amino acids crucial for signaling (Billack, 2006). In contrast, the extracellular portions of the TLRs contains a leucine-rich repeat (LRR) motifs. These LRR domains are directly involved in the recognition of a variety pathogens (Akira, 2003). The major ligands recognized by individual TLRs are summarized in Fig. 5. Among TLR

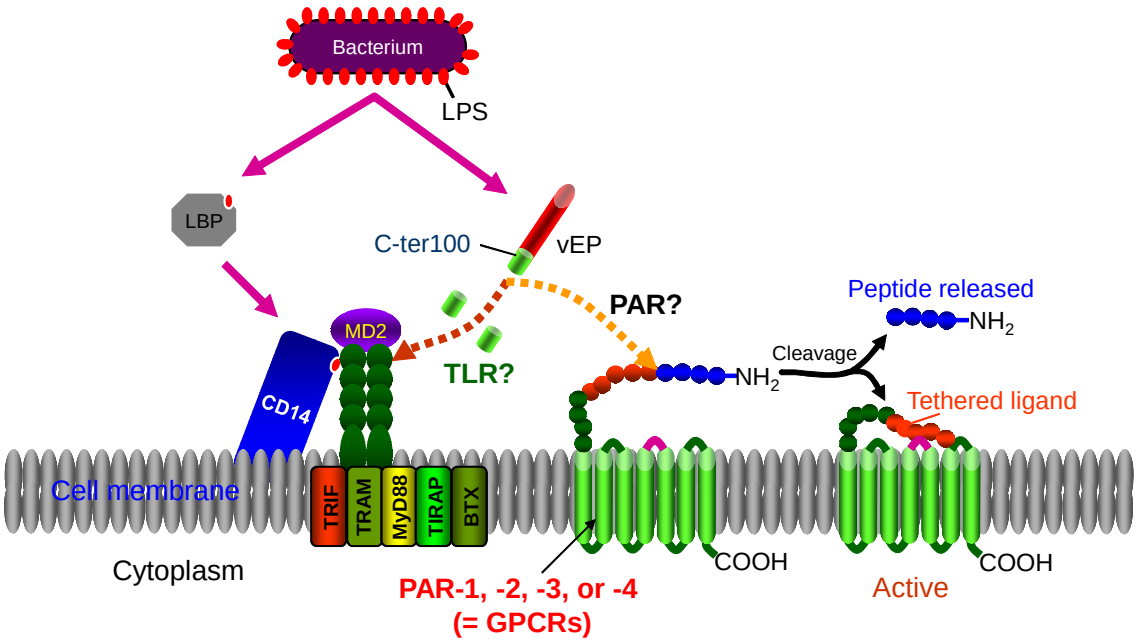


Fig. 4. Possible involvement of vEP in the production of inflammatory mediators through Toll-like receptors (TLRs) or protease-activated receptors (PARs). LBP, LPS binding protein; PAR, Protease-activated receptor.

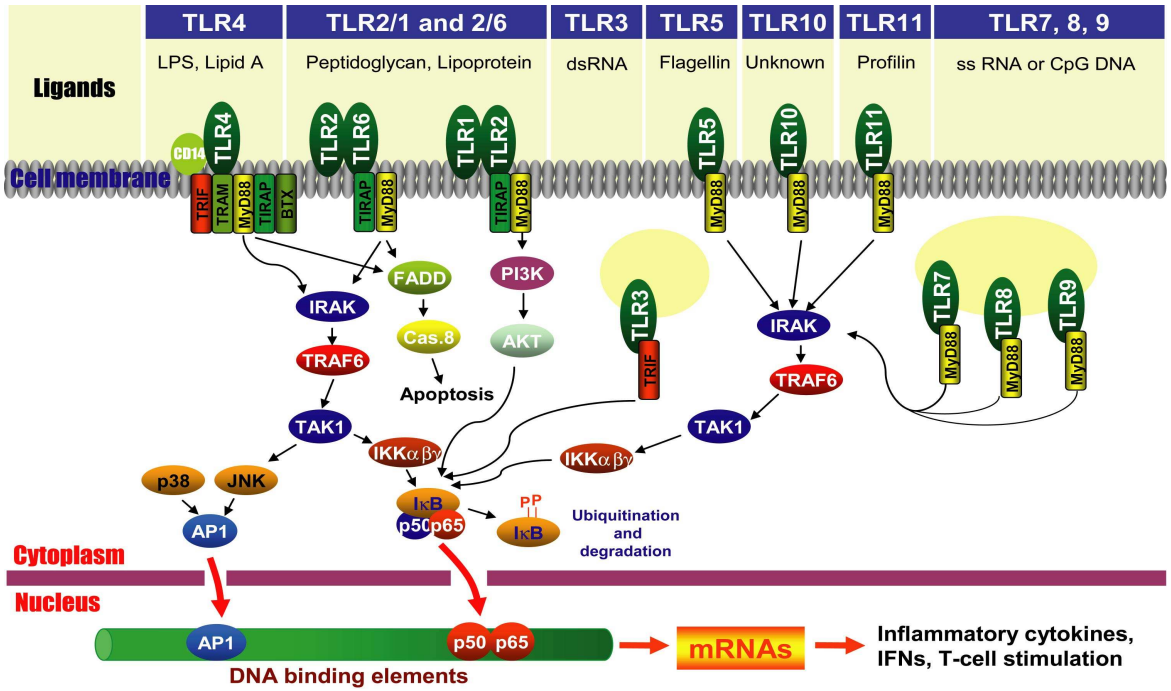


Fig. 5. Signaling pathway and ligands recognized by TLR family.

family members, both TLR2 and TLR4 have been shown to recognize bacterial components (Takeuchi *et al.*, 1999; Takeuchi *et al.*, 2000; Hirschfeld *et al.*, 2001; Xu *et al.*, 2007). The observation that a mutation in the *Tlr*4 gene is responsible for the phenotype of the C3H/HeJ mouse strain, which is unresponsiveness to LPS, a component of the outer membrane of Gram-negative bacteria (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999; Takeuchi *et al.*, 1999). In contrast, TLR2 is implicated in the recognition of Gram-positive bacterial components, bacterial lipoproteins, and zymosan (Schwandner *et al.*, 1999; Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999). The stimulation of TLR leads to the sequential activation of the adapter protein myeloid differentiation factor 88 (MyD88), the IL-1 receptor-associated kinase (IRAKs), TRAF6, and eventually, the IκB kinase complex (IKK-α, -β and –γ) (Matsumura *et al.*, 2003). The NF-κB/Rel family of transcription factors is maintained in the cytoplasm as inactive complexes with inhibitory proteins, called IκBs (Hayden *et al.*, 2004; Xie *et al.*, 1994). The phosphorylation and subsequent ubiquitination of IκB proteins release the free NF-κB dimers which can then readily translocate the nuclear envelope and activates a number of other genes including those responsible for producing cytokines and adhesion molecules (Simeonidis *et al.*, 1999; Yamazaki *et al.*, 2001; Chu *et al*., 2003; Lee *et al*., 2005) (Fig. 5).

PARs belong to a new subfamily of G-protein coupled receptors (GPCRs) with seven transmembrane domains are activated by proteolytic cleavage within the amino terminus exposing a tethered ligand domain that binds and activates the receptors (Dery et al., 1998; Coughlin, 2000; Macfarlane et al., 2001; Hollenberg, 2002) (Fig. 4 and 6). Until now, four members of PARs are known in mouse and human. PAR1, PAR3, and PAR4 are targets for thrombin (Kahn *et al.*, 1998). In contrast, PAR2 is resistant to thrombin, but

can be activated by trypsin (Molino *et al.*, 1997; Camerer *et al.*, 2000). These activation process is followed the trigging of a variety of downstream signal transduction pathways (Fig. 6).

The mitogen activated protein kinases (MAPK) are also activated in macrophages upon the binding of inflammatory cytokines to specific receptors. The MAP kinase family is composed of the ERK 1/2, p38 and SAPK/JNK pathways (Fig. 7). Although the activation pathways are different each other, there is considerable co-operation between these kinases and many substrates (Cobb, 1999). All MAP kinases are highly conserved serine-threonine kinases that are activated by upstream MAPK kinases through a Thr-XXX-Tyr phosphorylation motif (Martin-Blanco, 2000). In general, the ERK 1/2 pathway is activated by growth factors, mitogenic stimuli and tumor promoters (Sung *et al*., 2007), whereas environmental stresses and inflammatory cytokines stimulate the p38 and SAP/JNK pathways (Zu *et al.*, 1998; Bellmann *et al.*, 2000; Kovalovsky *et al.*, 2000).

As mentioned previously, *V. vulnificus* infection is usually accompanied with inflammation. Therefore, it could be expected as a matter of course that vEP should induce an inflammatory response by producing various pro-inflammatory cytokines. Under this expectation, the present study was performed to reveal the implication of vEP in the induction of the cytokines in cultured macrophage cells. This study demonstrate that (1) vEP can activate Toll-like receptors TLR2 and TLR4; (2) vEP binds to the TLRs through its C-terminal region; (3) The TLRs activated by vEP can induce IκB

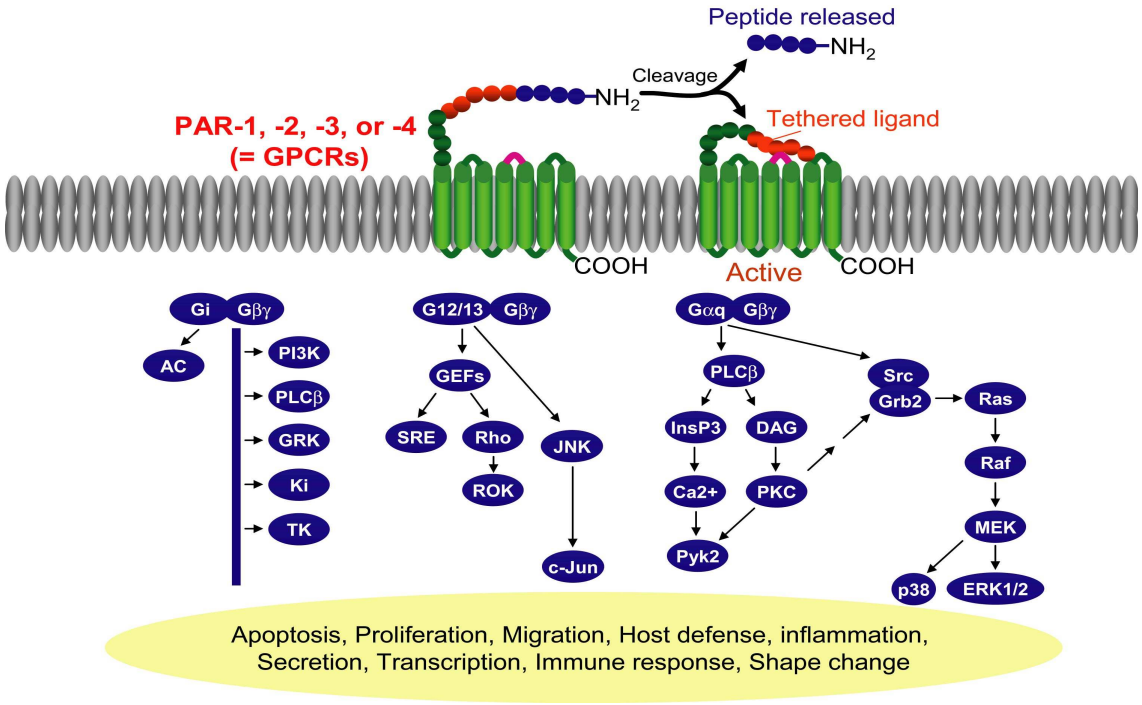


Fig. 6. Signaling pathway by Protease-activated receptors (PARs).

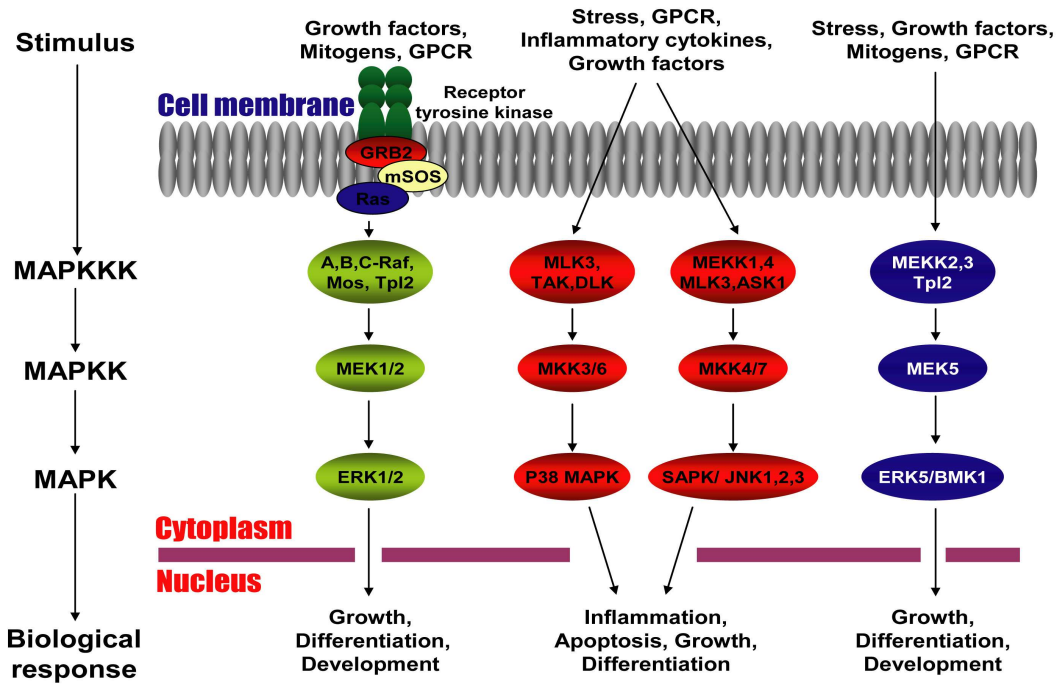


Fig. 7. Signaling pathway by MAPK cascade.

degradation, leading translocation of NF-κB protein into nucleus; (4) the translocated NF-κB proteins can upregulate the expression of various cytokine genes such as TNF-α and IL-1β in their transcription levels; (5) the C-terminal 99 amino acid stretch of vEP plays a critical role in the production of the cytokines.

II. MATERIALS AND METHODS

II-1. Materials

HiPrep 16/10, Phenyl FF (high sub), Superdex 75 10/300 GL, Source 15 Q 4.6/100PE, CNBr-activated Sepharose 4 Fast Flow and PD-10 columns were purchased from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Amylose column and anti-FLAG M2 affinity gel were purchased from New England BioLabs (Inc., USA) and Sigma (St. Louis, MO, USA). Protein molecular weight markers were obtained from Fermentas (Darmstadt, Germany). Bradford protein assay kit, protein A agarose, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), agarose, TEMED, 1,10-phenanthroline (1,10-PT), ammonium sulfate, trizma base, azocasein, N,N-methylene-*bis*-acrylamide, SDS, and other chemicals were obtained from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from BioWhittaker (Waikersville, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kit for tumor necrosis factor-α (TNF-α) was obtained from R&D systems (Minneapolis, MN). The plasmids pFLAG-ATS and pMAL-c2X were obtained from Sigma and New England BioLabs (Inc., USA), respectively. The p*Silencer*™ 4.1-CMV neo plasmid was obtained from Ambion (Texas, USA). The antibodies raised against GAPDH, TNF-α, IκBα, TLR2, and TLR4 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody raised against phospho-NF-κB p65 was obtained from Cell Signaling

Technology (Beverly, USA). Polyvinylidene fluoride (PVDF) membrane was obtained from Bio-rad (Richmond, CA). Peroxidase-conjugated and fluorescein (FITC)- or rhodamine-conjugated immunoglobulin Gs (IgGs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Jackson Immuno Research (West Grove, PA). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). An agarose gel extraction kit, PCR purification kit and i*pfu* polymerase obtained from iNtRON Biotechnology (Seonnam, Korea).

II-2. Cultivation of bacterial strains and Raw 264.7 cells

Vibrio vulnificus ATCC29307 and *E. coli* DH5α cells were cultured in LB media containing 0.5% and 1% NaCl, respectively. These bacterial cells were grown at 37℃ under aerobic conditions. Raw 264.7 cells that were normally used for examining the induction of inflammatory response by vEP were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37℃ under 5% CO2 condition.

II-3. Cloning of genes-encoding vEP, mutant vEPs

The entire coding region of *vEP* gene was amplified by PCR from the chromosomal DNA of *V. vulnificus* ATCC29307 using two primers as follows: 5'-CTTCTCGAGATGAAACTCAATCAACGT-3' and 5'-CGGGGTACCTCAATATT

GCAGCTTTAA-3' as a forward and reverse primers, respectively. (The underlined bases in the primers indicate the introduced restriction sites, *Xho* I in forward primer and *Kpn* I in reverse primer.) PCR for 30 cycles was performed for 1 min at 94℃, 45 sec at 50℃, and 2 min at 72℃ using Applied Biosystem 9700 thermal cycler. The amplified PCR product (420 bp) was doubly digested with *Xho* I and *Kpn* I and then ligated with *Xho* I/*Kpn* I-cut pFLAG-ATS vector to give the construct pVEP. To construct vEP without the C-terminal propeptide (ΔC99), the plasmid pVEP was used as a template for the PCR. The ΔC99-encoding region was amplified using the same forward primer and using reverse primer as follow: 5'-GTGATGGTGAT GGTGATTACCACTTGGCGGCGT-3'. The same PCR program was used. After purification of PCR product, the DNA was used as a template for a second PCR using the same forward primer and using reverse primer as follow: 5'-CGGGGTACCTTA*ATGGTGATGGTGATGGTG*ATT-3'. (The underlined bases in the primers indicate the introduced restriction sites, *Kpn* I in reverse primer and the six histidine codons are italicized). The amplified PCR product (1.5 kb) was doubly digested with *Xho* I and *Kpn* I and then ligated with *Xho* I/*Kpn* I-cut pFLAG-ATS vector to give the construct pΔC99.

The construction of mutant G202D was made by PCR using megaprimers. The outline of mutagenesis was showed in Fig. 8. The plasmid pVEP was used as a template and using two primers as follows: 5'-AAGTCGAATGACGGTTTACGCTAC-3' (The underlined bases in the primers indicate changed base.) and the reverse primer for amplified the entire vEP. The PCR product was purified and used as reverse primer in the second PCR. The second PCR was used as forward primer for amplified the

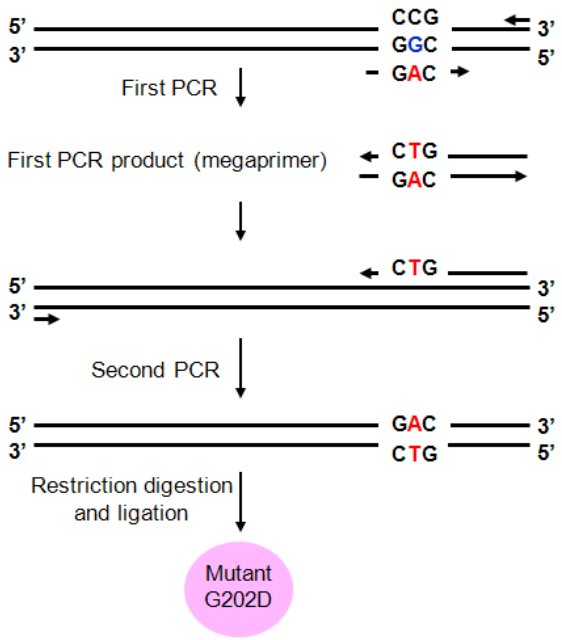


Fig. 8. Outline of mutagenesis protocol used for the construction of G202D mutant.

entire vEP. The second PCR product which was doubly digested with *Xho* I and *Kpn* I and the ligated with *Xho* I/*Kpn* I-cut pFLAG-ATS vector to give the construct pG202D.

II-4. Cloning of genes-encoding vEP-derived peptides (N-ter139 and C-ter100)

The entire coding region of *N-ter139* gene was amplified by PCR from the chromosomal DNA of *V. vulnificus* ATCC29307 using two primers as follows: 5'-TCAGCCTTCGGAGCGCAAGCAGACGGCACTGGA-3' and 5'-GACTCTAGAGGACAACGGATAGAAGGTAGACGC-3' as a forward and reverse primers, respectively. (The underlined bases in the primers indicate the introduced restriction sites, *Eco*R I in forward primer and *Xba* I in reverse primer.) PCR for 35 cycles was performed for 30 sec at 94℃, 30 sec at 60℃, and 40 sec at 72℃ using Applied Biosystem 9700 thermal cycler. The amplified PCR product (420 bp) was doubly digested with *Eco*R I and *Xba* I and then ligated with *Eco*R I/*Xba* I-cut pMAL-c2X vector to give the construct pN-ter139.

A gene for C-terminal region of vEP was amplified from the chromosomal DNA of *V. vulnificus* ATCC29307 by PCR using two primers as follows: 5'-GTCAAGCTTAATGTGTTGAAAAACAACACGCCA-3' and 5'-CGGGGTACCTCAATATTGCAGCTTTAA-3' as a forward and reverse primers, respectively. (The underlined bases in the primers indicate the introduced restriction sites, *Hin*d III in forward primer and *Kpn* I in reverse primer.) The

PCR condition was as follow: 35 cycles of 94℃ for 30 sec, 50℃ for 30 sec, 72℃ for 40 sec. The amplified PCR product (300 bp) was doubly digested with *Hin*d III and *Kpn* I and then ligated with *Hin*d III/*Kpn* I-cut pFLAG-ATS to give the construct pC-ter100.

II-5. vEP and mutant vEPs enzyme expression and purification in *E. coli*

E. coli DH5α cells were used as host cell for the expression of *vEP, ΔC99, and G202D* genes. DH5α cells harboring pvEP or pΔC99 were inoculated into 50 ml of LB broth plus 100 μg/ml ampicillin and were grown overnight at 37℃. Ten milliliters of this overnight culture was used to inoculate 500 ml of fresh LB broth plus ampicillin and was grown at until the A_{600} reached 0.8. A total of 2 liters of cultures were prepared. The target proteins were induced by the addition of 0.2 mM IPTG followed by overnight incubation at 20℃. The cells were harvested by centrifugation, and the periplasmic proteins were isolated by an EDTA-lysozyme treatment. The harvest cells were resuspended in 100 ml of a resuspension buffer [25 mM Tris-HCl (pH 8.0), 20% sucrose, 1 mM EDTA, 0.3 mg/ml lysozyme, 1 mM PMSF] and then incubated at 4℃ for 30 min. The cell suspension was harvested by centrifugation at 18,000 x*g* for 20 min. The supernatant, which contained the periplasmic proteins, was subjected to ammonium sulfate precipitation at 70% saturation. The resulting protein pellet was collected by centrifugation at 35,000 x*g* for 30 min at 4℃, dissolved in 25 mM Tris-HCl

(pH 7.5) containing 1 M NaCl, and then applied to a PD-10 column equilibrated in the same buffer to remove residual $(NH_4)_2SO_4$. The sample was then applied to a phenyl Sepharose HP column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and 1 M NaCl. After a wash with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 1 to 0 M in the same buffer. Fractions were assayed for protease activity using azocasein as a substrate, and those fractions containing major protease activities were pooled and further chromatographed on a Superdex 75 10/300 GL column equilibrated in 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and 0.15 M NaCl.

To obtain G202D enzyme, a single colony harboring pG202D was inoculated into 50 ml of LB broth containing 100 μg/ml of ampicillin and incubated at 37℃ overnight. Ten milliliters of this overnight culture was used to inoculate 500 ml fresh LB broth plus ampicillin. The cultures were incubated with vigorous shaking (200 rpm) at 37℃ until the A_{600} of about 0.8 was reached. IPTG was added to the culture to a final concentration of 0.2 mM and incubated for 6 h at 20℃. The cells were harvested from the cultures by centrifugation at 4,000 x*g* for 15 min and the pellet was resuspended in 100 ml of buffer containing 30 mM Tris-HCl (pH 8.0), 20% sucrose, 1 mM EDTA, 0.3 mg/ml lysozyme, and 1 mM PMSF. The cell suspension was inoculated on a shaker at moderate speed for 30 min at 4℃. The cell suspension was harvested by centrifugation at 18,000 x*g* for 20 min. The supernatant, which contained the periplasmic proteins, was subjected to ammonium sulfate precipitation at 70% saturation. The resulting precipitate was collected by centrifugation at 16,000 x*g* for 40 min at 4 ℃.

The pellet was dissolved in 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and then applied to a PD-10 column equilibrated in the same buffer to remove residual $(NH_4)_2SO_4$. The sample was applied to a HiPrep 16/10 Q FF column pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes equilibration buffer, the column was eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. Fractions were assayed for protease activity using azocasein as a substrate, and those fractions containing major protease activities were pooled, concentrated by ultra-filtration using an Amicon YM10 membrane (Millipore, Billerica, MA), and then further fractionated on a Source 15 Q 4.6/100 PE column (Amersham Biosciences) pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of equilibration buffer, the column was eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. Fractions with major protease activities were pooled, concentrated, and used as the purified enzyme and stored in small aliquots at -20℃.

II-6. Protease activity assay

Protease activity was routinely assayed with azocasein as a substrate. Reaction sample (total 200 ml) containing enzyme, 50 mM Tris-HCl (pH7.5), and 0.25% azocasein was incubated at 37℃ for 15 min. The reaction was stopped by addition of 100 ml of 10% (w/v) trichloroacetic acid and then centrifuged at 10,000 xg for 10 min. Two hundred micoliters of the

supernatant was taken and the absorbance at 440 nm was measured in a 96 well plate reader (Molecular Devices Corporation, California, USA).

II-7. N-ter139 and C-ter100 peptide expression and purification in *E. coli*

E. coli DH5α cells were also used as host cell for the expression of *N-ter139* and *C-ter100* genes. Briefly, the *E. coli* cells were transformed with the appropriate construct as described elsewhere. A single colony harboring pN-ter139 was inoculated into 50 ml of LB broth containing 100 μg/ml of ampicillin and incubated at 37℃ overnight. Ten milliliters of this overnight culture was used to inoculate 500 ml fresh LB broth plus ampicillin and grown at 37℃ until the A_{600} of about 0.5 was reached. A final concentration of 0.3 mM of isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce and the culture was continued for 4 h at 37℃. The cells were harvested by centrifugation at 4,000 x*g* for 15 min and the resulting pellet was resuspended in 50 ml of a lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 mM EDTA, 0.5 mM DTT and 10% glycerol) and sonicated for 30 sec several times. The cell lysate was centrifuge at 10,000 x*g* for 30 min at 4℃ and the supernatant was collected. The sample was applied to an amylose column pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of the same buffer, the column was eluted with an elution buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 mM EDTA, 0.5 mM DTT, 10% glycerol and 10 mM maltose). Fractions were

concentrated by ultra-filtration using an Amicon YM10 membrane from Millipore (Billerica, MA).

To obtain C-ter100 peptide, *E. coli* DH5α cells harboring pC-ter100 were inoculated into 50 ml of LB broth containing 100 μg/ml of ampicillin and incubated at 37℃ for overnight. Ten milliliters of this overnight culture was used to inoculate 500 ml fresh LB broth plus ampicillin and the cells were grown at 37℃ until the A_{600} of about 0.8 was reached. IPTG was then added to the culture to a final concentration of 0.2 mM and incubated for overnight at 20℃. The cells were harvested from the cultures by centrifugation at 4,000 x*g* for 15 min and the resulting pellet was resuspended in 50 ml of a lysis buffer (30 mM Tris-HCl, pH 8.0, 20% (w/v) sucrose, 1 mM EDTA, 0.3 mg/ml lysozyme and 1 mM PMSF). The cell suspension was incubated on a shaker at moderate speed for 30 min at 4℃. It was then centrifuged at 6,000 x*g* for 20 min at 4℃ and the supernatant was collected as a cell-free extract. Ammonium sulfate was added to the cell-free extract to give 20% saturation and the protein precipitate was removed by centrifugation at 16,000 x*g* for 30 min at 4℃. The supernatant was collected and the ammonium sulfate concentration was increased to 70% saturation. The resulting precipitate was collected by centrifugation at 16,000 x*g* for 40 min at 4℃. The pellet was dissolved in 25 mM Tris-HCl buffer (pH 7.5) followed by desalting on PD-10 columns equilibrated with the same buffer. The desalted sample was applied to an anti-FLAG M2 affinity gel pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of the same buffer, the column was eluted with 0.1 M glycine-HCl buffer (pH 3.5). Fractions were

neutralized with 1 M Tris-HCl buffer (pH 8.0) and concentrated by ultra-filtration using an Amicon YM10 membrane. The final purified enzyme was stored in small aliquots at -20℃.

II-8. Preparation of rabbit anti-N-ter139 serum and purification of anti-vEP IgGs

Rabbit anti-N-ter139 serum was produced by Peptron (Daejeon, Korea). Briefly, three boostings were made with 50 μg of N-ter139 peptides and the anti-serum were obtained. The titers of the pre-immune and the anti-N-ter139 sera were 0.199 and 1.959, respectively. To obtain anti-vEP IgGs, 2 mg of N-ter139 were coupled to CNBr-activated matrices in a coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The column was treated with 1 M ethanolamine for 4 h at room temperature to block the non-bound sites. After washing with 10 column volumes of the binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), the rabbit plasma was loaded on CNBr-activated sepharose 4 column pre-equilibrated with the same binding buffer. The bound proteins were eluted with elution buffer (100 mM CH_3COONa, pH 4.0, 500 mM NaCl).

II-9. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). Samples to be analyzed were mixed with an equal volume of 2x SDS-PAGE sample buffer, heated at 100℃ for 1 min, and then loaded onto either 10 or 12% gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie blue. Molecular weight markers used consisted of β-galactosidase (*E. coli*, 116 kDa), bovine serum albumin (bovine plasma, 66 kDa), ovalbumin (chicken egg white, 45 kDa), lactate dehydrogenase (porcine muscle, 35 kDa), restriction endonuclease Bsp98I (*E. coli*, 25 kDa), β-lactoglobulin (bovine milk, 18.4 kDa), lysozyme (chicken egg white, 14.4 kDa).

II-10. Enzyme-linked immunosorbent assay (ELISA)

The 1 x 10^5 cells were plated on 48-well plates the day before vEP stimulation. Cells were treated with LPS, vEP, ΔC99 or C-ter100 (1 μg/ml) for 3 h at 37 ℃. The TNF-α protein levels were determined using ELISA kit (R&D system, Minneapolis, USA) according to manufacturer's instruction. Absorbance at 450 nm was read with a 96-well microplate spectrophotometer (spectraMAX 190, Molecular Devices). Sample concentrations were determined by interpolation from a TNF-α standard curve.

II-11. Total RNA purification and cDNA synthesis

The 5 x 10^6 cells were plated on 100 mm culture dishes the day before vEP stimulation. Cells were treated with LPS, vEP, ΔC99 or C-ter100 (1 μg/ml) for different time periods (0 ~ 12 h). After treatment with LPS, vEP, ΔC99 or C-ter100, the cells were lysed and total RNA was isolated using easy spin from iNtRON Biotecnology (Seongnam, Korea) according to the manufacturer's instruction. cDNA was made from 1 μg of RNA using oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase from Bioneer (Daejeon, Korea). Cycling conditions were 25℃ for 5 min, 42℃ for 1 h and 70℃ for 15 min.

II-12. Real-time PCR

Real-time PCR was performed using SYBR Green kit. Cycling conditions were as follows: denaturation for 20 sec at 94℃, annealing for 20 sec at 50℃, and extension for 30 sec at 72℃ for 40 cycles. The sequences of the specific primers used were as follows: 5'-TCAGCAATGCCTCCTGCAC CAA-3' and 5'-TGCCAGTGAGCTTCCCGTTCAG-3' for GAPDH; 5'-TTTCCTCC CAATACCCCTTC-3' and 5'-AGTGCAAAGGCTCCAAAGAA-3' for TNF-α; 5'-TGTGAAATGCCACCTTTTGA-3' and 5'-GTAGCTGAAACAGCTTCTCC-3' for IL-1β. Real-time assay were run on a Roter Gene 3000 cycler (Corbett research, Mortlake, Australia).

II-13. RT-PCR

The cDNA products were then used as PCR templates to amplify its target genes. The specific primers and cycling conditions used are listed in Table 2. PCR amplification using 0.5 μg of cDNA aliquots were performed by adding 2.5 mM dNTPs, 2.5 units *Taq* DNA polymerase, and 10 pmol each of forward and reverse primers in a PCR buffer [1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3)]. Reactions were processed in a DNA thermal cycler (Eppemdorf, NY, USA) through 30 cycles of 30 sec of denaturation at 94℃, 30 sec of different temperatures (table 2) and followed by 40 sec elongation at 72℃. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide (EtBr), and visualized using a UV transilluminator. The band densities were quantified using Scion image program (Scion Corp., Frederick, MD).

II-14. Western blot analysis

Cells were washed in PBS and then resuspend in a lysis buffer (20 mM HEPES-KOH, pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.25% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM DTT). After incubation for 20 min on ice with shaking, the samples were centrifuged at 15,000 x*g* for 10 min at 4℃. Equal amounts of protein (approximately) were loaded onto a 12%

Table 2. The primer sequences and cycling conditions used in RT-PCR.

Gene		Primer sequence (5' to 3')	Expected size (bp)	Annealing temperature (℃)	GeneBank Accession No.
TNF-α	Forward	TTTCCTCCCAATACCCCTTC	197	48	Y00467
	Reverse	AGTGCAAAGGCTCCAAAGAA			
IL-1β	Forward	TGTGAAATGCCACCTTTTGA	205	50	AK168047
	Reverse	GTAGCTGCCACAGCTTCTCC			
IL-6	Forward	AATTTCCTCTGGTCTTCTGG	220	50	M24221
	Reverse	TAGCCACTCCTTCTGTGACTC			
Cox-2	Forward	CAGCAAATCCTTGCTGTTCC	658	50	BC052900
	Reverse	CCATCCTTGAAAAGGCGCAG			
iNOS	Forward	GTGTTCCACCAGGAGATGTTG	576	53	BC062387
	Reverse	CTCCTGCCCACTGAGTTCGTC			
PGEs	Forward	ATGCCTTCCCCGGGCCTG	462	48	AK150013
	Reverse	TCACAGATGGTGGGCCAC			
MIP-2	Forward	AACAAAGGCAAGGCTAACTGA	203	53	X53798
	Reverse	AACATAACAACATCTGGGCAAT			
TLR2	Forward	GGCCAGGTTCCAGTTTTC	794	55	NM_011905
	Reverse	GGAACAACGAAGCATCTGGG			
TLR4	Forward	ATACATTCCTGTAAGTTACCTG	546	48	NM_021297
	Reverse	CTGCTTAAGTTGACATCTAATGAT			
GAPDH	Forward	TCAGCAATGCATCCTGCACCAC	252	55	BC082592
	Reverse	TGCCAGTGAGCTTCCCGTTCAG			

SDS-polyacrylamide gel and transferred to PVDF membranes (Biorad). The membranes were washed in TBS-T buffer (20 mM Tris-HCl, pH 7.5, 0.1% Tween 20) and non-specific binding sites were blocked in block reagent (5% fat free milk in TBS-T) for 3 h at room temperature on a shaker. The membrane was incubated overnight at 4℃ with specific antibodies raised against TNF-α and IκBα in the blocking buffer. After incubation, the membranes were washed in TBS-T buffer incubated with secondary antibodies for 1 h at room temperature and again washed. Bound antibodies were detected by West-Zol plus detection kit and exposed on X-ray film.

II-15. Determination of NO synthesis

The 5 x 10^5 cells were plated on 48-well plates the day before vEP stimulation. Cells were treated with LPS, vEP, ΔC99 or C-ter100 (1 μg/ml) for 18 h at 37 ℃. The amount of NO was estimated from the culture supernatant by measuring of stable NO metabolite nitrite in the medium by Griess assay (Green *et al.*, 1982). Briefly, equal volumes (90 μl) of culture supernatant and Solution I (1% sulfanilamide and 5% phosphoric acid) incubated at room temperature for 10 min in dark place. And then equal volume of Solution II (0.1% N-[nafthyl] ethyl-enediamine dihydrochloride in 2.5% H_3PO_4) was mixed and incubated at room temperature for 10 min in dark place. The absorbance was determined at 550 nm using a microplate reader.

II-16. Electrophoretic mobility shift assay (EMSA) and supershift assay

The treated cells were washed twice with PBS and resuspended in lysis buffer [10 mM HEPES, pH 7.9, 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT and 0.2 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells were left on ice for 5 min and centrifuged at 15,000 x*g* for 5 min. The resulting pellet was resuspended in 25 ml of high salt buffer [20 mM HEPES buffer, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF] and centrifuged at 15,000 x*g* for 20 min. The supernatant was used as the nuclear extract and stored in small aliquots at -20℃.

A double-stranded oligonucleotide containing the NF-κB binding site (5'-AGCTTGGGGACTTTCC-3') was end-labeled with [γ^{32}P]-ATP (50 μCi at 3,000 Ci/mmol, Amersham Pharmacia Biotech Co. Uppsala, Sweden) and 5 U of T_4 nucleotide kinase at 37℃ for 10 min. The DNA probe was used for each binding assay with the nuclear extracts. Briefly, 10 μg of nuclear extract were incubated for 20 min at room temperature with 0.5 pmol of the radio-labeled oligonucleotides and 250 ng of poly (dI·dC) in a buffer (5% glycerol, 1 mM EDTA, 1 mM DTT) in a final volume of 20 μl. The DNA-protein complexes were separated on native 6% polyacrylamide gel in 0.5% Tris-borate-EDTA buffer. Supershift assay were carried out after incubation of the nuclear extract with 2 μg of anti-p65 antibody for 1 h at 4℃ followed by EMSA.

II-17. Co-immunoprecipitation

Cells were resuspended in a lysis buffer containing 20 mM HEPES, 100 mM NaCl, 1 mM EDTA and 1 mM PMSF. Nonidet P40 was added to the sample to achieve a final concentration of 1% and then incubated at 4℃ for 1 h. The sample was centrifuged at 15,000 x*g* for 20 min to remove insoluble proteins and the resulting supernatant containing membrane proteins were collected. The 10 μg of membrane proteins were incubated with 1 μg of corresponding enzymes (or peptide) to be tested 1 μg of anti-TLR2 or -TLR4 antibody coupled to protein A agarose at 4℃ for 1 h. The precipitates were washed twice in lysis buffer without detergent. Samples from immunoprecipitates were separated by 12% SDS-PAGE and transferred to PVDF membrane, and probed with anti-vEP or -FLAG antibodies.

II-18. Immunolocalization of vEP and TLRs by confocal microscopy

Raw 264.7 cells stimulated with 1 μg of LPS, vEP, ΔC99 or C-ter100 were washed with ice-cold PBS and fixed for 10 min with 4% paraformaldehyde. The fixed cells were blocked for 1 h with a blocking solution (3% bovine serum albumin in PBS) and then treated with a 1:50 dilution of anti-TLR2, -TLR4 or -FLAG antibodies for overnight. After washing

two times with PBS, the cells were incubated with fluorescein or rhodamine-conjugated IgG antibody diluted 1:200 in PBS for 1 h. Cells were stained with 4', 6-diamidino-2-phenylindole and observed using a Zeiss (Le Pecq, France) LSM 510 confocal microscope.

II-19. Design of RNAi molecules and plasmid construction

Based on the complete genome of TLR2 and TLR4 (GeneBank Accession No. NM_011905 and NM_021297) RNAi duplexes were designed. To make the RNAi duplexes p*Silencer*^{TM} 4.1-CMV neo plasmid (Ambion, Austin, USA) was used. This siRNA expression vector was linearized with both *Bam*H I and *Hin*d III to facilitate directional cloning. siRNAs duplexes were synthesized by Bioneer (Daejeon, Korea). The sense and the anti-sense strands of siRNAs for TLR2 were as follows: 5'-GATCCGTCAGCTCACCGATGAAGATTCAAGAGATCTTCATCGGTGAGCTGACTT-3' and 5'-AGCTTAAGTCAGCTCACCGATGAAGATCTCTTGAATCTTCATCGGTGAGCTGACG-3' as a sense and an anti-sense strands. The sense and the anti-sense strands of siRNAs for TLR4 were as follows (Xu *et al.*, 2007): 5'-GATCCCGACTTACAGTTTCTACGTTTCAAGAGAACGTAGAAACTGTAAGTCGTTA-3' and 5'-AGCTTAACGACTTACAGTTTCTACGTTCTCTTGAAACGTAGAAACTGTAAGTCGG-3' as a sense and an anti-sense strands. The siRNA for TLR4 was described previously. The synthesized siRNA constructs were cloned into the *Bam*H I/ *Hin*d III-cut p*Silencer*^{TM} 4.1-CMV neo plasmid.

II-20. Transfection of RNAi constructs into Raw 264.7 cells

Raw 264.7 cells were transfected with each plasmid in a 6-well plate by lipofectamine method (Invitrogen) according to the manufacture's instructions. The 1 x 10^6 cells were plated on 24-well plates the day before RNAi transfection. The 500 ng each of plasmid diluted in 100 μl serum free media and mix with 1.25 μl of lipofectamine. The sample was incubate for 30 min at room temperature and added to the well containing cells. The transfected cells were incubated at 37℃ in a CO_2 incubator for 12 h and 600 μg/ml of G418 was added to obtain stable cell populations.

III. RESULTS

III-1. Molecular cloning and purification of vEP, mutant vEPs, and vEP-derived peptides

III-1-1. Molecular cloning of genes-encoding vEP, mutant vEPs, and vEP-derived peptides

The *vEP* gene encodes 609 amino acids proenzyme (Fig. 9) consisting of a signal peptide, an N-terminal propeptide and a mature region displaying catalytic activity (Fig. 2). Two forms of vEP constructs (pvEP and pΔC99) were cloned and expressed in *E. coli* as described previously (Chang *et al.*, 2007). Computer modeling showed that vEP might have a structure homology with a Zn^{2+}-metalloprotease thermolysin (data not shown). In thermolysin, a residue Asp^{200} plays a critical role in thermal stability of the enzyme. Due to the high homology between vEP and thermolysin, a residue Gly^{202} in vEP, that corresponds to Asp^{200} in thermolysin, was chosen for mutagenic site to improve the thermal stability of vEP. PCR-based site-directed mutagenesis was performed to make mutant G202D as shown in Fig. 10. The cloning of *N-ter139* and *C-ter100* genes were also achieved as shown in Fig. 10.

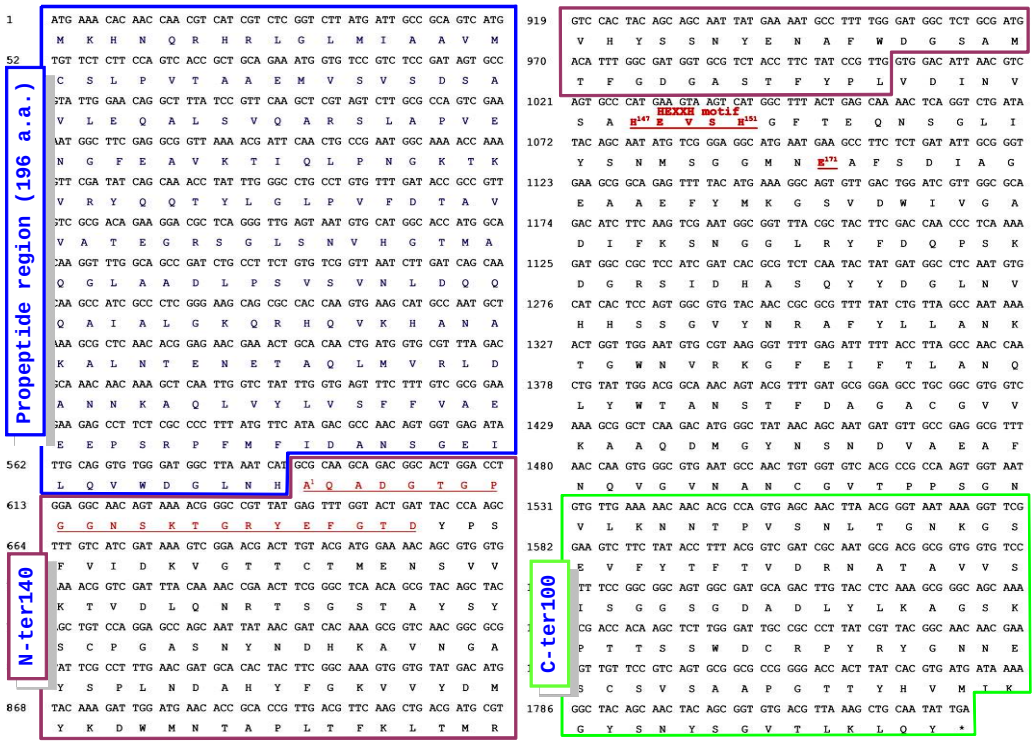


Fig. 9. The nucleotide sequence of vEP-encoding gene and its deduced amino acid sequence. The N-terminal sequence of mature enzyme was AQADGTGPGGNSKTGRYEFGTD. The C-ter100 and N-ter139-encoding sequences are in boxes. vEP contains a typical HEXXH motif that is often found in Zn^{2+}-metalloproteases. The three residues (H^{147}, H^{151}, and E^{171}) are seemed to be coordinated with Zn^{2+} ion.

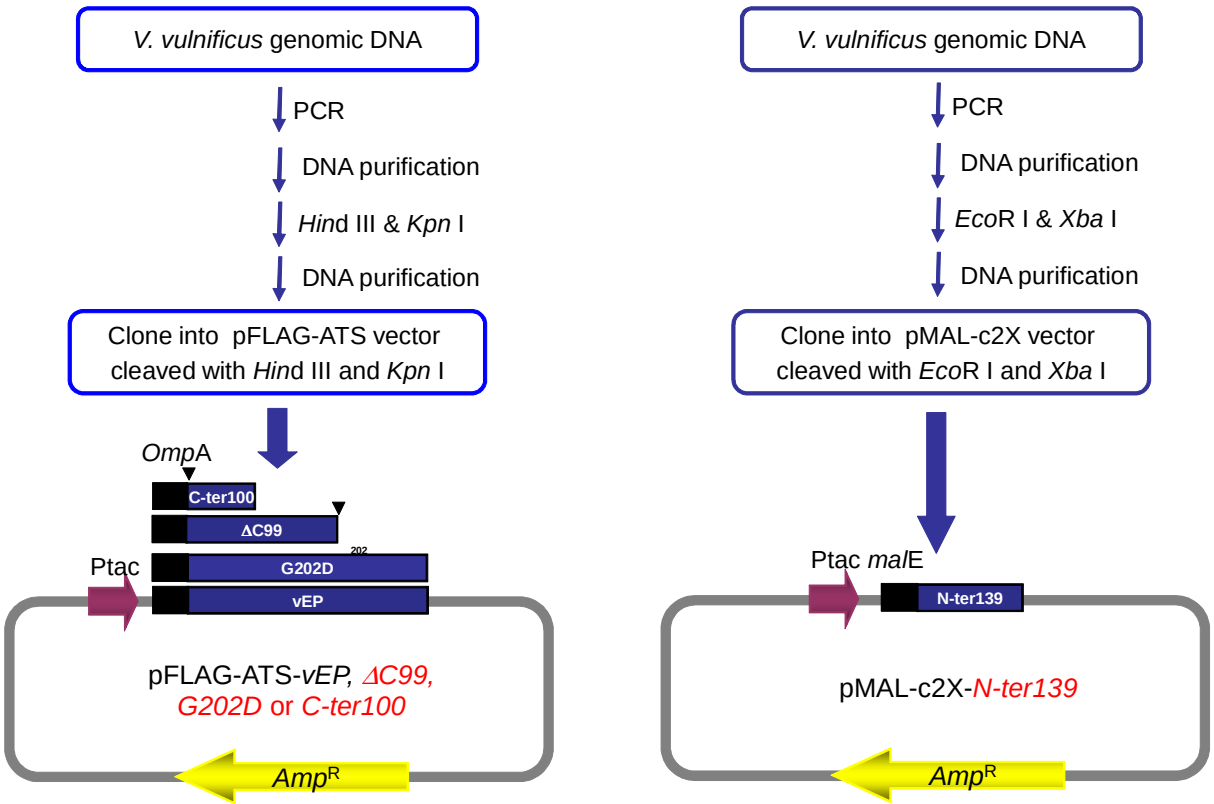


Fig. 10. Cloning step and physical map of pvEP, pΔC99, pG202D, pC-ter100, and pN-ter139.

III-1-2. Expression and purification of vEP, mutant vEPs, and vEP-derived peptides

As pFLAG-ATS is a periplasmic-based expression vector, the expressed proteins are targeted to the periplasmic region. The active recombinant vEP and mutant vEPs (ΔC99 and G202D) could be purified from the periplasmic proteins of the *E. coli* transformants (Fig. 11A). vEP (16,100 U/mg) and ΔC99 (16,200 U/mg) showed similar specific activities as assayed with azocasein, suggesting that the loss of the C-terminal propeptide has a little effect on enzyme activity (Fig. 11B). The purification yield of ΔC99 enzyme was more than twice that of vEP enzyme (Table 3). A mutant protease G202D that has a substitution of glycine^{202} to aspartate^{202} exhibited approximately 58% lower specific activity than that of wild type enzyme (Fig. 11B). The 45 kDa wild type vEP could be actively autoprocessed to make a 35 kDa species during the course of electrophoresis as described previously (Chang *et al*., 2005) (Fig. 11A).

The C-terminal 100 a.a.-encoding region of vEP was also cloned into pFLAG-ATS vector, expressed in *E. coli* and designated to as C-ter100. The peptide was isolated from periplasmic proteins and then purified using anti-FLAG M2 affinity column chromatography. Another vEP-derived peptide N-ter139 was purified using pMAL-c2X vector system shown in Fig. 10. The pMAL-c2X vector has a tac promoter that can be activated by an addition of IPTG and the malE translation initiation signals to give high-level expression. N-ter139 proteins were expressed as a maltose-binding protein (MBP) fusion protein and existed as a soluble protein, which enabled the easy purification

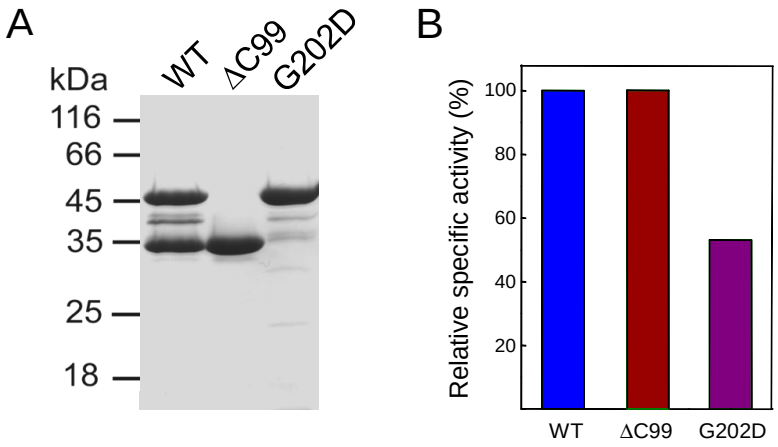


Fig. 11. SDS-PAGE and protease activity assay of purified enzymes. (A) Purified enzymes resolved from a Superdex 75 10/300 GL or Source Q columns were eletrophoresed on a 12% SDS-polyacrylamide gel under reducing condition. (B) The proteolytic activity of purified enzymes were assayed with azocasein as a substrate.

Table 3. Purification summary of vEP, ΔC99, and G202D.

Enzyme	Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)[b]
Wild type vEP	Periplasmic protein	75	282,000	3,750	100
	Phenyl sepharose HP	13	194,000	14,900	69
	Superdex75 10/300 GL	4.7	75,900	16,100	27
ΔC99	Periplasmic protein	118	545,000	4,620	100
	Phenyl sepharose HP	46	509,000	11,700	93
	Superdex75 10/300 GL	23	373,000	16,200	68
G202D	Periplasmic protein	40.7	146,738	3,605	100
	HiPrep Q	7.7	57,228	7,432	39
	Source Q	4.4	41,087	9,338	28

[a] One unit of enzyme activity is defined as the amount of enzyme that catalyzes the proteolysis of 1 μg of azocasein per min at 37℃.
[b] The total activity in the periplasmic region was assigned the value of 100%.

of the protein by one-step chromatography using an amylose column as described in Materials and Methods. The apparent molecular weights of N-ter139 and C-ter100 were found to be 55,400 and 14,000 dalton, respectively as judged by SDS-PAGE (Fig. 12). The purifications of N-ter139 and C-ter100 are summarized in Table 4. The purification yields of N-ter139 and C-ter100 were 19% and 1.8%, respectively. Overall schematic structures of vEP, mutant vEPs, and vEP-derived peptides used in this study are represented in Fig. 13.

III-1-3. Preparation of rabbit anti-N-ter139 serum and purification of anti-vEP IgGs

To obtain anti-vEP IgGs, CNBr-activated matrices were coupled to N-ter139. The bound proteins were eluted with a linear pH gradient ranging from 7.5 to 4.0 (Fig. 14A). The purified protein was designated anti-vEP anti-body.

III-2. vEP can induce the production of inflammatory mediators in macrophage Raw 264.7 cells

III-2-1. Effect of vEP on TNF-α release from macrophages

To assess the effects of vEP on TNF-α production, Raw 264.7 cells were incubated with vEP and the level of TNF-α secreted in culture

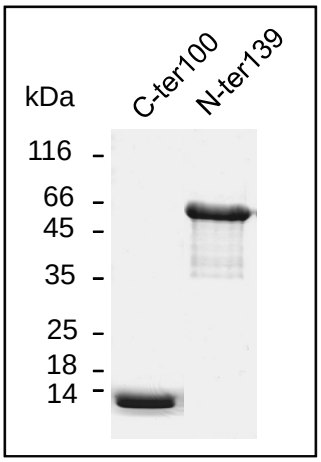


Fig. 12. Analysis of the purified enzymes on 15% SDS-polyacrylamide gel. The electrophoresed proteins were stained with Comassie brilliant blue dye.

Table 4. Purification of summary of C-ter100 and N-ter139.

Enzyme/Peptide	Purification step	Total protein (mg)	Yield (%)[a]
C-ter100	Periplasmic protein	130	100
	FLAG agarose	2.32	1.8
N-ter139	Cell extract	131.5	100
	Amylose column	25.5	19

[a] The total activity in the first purification step was assigned the value of 100%.

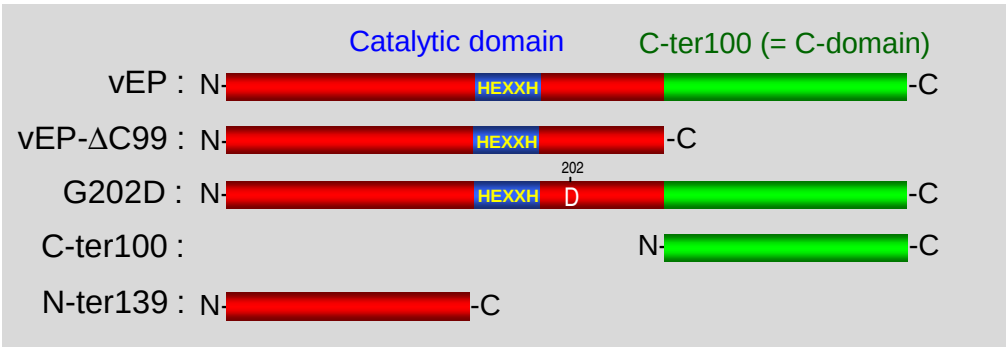


Fig. 13. Overall schematic structures of vEP, mutant vEPs, and vEP-derived peptides.

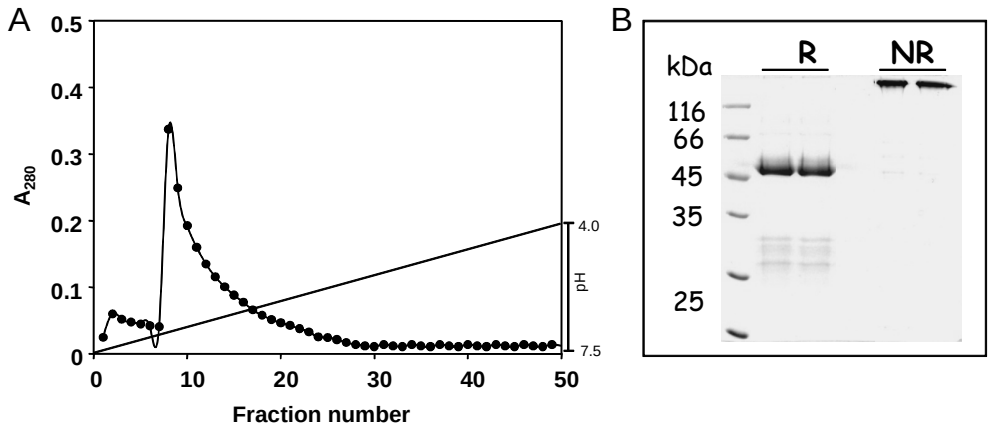


Fig. 14. Affinity chromatography on CNBr-activated sepharose 4 column. IgGs were eluted with a linear pH gradient ranging from 7.5 to 4.0. Flow rate was adjusted to 1 ml per minute and 1 ml sample per fraction was collected. The content of protein was measured at 280 nm (A) and IgGs electrophoresed on 10% SDS-polyacrylamide gel (B). R, reducing; NR, non-reducing conditions.

supernatant was monitored by ELISA. As shown in Fig. 15, TNF-α production clearly increased upon treatment with vEP. When 1 μg/ml of vEP was treated for 3 h, 822.5 pg/ml of TNF-α were produced (Fig. 15). However, 3 μg/ml of vEP could produce TNF-α to about half level compared to that by 1 μg/ml of vEP, due to TNF-α degradation by vEP. Very interestingly, ΔC99 deficient in 99 a.a. residues from C-terminal region of vEP showed no effect on the production of TNF-α (Fig. 15). These results suggest that vEP can induce TNF-α production through by its C-terminal region.

III-2-2. vEP up-regulates the transcription levels of inflammatory regulators

The inflammatory cytokines, TNF-α and IL-1β have been strongly implicated in the pathophysiology of septic shock and the systemic inflammatory response syndrome (SIRS). TNF-α and IL-1β are sensitive indicators of immune stimulation that can help to monitor the levels of cellular activation induced by different biomolecules. To examine whether vEP can induce an inflammatory response, mRNA expression levels of pro-inflammatory cytokines such as TNF-α and IL-1β were measured in Raw 264.7 cells. Treatment of Raw 264.7 cells with vEP led to the induction of TNF-α and IL-1β expressions in a time-dependent manner as shown by real-time PCR and RT-PCR (Fig. 16). The expression levels of TNF-α and IL-1β in their transcriptions were increased significantly at 3 h treatment time, but decreased to almost basal level after 12 h. However, ΔC99 had no effect

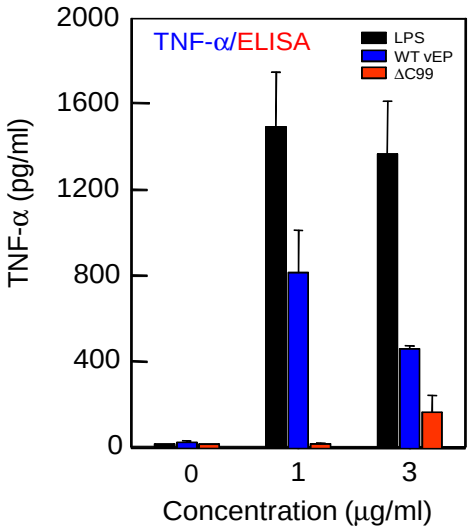


Fig. 15. Production of TNF-α by vEP in Raw 264.7 cells. 1 x 10^5 cells were cultured and treated with different concentrations of LPS, vEP, or ΔC99 for 3 h. TNF-α concentrations in the culture supernatant were measured using an ELISA kit for TNF-α. Results represent the mean ± SD of duplicate determinations from three different experiments.

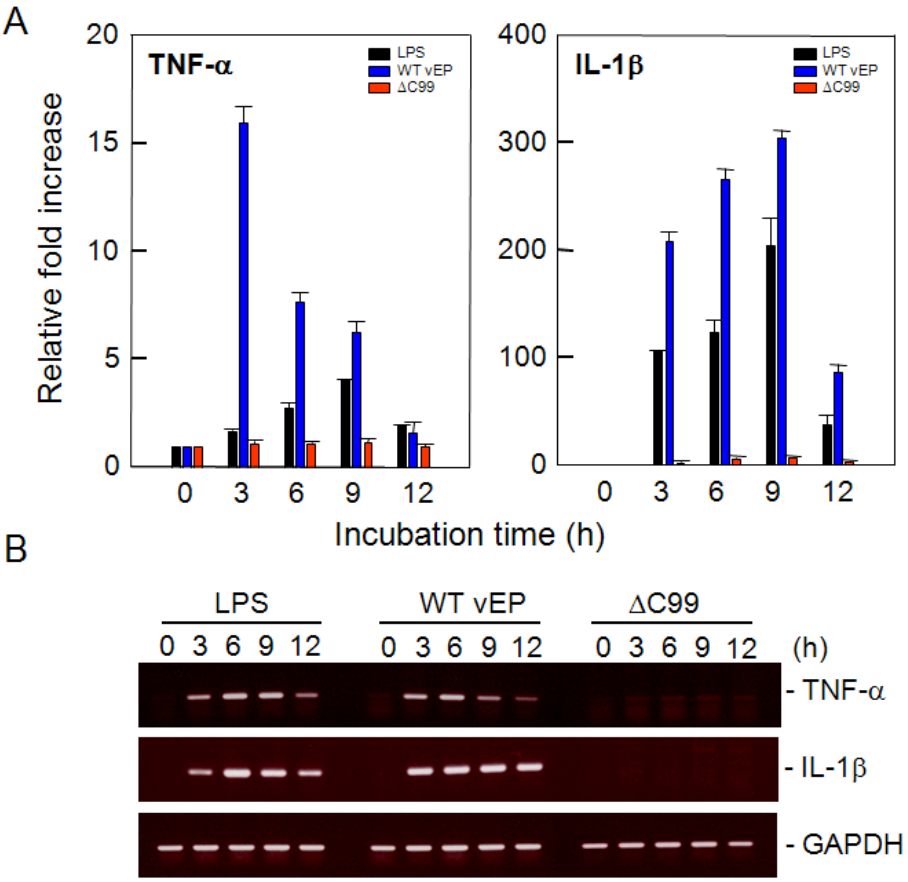


Fig. 16. Effects of vEP and ΔC99 on the pro-inflammatory cytokines mRNA levels in Raw 264.7 cells. Raw 264.7 cells were treated with LPS (1 μg/ml), vEP (1 μg/ml), and ΔC99 (1 μg/ml) for various time periods. Total RNAs were isolated and real-time and RT-PCR were performed using primers specific for TNF-α and IL-1β (A). The PCR products were analyzed on 1.2% agarose gel (B). Each bars are representative of mean value of two separation experiments (A).

on the transcription level of pro-inflammatory cytokines.

iNOS, Cox-2, and their associated products such as NO and prostaglandins E2 synthase (PGEs) are implicated in the development of inflammation (Nathan, 1997; Sautebin, 2000). To investigate the involvements of vEP in the induction of iNOS, PGEs, and Cox-2, their transcription levels were also examined. As shown in Fig. 17, the transcription levels of iNOS, PGEs, and Cox-2 were increased by LPS and wild type vEP, but not by ΔC99 as determined by RT-PCR. Therefore, the increased mRNA expression of pro-inflammatory cytokines (TNF-α and IL-1β) and regulators (Cox-2, PGEs, and iNOS) by vEP further supports a role for vEP as an inflammatory initiator. In addition, these results suggest that C-terminal of vEP is an important region of vEP to induce an inflammatory response.

III-2-3. C-terminal region of vEP plays a critical role in inflammatory response

Based on the previously results (ELISA, RT-PCR, and real-time PCR), it could be expected that the C-terminal region of vEP may play a critical role in an inflammatory response by vEP. As shown in Fig. 18, the C-ter100 only was sufficient to produce TNF-α and also the peptide could restore the inability of ΔC99 in induction of TNF-α, as judged by ELISA. The transcription levels of TNF-α and IL-1β were also clearly increased by LPS, wild type vEP and C-ter100 as determined by RT-PCR. The increased TNF-α transcription level by vEP and C-ter100 were estimated to be 4.12- and

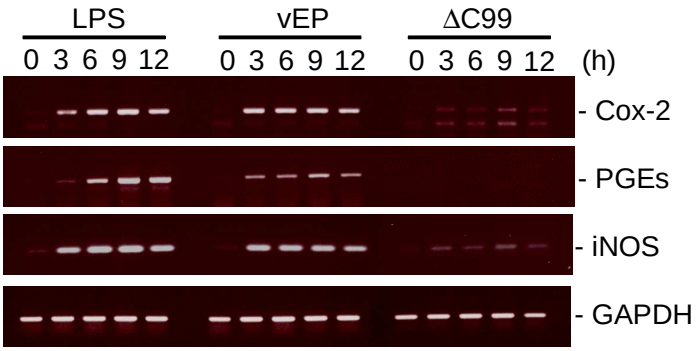


Fig. 17. Up-regulation of Cox-2, PGEs, and iNOS mRNA expression by vEP. Raw 264.7 cells were treated with LPS (1 μg/ml), vEP (1 μg/ml), and ΔC99 (1 μg/ml) for various time intervals. Total RNA were isolated and RT-PCR were performed with primers specific for Cox-2, PGEs, and iNOS. The PCR products were analyzed on 1.2% agarose gel. GAPDH was used as an internal control.

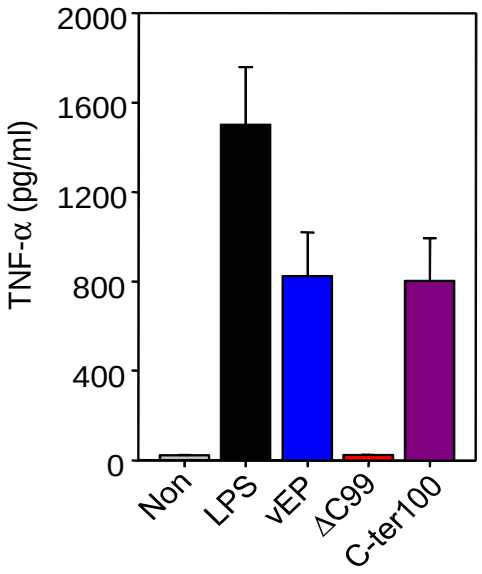


Fig. 18. Production of TNF-α by C-ter100. Raw 264.7 cells (1 x 10^5) were treated with 1 μg/ml each of LPS, wild type vEP, ΔC99 or C-ter100 for 3 h. TNF-α concentration in the culture supernatant was measured using an ELISA kit for TNF-α. Results represent the mean ± SD of duplicate determinations from three different experiments.

3.61-folds, compared with that of non-treated control at 6 h. The transcription levels of IL-1β were also increased to 3.43- and 3.39-folds by vEP and C-ter100, respectively (Fig. 19). Intracellular level of TNF-α in the cell treated with vEP and C-ter100 was also examined using western blotting (Fig. 20). As shown in Fig. 20, TNF-α was clearly produced by vEP and C-ter100, but not by ΔC99. The TNF-α was maximally produced by vEP and C-ter100 at 3 h and decreased to non-treated control level at 9 h. These results strongly suggest that 99 amino acid stretch comprising the C-terminal region of vEP plays a critical role in the induction of pro-inflammatory cytokines and mediators.

III-2-4. Expression of other inflammatory mediators by vEP

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the formation of prostaglandins by cyclooxygenase-2 (Cox-2) (Sautebin, 2000; Tsatsanis *et al.*, 2006). Therefore, increased expression of Cox-2, PGEs, and iNOS are commonly associated with inflammation. As shown in Fig. 20, NO production level in Raw 264.7 cells were significantly enhanced when the cells were treated with vEP. However, ΔC99 appeared to have no effect (Fig. 21). The transcription levels of Cox-2, PGEs, and iNOS genes by vEP and C-ter100 were also investigated (Fig. 22). vEP and C-ter100 could up-regulate Cox-2 transcription level to 2.84- and 3.08-folds, respectively. In addition, the levels of iNOS were increased to 2.57- and 2.54-folds by vEP and C-ter100, respectively.

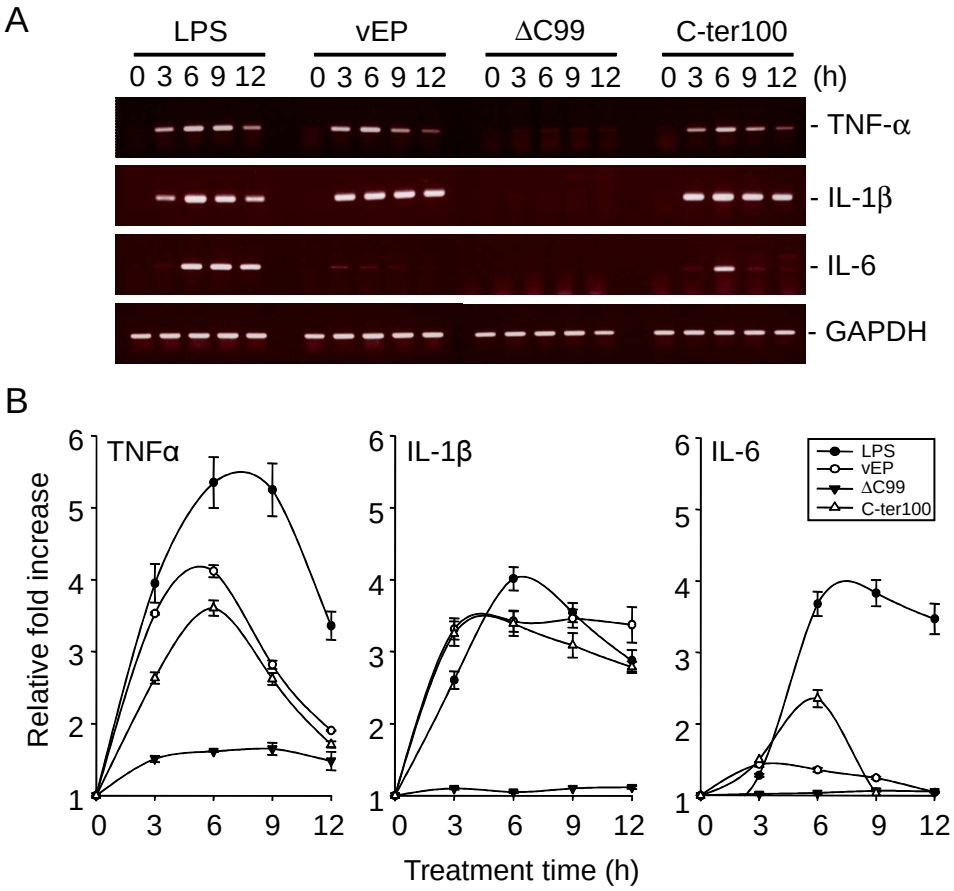


Fig. 19. Transcription levels of pro-inflammatory cytokines by vEP and C-ter100. Raw 264.7 cells were treated with 1 μg/ml each of LPS, vEP, ΔC99, or C-ter100 and then total RNAs were isolated. RT-PCR was performed using primers specific for TNF-α, IL-1β, IL-6, and GAPDH. The PCR products were analyzed on 1.2% agarose gel (A) and the band intensity was measured using Scion image software (B). Each plots are representative of mean value of two separation experiments (B).

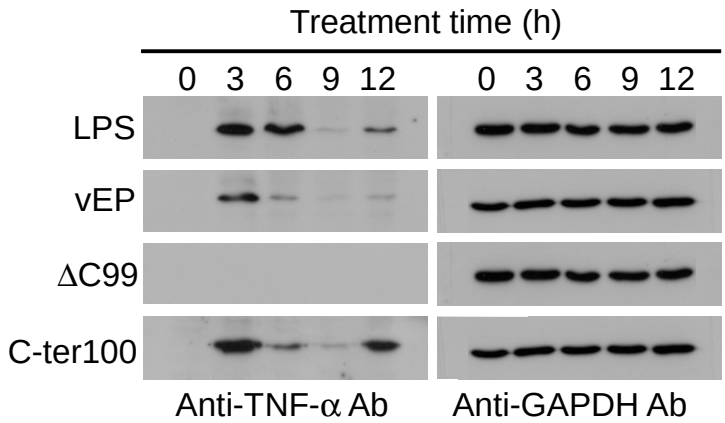


Fig. 20. Western blot analysis of TNF-α in Raw 264.7 cells. After treatment with 1μg/ml each of vEP and C-ter100, cells were lysed in a lysis buffer. The 50 μg of proteins were electrophoresed on 12% SDS-polyacrylamide gel and western blot analysis was performed with anti-TNF-α monoclonal antibody as described in Materials and Methods.

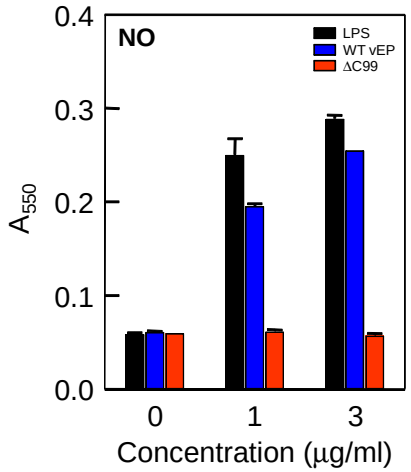


Fig. 21. The production of NO by vEP and C-ter100 in Raw 264.7 cells. Raw 264.7 cells (1 x 10^5) were treated various concentrations of LPS, vEP, or ΔC99 for 18 h and then NO level in the culture medium was measured using Griess reagents as described in Materials and Methods. Results represent the mean of duplicate determinations from at three different experiments.

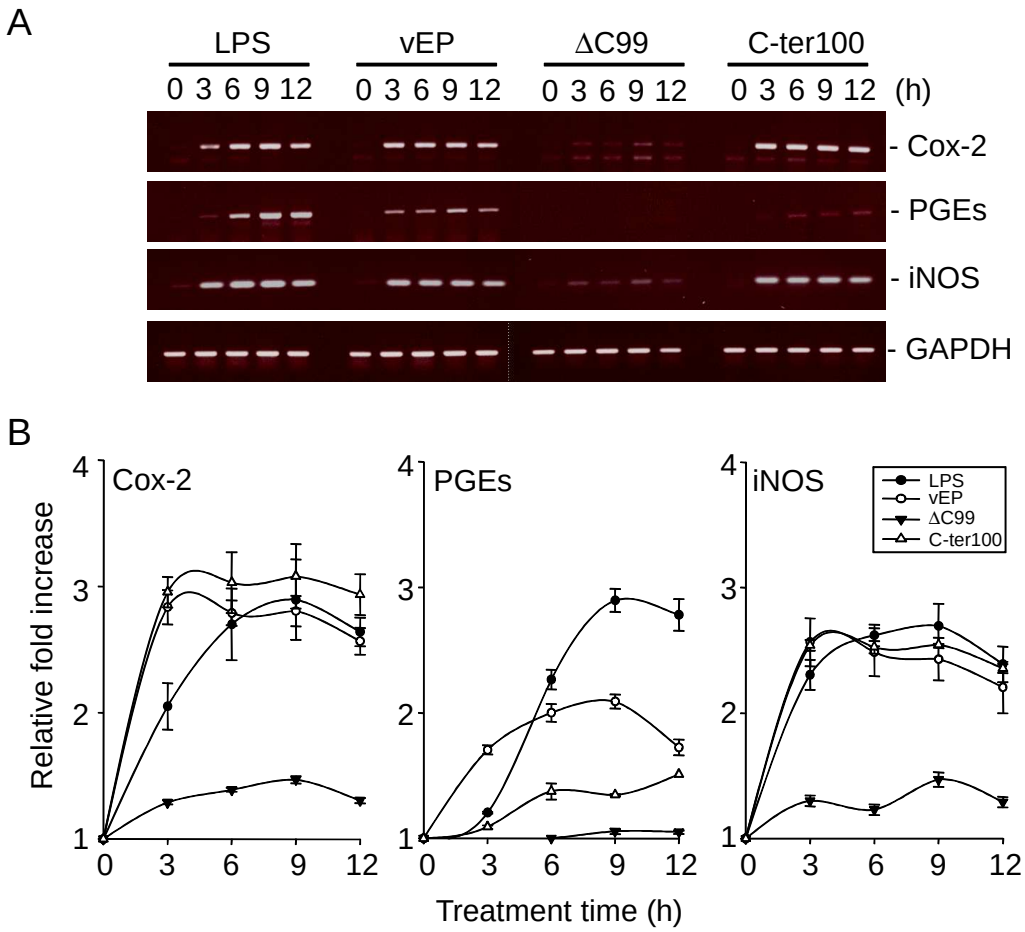


Fig. 22. Effect of vEP and C-ter100 on transcription levels of inflammatory regulators. Raw 264.7 cells were treated with 1 μg/ml each of LPS, vEP, ΔC99, and C-ter100 for indicated time periods and RT-PCRs were performed as described in Materials and Methods. The PCR products were analyzed on 1.2% agarose gel (A) and the band intensity was measured using Scion image software (B). Each plots are representative of mean value of two separation experiments (B).

The transcription level of PGEs was also clearly increased to 1.71-fold by vEP and 1.38-fold by C-ter100 (Fig. 22).

III-2-5. Expression of MIP-2 by vEP

A macrophage inflammatory protein-2 (MIP-2) belongs to the member of chemokine subfamilies. It has been well known that the expression of MIP-2 contributes to the significant neutrophils recruitment to amplify the inflammatory process (De Plaen *et al.*, 2006). As shown in Fig. 23, the transcription levels of MIP-2 was also increased to 2.24-, 2.02-, and 2-folds by LPS, wild type vEP and C-ter100, respectively, as determined by RT-PCR, but not by ΔC99.

III-2-6. vEP and C-ter100 mediate the degradation of IκBα

Degradation of IκBα is a tightly regulated event that is initiated upon specific phosphorylation by activated IKK. Phosphorylation of IκBα leads to poly-ubiqutination in the cytoplasm (Valerio *et al.*, 2006). In this study, it was also examined that vEP and C-ter100 could induce IκB degradation. As shown in Fig. 24, vEP and C-ter100 could clearly induce the IκB degradation at 30 min, but ΔC99 could not. These results show that vEP can lead an IκB degradation through its C-domain.

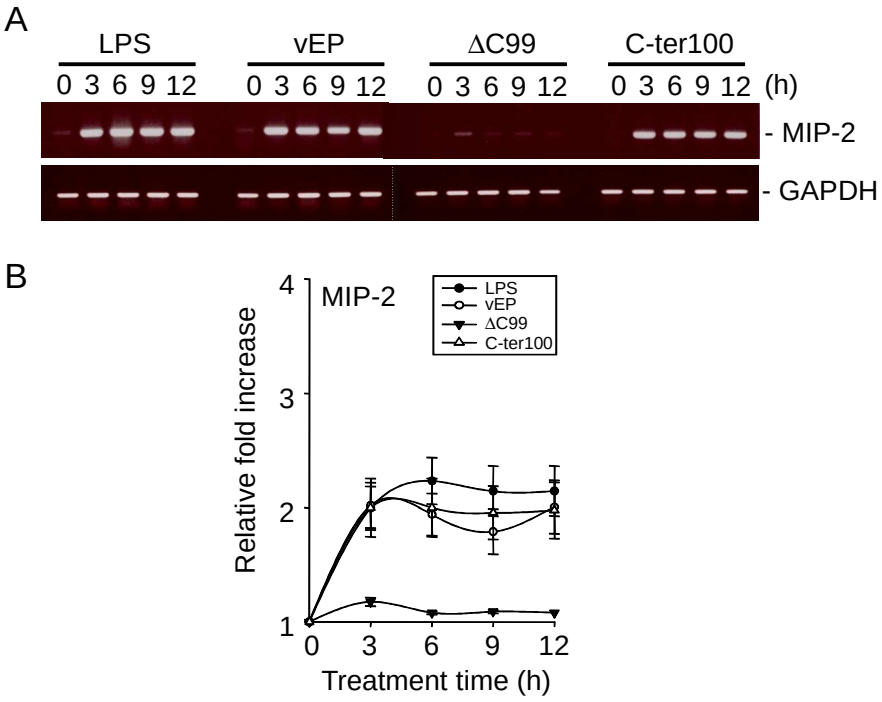


Fig. 23. MIP-2 mRNA expression by vEP and C-ter100 in cultured Raw 264.7 cells. Cells were treated with 1 μg/ml each of LPS, vEP, ΔC99, and C-ter100 for indicated time periods and RT-PCRs were performed as described in Materials and Methods. The PCR products were analyzed on 1.2% agarose gel (A) and the band intensity was measured using Scion image software (B). Each plots are representative of mean value of two separation experiments (B).

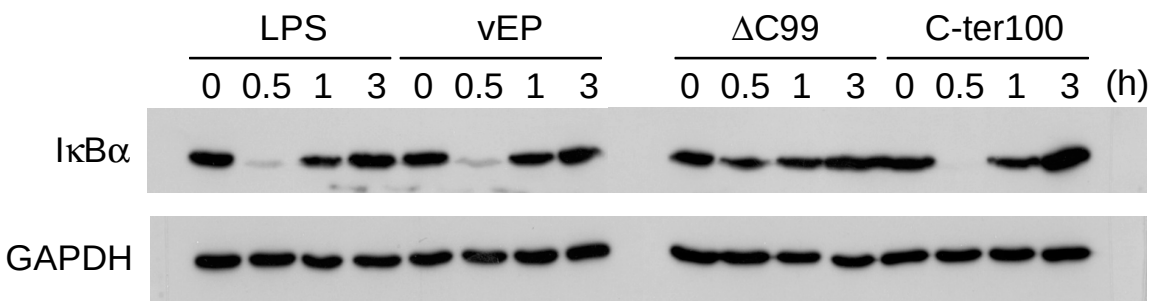


Fig. 24. vEP and C-ter100 mediate the degradation of IκBα. Western blot analysis of intracellular IκBα levels in Raw 264.7 cells treated with 1 μg/ml each of vEP for indicated time periods. Equal amount (50 μg) of cell lysates were separated by SDS-polyacrylamide gel and western blotting was performed with anti-IκBα or anti-GAPDH anti-bodies.

III-2-7. Activation of NF-κB by vEP

IκBα degradation give a free NF-κB proteins from the inactivated complex that is composed of IκB and NF-κB and located in cytoplasm and the liberated NF-κB proteins then translocate into nucleus (Hayden *et al.*, 2004; Fan *et al.*, 2004; Valerio *et al.*, 2006; Salazar-Montes *et al.*, 2006). In nucleus, NF-κB proteins bind to the upstream regions of targets genes to up-regulate their transcription (Zen *et al.*, 1998). Therefore, NF-κB activation by vEP was examined using EMSA and supershift assay (Figs. 25 and 26). As shown in Fig. 25, vEP could induce the NF-κB binding to its consensus sequence element like LPS, but ΔC99 could not. Together with the results of Fig. 24, these results suggest that vEP can activate NF-κB proteins by inducing IκB degradation, in which the C-domain is directly involved. The results also represent that the NF-κB proteins activated by vEP can translocate into nucleus to upregulate the target genes such as TNF-α and IL-1β in their transcription levels. The translocation of NF-κB into nucleus and their binding to the consensus sequence, that are all initiated by vEP, were also confirmed by super-shift assay with anti-p65 antibody (Fig. 26). As shown in Fig. 26, the nuclear extracts prepared from Raw 264.7 cells treated with vEP and C-ter100 could make a supershifted band, together with the shifted band of the consensus sequence element. These results clearly show that vEP can activate p50/p65 NF-κB through its C-domain.

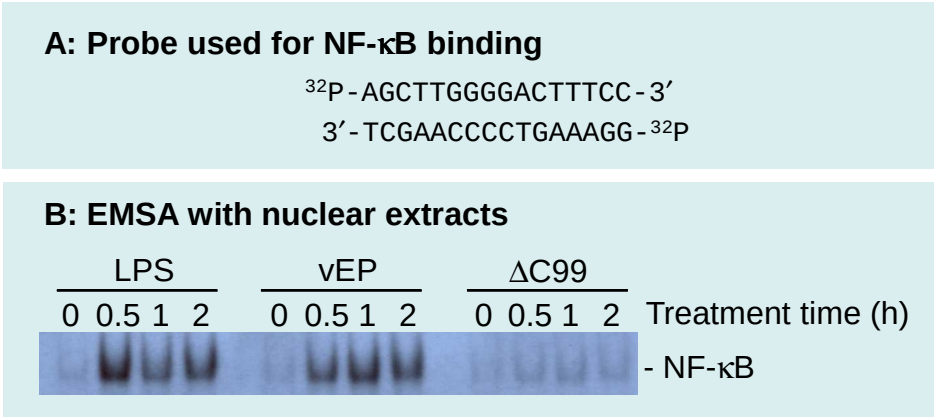


Fig. 25. Activation of NF-κB by vEP. Raw 264.7 cells were treated with 1 μg/ml each of LPS, vEP, and ΔC99 for indicated time periods and nuclear extracts were prepared as described in Materials and Methods. Ten μg of nuclear extracts and [γ-^{32}P]–labeled consensus NF-κB binding element (A) were incubated at room temperature for 20 min and the complexes formed were separated on 6% native polyacrylamide gel. The gel was dried and autoradiographied on X-ray film (B).

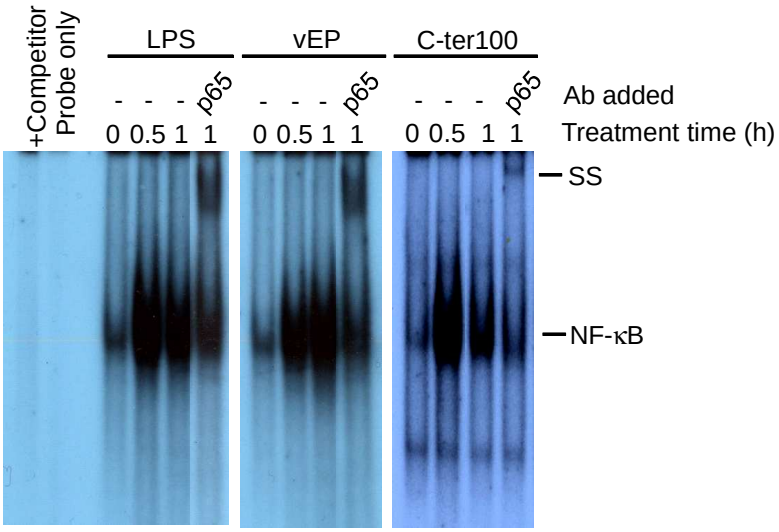


Fig. 26. Supershift assay of NF-κB activated by vEP and C-ter100. Raw 264.7 cells were treated with 1 μg/ml each of LPS, vEP, and C-ter100 for indicated time periods and nuclear extracts were prepared as described in Materials and Methods. Ten μg of nuclear extracts used in Fig. 25 (B), [γ-^{32}P]–labeled consensus NF-κB binding element, and anti-p65 antibodies were co-incubated at room temperature for 1 h and the complexes formed were separated on 6% native polyacrylamide gel. The gel was dried and autoradiographied on X-ray film (B). SS, supershift band.

III-3. Toll-like receptors 2 and 4 can be activated by vEP

III-3-1. Expression of TLR2 and TLR4 by vEP

To determine whether TLRs could be expressed in the response to vEP, the mRNA levels of TLR2 and TLR4 in vEP-treated cells were examined by RT-PCR (Fig. 27). When Raw 264.7 cells were challenged with 1 μg/ml each of vEP or C-ter100 for 30 min, the mRNA levels of TLR2 and TLR4 were significantly increased (Fig. 27). The transcription levels of TLR2 and TLR4 by vEP were increased to 2.29- and 2.57-folds, respectively, compared to those of non-treated control. The mRNA levels of TLR2 and TLR4 were increased to 1.6- and 1.64-folds, respectively, upon treatment with C-ter100. However, ΔC99 could not affect on the TLRs expression (data not shown). These results suggest that TLR2 and TLR4 can be involved in the induction of pro-inflammatory cytokines and the production of inflammatory mediators by vEP protease, in which C-domain of vEP can directly bind to the receptors.

III-3-2. C-ter100 binds to the receptors TLR2 and TLR4

The direct interaction of TLRs and vEP was examined using co-immunoprecipitation and immunofluoresence assay. Protein A agarose beads coupled to anti-TLR2 or -TLR4 anti-bodies were incubated with the membrane fraction of Raw 264.7 cells together with vEP, ΔC99, G202D or C-ter100. The precipitated samples were load on 12% SDS-polyacrylamide

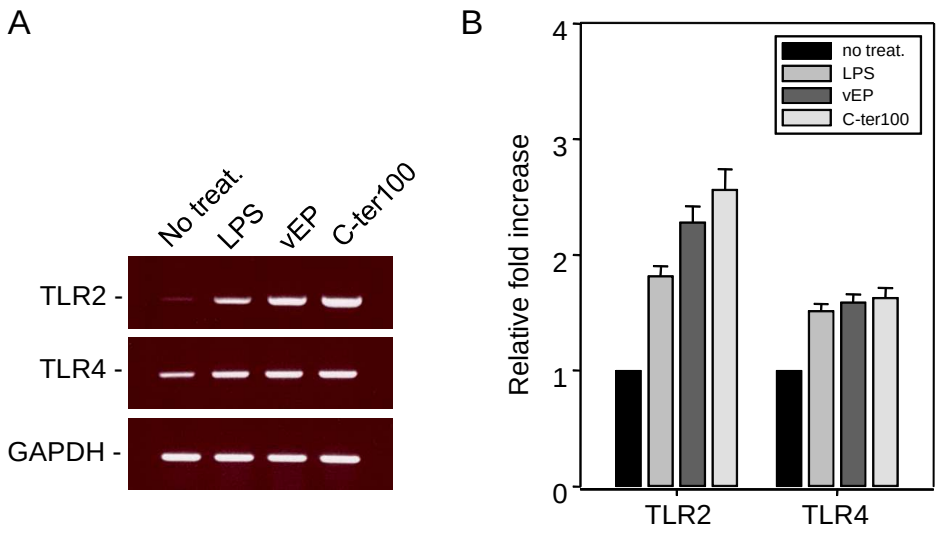


Fig. 27. Up-regulation of TLR2 and TLR4 mRNA expression by vEP and C-ter100. Cells were stimulated with 1 μg/ml each of vEP, C-ter100 or LPS for 30 min and total RNAs were prepared. The mRNA levels of TLR2 and TLR4 were examined by RT-PCR as described in Materials and Methods. The PCR products were analyzed on 1.2% agarose gel (A) and the expression levels were expressed as a relative fold increase (B). Each bars represent mean value of two separate experiments (B).

gel and western blottings were performed with anti-vEP or -FLAG anti-bodies (Figs. 28 and 29). As shown in Fig. 28, vEP and G202D could be precipitated with TLR2 and TLR4, but ΔC99 could not. These results suggest that vEP can bind to TLR2 and TLR4 directly through its C-domain region. If it is true, C-ter100 only should also bind to the receptors. To confirm this, co-immunopricipitation and western blotting were performed (Fig. 29). As shown in Fig. 29, protein A agarose beads coupled to anti-TLR2 or -TLR4 antibodies were incubated with the membrane fraction obtained from Raw 264.7 cells and C-ter100 fused to FLAG peptide. The complexes formed were precipitated and subjected to SDS-PAGE. The western blottings showed that anti-FLAG, -TLR2 or -TLR4 antibodies could bind to all the protein samples that had been immunoprecipitated with anti-TLR2 or -TLR4 antibodies (Fig. 29). In addition, anti-TLR2 or -TLR4 antibodies could make the specific bands on the western blotting with the proteins samples precipitated by only their own antibodies (Fig. 29). These results suggest that C-ter100 directely binds to TLR2 or TLR4 receptors.

To examine that C-ter100 can bind to TLR2 and TLR4, immunostaining assay was performed using confocal microscopy (Fig. 30). Visualization of the C-ter100 and TLRs were carried out using fluorescein- and rhodamine-conjugated secondary antibodies. As shown in Fig. 30, C-ter100 and TLRs were co-localized on the cell surface, suggesting that C-ter100 directely binds to TLR2 and TLR4 receptors.

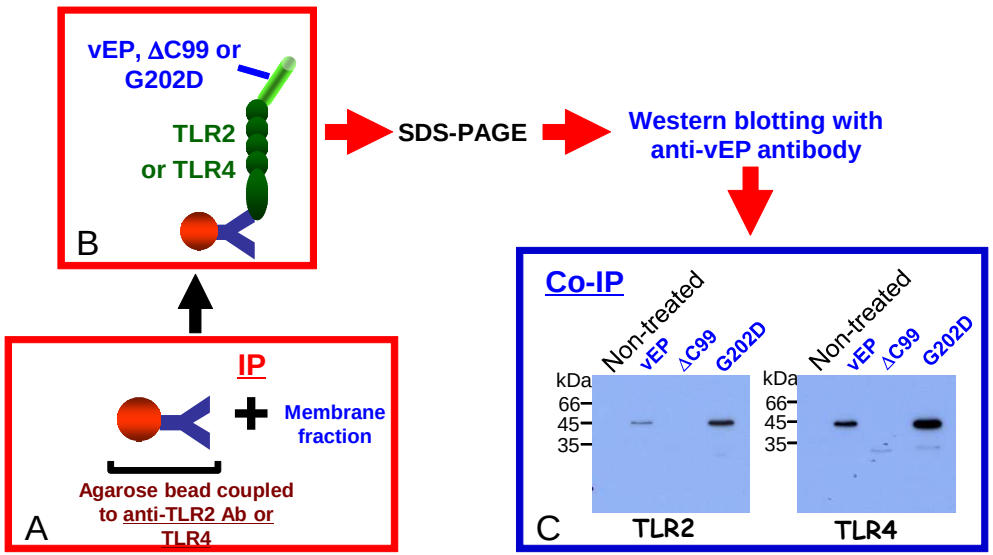


Fig. 28. Direct binding of vEP protease to TLR2 and TLR4 as revealed by co-immunoprecipitation and Western blotting. Agarose beads couple to anti-TLR2 or anti-TLR4 antibodies were incubated with 50 μg of membrane fraction of Raw 264.7 cells (A) and 1 μg each of vEP, ΔC99 or G202D at 4℃ for overnight (B). The precipitated proteins were loaded on SDS-polyacrylamide gel and western blottings were performed with anti-vEP antibody (C).

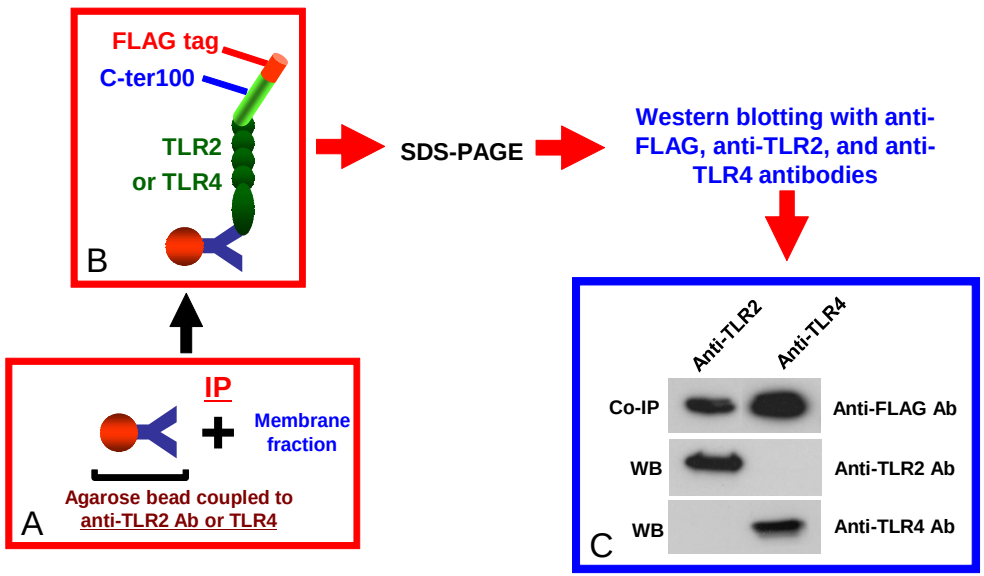


Fig. 29. Co-immunoprecipitation and Western blotting of TLRs and C-ter100 peptide. Agarose beads couple to anti-TLR2 or -TLR4 antibodies were incubated with 50 μg of membrane fraction prepared from Raw 264.7 cells (A) and 1 μg of C-ter100 at 4℃ for overnight (B). The precipitated proteins were loaded on SDS-polyacrylamide gel and western blottings were performed with anti-FLAG, -TLR2, and -TLR4 anti-bodies (C).

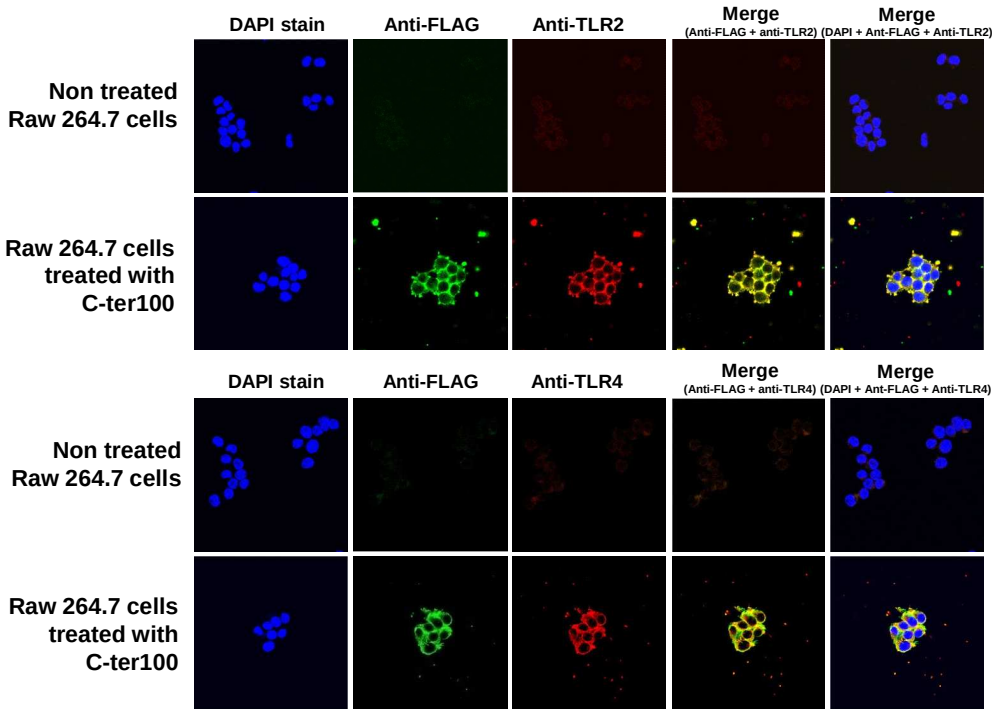


Fig. 30. Immunofluoresence assay of TLRs and C-ter100 by confocal microscopy. The visualized colors were from fluorescein (green)- and rhodamine (red)-conjugated secondary antibodies. The blue color was stained for nucleus with DAPI.

III-3-3. Down-regulation of transcription levels of TLR2 and TLR4 by their RNAi

Based on the previously results (co-immunoprecipitation and immunofluoresence assay), it could be expected that TLR2 and TLR4 may play a critical role in inflammatory response by vEP and C-ter100. Therefore, the TLR2- or TLR4-suppressed cell lines were made by RNA interference (RNAi) technique. As shown in Fig. 31, the RNAi oligos for TLR2 or TLR4 were cloned into p*Silencer*^{TM} 4.1-CMV neo plasmid and the recombinant plasmids were transfected into Raw 264.7 cell for 24 h. To determine whether TLRs could be down-regulated by the RNAi molecules, the mRNA levels of TLR2 and TLR4 in the vEP- or C-ter100-treated cells were examined by RT-PCR. When Raw 264.7 cells were challenged with 1 μg/ml each of vEP or C-ter100 for 30 min, the mRNA levels of TLR2 and TLR4 were not increased compared to those of non-transfected cells (Fig. 32). These results suggest that the transcription levels of TLR2 and TLR4 can be effectively down-regulated by RNAi expressions.

III-3-4. Effect of RNAi on production of TNF-α and NO

To examine the effect of RNAi against TLR2 and TLR4 on TNF-α production, the RNAi transfected-cells were treated with vEP and C-ter100 for 3-18 h and the amounts of TNF-α secreted in culture supernatant were monitored by ELISA. As shown in Fig. 33A, TNF-α production was clearly decreased on treatment with vEP and C-ter100. To further investigate

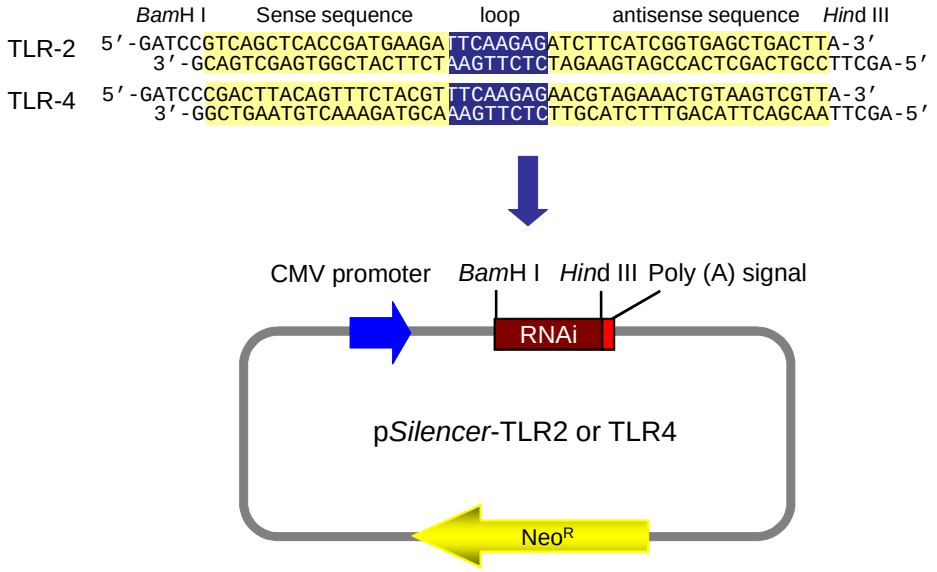


Fig. 31. Constructions of recombinant plasmid expressing RNAi against TLR2 and TLR4. Targeting sequences specific for TLR2 and TLR4 were synthesized as described in Materials and Methods and cloned into p*Silence*™ 4.1-CM neo plasmid to make two recombinant plasmids designated to as p*Silence*-TLR2 expressing TLR2 RNAi and p*Silence*-TLR4 expressing TLR4 RNAi.

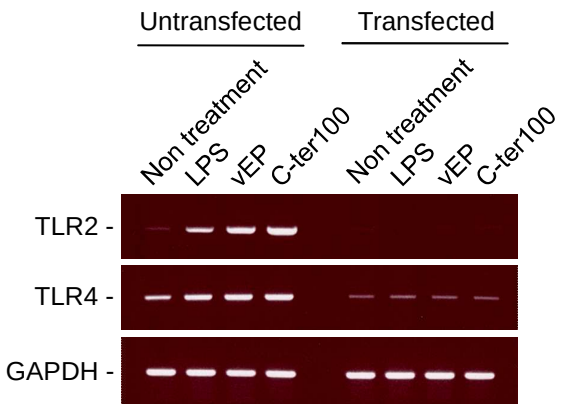


Fig. 32. Expression of TLR2 and TLR4 receptors in Raw 264.7 cells expressing TLR2- and TLR4-RNAi molecules. The cells were transfected with p*Silence*-TLR2 of p*Silence*-TLR4, incubated for 24 h, and treated with 1 μg/ml each of LPS, vEP, and C-ter100 for 30 min. RT-PCR was performed as described in Materials and Methods. The PCR products were analyzed on 1.2% agarose gel.

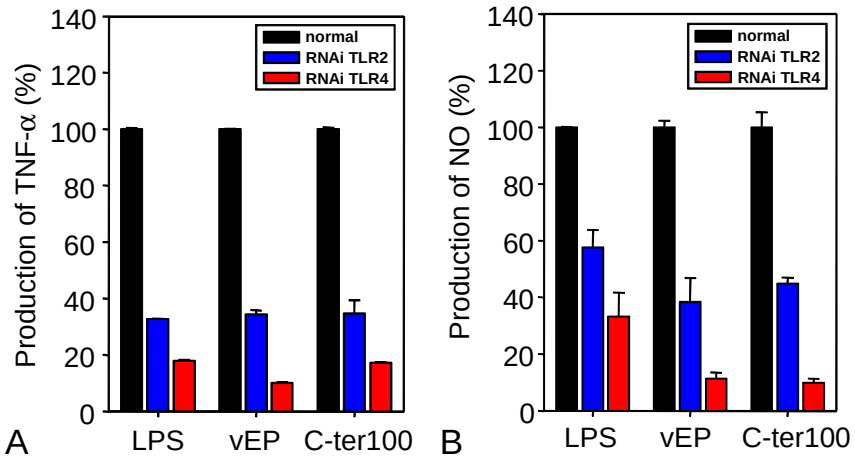


Fig. 33. Effect of RNAi on production of TNF-α and NO. After 24 h transfection, the cells were treated with 1 μg/ml each of LPS, vEP, or C-ter100 and then TNF-α (A) and NO (B) levels in the culture medium were measured using ELISA and Griess reagent as described in Materials and Methods. Results represent the mean of duplicate determinations from at three different experiments.

whether TLR2 and TLR4 RNAi molecules can influence on the secretion of NO in Raw 264.7 cells, the amount of NO in culture supernatant was also measured. As shown in Fig. 33B, NO production levels were also significantly reduced by both in vEP- and C-ter100-treated cells. These results suggest that TLR2 and TLR4 are important receptors in the induction of inflammatory response by vEP.

IV. DISCUSSION

The purpose of the present investigation was to determine the inflammatory potential of vEP in Raw 264.7 cells. Since Vibrio infection is usually associated with inflammation and septic shock, it becomes important to determine if vEP could have a role in the induction of inflammation through activating pro-inflammatory cytokines.

This study describes the expression and purification of wild type vEP and mutants vEP (ΔC99 and G202D). vEP and G202D can be obtained as 45 and 35 kDa forms. The ΔC99 which lacks the C-terminal 99 amino acids appeares only as a 35 kDa form (Fig. 11). As for a mutant ΔC99 enzyme, the deletion was chosen on the basis of the putative cleavage site of vEP resulting from autoprocessing. This site was determined to be at the carboxyl site of Asn^{314} based on the data obtained from mass spectrometry analysis of putatived native vEP (Chang *et al*., 2005). As in other investigations, the absence of C-terminal propeptide results in a loss of efficiency at cleaving insoluble proteins such as fibrin and elastin (Miyoshi *et al.*, 2001; Chang *et al*., 2007). However, this study shows that another property of C-terminal region of vEP, which is demonstrated by ΔC99, G202D, and C-ter100.

LPS is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation (Raetz, 1990; Noursadeghi *et al.*, 2000). It activates monocytes and macrophages to produce pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8, and IL-12 (Johnson *et al.*, 1996; Powell *et al.*, 1997; Vogel *et al*.,

2001). In response to LPS, macrophages also secrete a wide variety of other biological response mediators including platelet-activating factors, prostaglandins, free radicals, and NO. The present work demonstrates vEP-induced up-regulation of TNF-α at a concentration as low as 1 μg/ml. LPS was a better inducer than vEP resulting in almost two folds more TNF-α production at the same concentration as vEP (Fig. 15). Since vEP is a protease with broad specificity, the drop in TNF-α may be caused by proteolysis. Incubation of vEP with recombinant TNF-α reduced in a reduction of TNF-α suggesting the degradation of TNF-α by vEP (data not shown).

The virulence of *V. vulnificus* is associated with a number of factors, such as capsular polysaccharide (CPS) and the pore forming protein known as cytolysin. *V. vulnificus* CPS could directly stimulate the expression and secretion of TNF-α and IL-6 in murine and human cells (Powell *et al.*, 2003). The roles on cytolysin in pathogenesis during *V. vulnificus* infection is controversial. However, because the enzyme has a strong effect on cells and it is regarded as one of the most probable candidates in the pathogenesis of diseases caused by *V. vulnificus* (Miyoshi, 2006) and also can induce the expression of iNOS and production of NO in macrophage in the presence of interferon (Kang *et al.*, 2002). The induced expression is associated with the activation of NF-κB.

Although the role of vEP in the pathogenesis of *V. vulnificus* infection has not been established, its broad specificity and diverse biological activity demonstrate that the enzyme might be a virulence factor secreted by *V. vulnificus*. This study shows for the first time the effect of vEP on cytokine

production. The induction of pro-inflammatory cytokine supports a role of vEP in activating signal transduction pathway and in triggering inflammation. As for induction of TNF-α by vEP might also be mediated by the activity of NF-κB (Figs. 18 and 25). NF-κB is kept in an inactive state by binding with its inhibitor, IκB and sequestered in the cytosol. Activation of NF-κB involves the phosphorylation of IκBα, which then undergoes ubiquitin-mediated proteolysis, thereby releasing NF-κB that is translocated to the nucleus where it activate transcription of a number of different genes, some of which produce pro-inflammatory cytokines. The present study demonstrates that vEP causes the degradation of IκBα (Fig. 24) leading the translocation of p50/p65 of the NF-κB subunits into nucleus (Fig. 26). The induction of iNOS and Cox-2 expression by vEP could be also resulted from the action of activated NF-κB (Figs. 17 and 25). Furthermore, the targets of vEP action *in vivo* have been identified, in that this metalloprotease can enhance a vascular permeability through generation of kallikrein from prekallikrein which then acts to liberate bradykinin from high-molecular weight kiniogen (Feletou *et al.*, 1996; Lerner *et al.*, 1987; Brattsand *et al.*, 1991). The vascular permeability caused by vEP resulted from the proteolytic degradation of type IV collagen in the vascular basement membrane (data not shown).

The inflammatory responses on macrophage can be initiated by the activation of PARs and/or TLRs on the cell surface (Fig. 4). Since vEP is protease, the production of cytokines could be mediated through its binding to receptors on cell surface. The C-terminal part of vEP has an important role in inflammatory response, which is demonstrated by ΔC99 and C-ter100.

The vEP and ΔC99 have similar protease activity (Fig. 11), but there is much difference in inflammatory responses (Figs. 15, 16, and 17). The transcription levels of TNF-α and IL-1β were also clearly increased by wild type vEP and C-ter100, but not by ΔC99 (Fig. 19). IκBα degradation and NF-κB activation is also occurred by vEP and C-ter100, but not by ΔC99 (Figs. 24, 25, and 26). Accordingly, the C-terminal region comprising 100 a.a. of vEP has an important role in up-regulation of the cytokine genes and activation of NF-κB signaling pathway.

The direct interaction of TLRs and vEP shows that vEP can induce an inflammatory response through by activating TLR2 and TLR4 receptors as shown by co-immunoprecipitation and immunofluoresence assay (Figs. 28, 29, and 30). The vEP and mutant vEPs (ΔC99 and G202D) are expressed as a fusion protein containing FLAG tag. The beginning co-immunopricipitaions are performed with anti-TLRs and anti-FLAG anti-bodies, but there is no band on the X-ray film. Although vEP is a broad specificity protease, it can generally cleave c-terminal side of hydrophobic residue (data not shown). Accordingly, the dissociation of the FLAG tag from the purified proteins cause by proteolytic activity of vEP.

The ability of vEP capable of activating TLR2 and TLR4 receptors also can be confirmed using RNAi technique (Figs. 31, 32, and 33). The transcription levels of TNF-α and the production of NO in the cells transfected with the plasmids expressing TLR2 and TLR4 RNAi molecules are dramatically reduced when the cells are treated with vEP (Fig. 33). In recently study, TLRs play central roles in the innate immune response by

recognizing conserved structural patterns in diverse microbial molecules. It has been reported that the structures of the TLR family and its ligand complexes (Jin *et al.*, 2008). Accordingly, the elucidation 3-D structure of C-ter100 will provide us the first opportunity to visualize C-terminal part of vEP at a molecular level. This observation suggests a possibility that bacterial-protease has a homeodomain for induced an inflammatory response. Therefore, the determination of complex structures (TLR2-C-ter100 and TLR4-C-ter100) may be important point in bacterial-proteases induced inflammation.

In summary, the activation of inflammatory response by vEP occurs through by the C-domain binding of vEP to TLR2 and TLR4. The TLR2 and TLR4 recpetors activated by vEP binding can lead the IκB degradation to release the NF-κB proteins. The activated NF-κB proteins traslocate into nucleus and bind to the upstream regions of its target genes (ex, TNF-α and IL-1β-encoding genes), leading the up regulation of their transcription levels. Through by these series of activation of signalling molecules, various pro-inflammatory cytokines and mediators can be produced (Fig. 34).

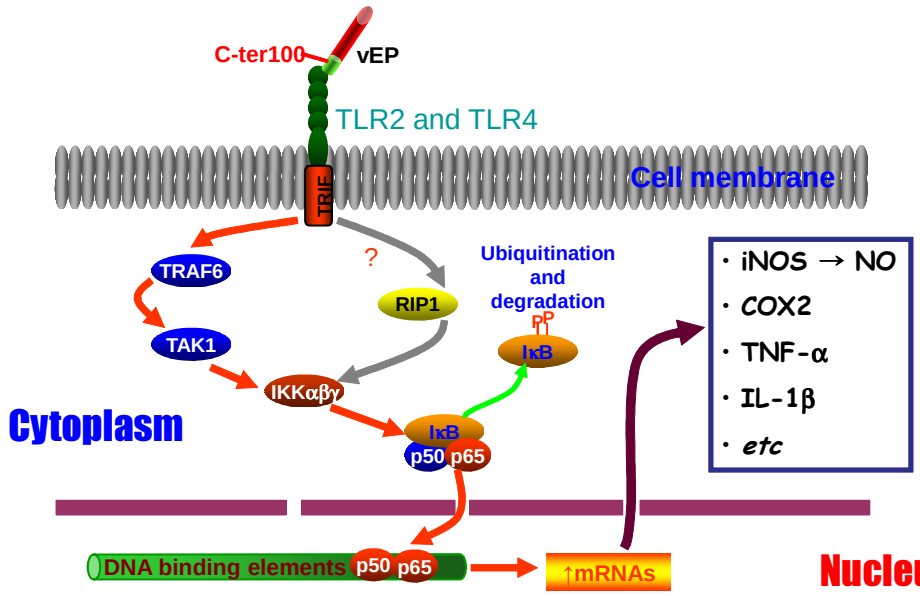


Fig. 34. vEP-mediated signaling pathway in part. The activation of inflammatory response by vEP occur through by the C-domain binding of vEP to Toll-like receptors 2 and 4. The NF-κB is liberated from IκB and then translocated into nucleus to increase the levels of transcription of target genes including for TNF-α and Cox-2.

V. 적 요

분비성 세균 단백질분해효소에 의한 톨-유사 수용체 활성화 및 염증반응 유도기작

박 정 은
지도교수: 이 정 섭
생물신소재학과
조선대학교 대학원

비브리오 패혈증균은 바다에 서식하는 그람음성 세균으로 사람에게 감염되면 장관독소와 함께 식중독을 유발하고 혈액내로 강력한 병원성 유발물질들을 분비함으로써 독소충격증후군(toxic shock syndrome)을 유발하는 것으로 알려져 있다. 일반적으로 비브리오와 같은 병원성 박테리아들은 금속함유 단백질 분해효소(metalloprotease)를 포함하여 capsular polysaccharide, lipopolysaccharide, haemolysin, cytolysin 등을 분비한다. 이중에서 분비성 metalloprotease의 감염 과정에서 역할은 영양성분의 획득, 숙주의 방어체계의 무력화, 숙주세포 감염을 위한 교두보 확보 등만 추측되고 있을 뿐 그 기능이 밝혀지지 않았으나 혈액응고계의 교란 및 세포사멸에도 관여한다는 사실이 본 연구실에 의해 밝혀진 바 있다. 선행연구를 통하여 *V. vulnificus* ATCC29307 균주로부터 분비되는 단백질분해효소를 암호화하는 유전자를 클로닝하여 염기서열을 분석하였으며, 유전자를 대장균에서 과발현시킨 후 순수분리하였다. vEP로 명명된 이 효소는

총 609개의 아미노산으로 구성되어 있었다. vEP는 자가분해능을 지니고 있어 45 kDa과 C-말단부위가 없는 35 kDa의 두가지 형태로 존재한다. vEP는 혈장 단백질들(프로트롬빈, 피브리노겐, 플라스미노겐 등)을 분해하고 프로트롬빈을 트롬빈으로 전환하는 등 혈액응고에도 관여할 뿐만 아니라 교차결합된 피브린을 분해함으로써 혈전을 용해시키는 활성도 지니고 있다 (Chang *et al.*, 2005). 또한 vEP의 활성은 효소 자체의 propeptide에 의해 저해된다(Chang *et al.*, 2007). 본 연구에서는 비브리오 감염 시 vEP의 역할을 규명하고자 대식세포에서 염증반응에 관여하는 여러 가지 사이토카인의 발현을 관찰하고 조절기작에 대한 연구를 수행하였다. 이를 위해 45 kDa 크기의 야생형 vEP 뿐만 아니라 vEP의 C-말단 99개 아미노산이 결실된 ΔC99, vEP의 N-말단 부위 139개의 아미노산으로 이루어진 N-ter139, vEP의 C-말단 100개의 아미노산으로 이루어진 C-ter100를 대장균에서 발현시킨 후 순수분리하여 본 연구에 사용하였다. 정제된 vEP와 ΔC99을 이용하여 염증반응 초기에 강하게 발현되는 대표적 사이토카인인 TNF-α의 생성량을 ELISA를 이용하여 측정한 결과, vEP를 처리할 경우, 높은 농도의 TNF-α가 생성됨을 알 수 있었다. 또한 vEP를 처리하였을 때, mRNA 발현량 역시 TNF-α는 16배, IL-1β는 300배 이상으로 증가하였고 염증반응의 조절인자로 알려져 있는 Cox-2와 iNOS의 발현량도 또한 크게 증가하였다. 흥미롭게도 이러한 모든 초기 염증 반응은 vEP를 처리한 경우에만 발생하고, ΔC99를 처리한 경우에는 어떠한 반응도 일어나지 않았다. 따라서 vEP의 C-말단부위가 염증반응을 유도하는 매우 중요한 부분으로 작용할 것으로 예상되었다. 따라서 C-ter100 재조합 단백질을 이용하여 염증반응의 유도를 관찰한 결과, C-ter100 자체만으로 TNF-α 생성 및 염증반응이 유도됨을 확인하였다. 또한 이러한 염증반응은 vEP에 의해 세포 외부의 수용체중 하나인 Toll-like receptor-2와 -4의 활성화에 의해 세포 내부로 전달됨을 immunopricipitation과 confocal imaging을 통해 확인하였다. vEP에 의한 염증반응 유도 사이토카인들의 생성은 전사인자로 잘 알려져 있는 NF-κB의 활성화를 통해 일어남을 EMSA와 supershift assay를 통해 확인하였다. 따라서 이러한 결과를 종합하여 볼 때,

vEP는 비브리오 감염 시 혈액내의 항상성을 교란 시킬 뿐만 아니라 염증반응의 개시자로 작용하는 것으로 판단된다.

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감사의 글...

실험실에 들어온지 어느덧 9년이 지나 10년째에 접어듭니다. 긴 시간을 보냈다고 생각했지만 막상 졸업을 앞두니 많은 아쉬움과 두려움이 앞섭니다. 먼저 9년 동안 부족한 저를 믿어주시고 포기하려고 할 때마다 “정은아~ 넌 여전히 할 수 있다”라는 말로 이끌어 주시고, 때론 부모님처럼 교훈이 담긴 좋은 글을 건네시며 삶의 가치관과 기준을 정립하게 해주신 이정섭 교수님께 깊은 감사를 드립니다. 또한 성격 탓에 많이 답례해 드리지 못했지만 항상 인자한 웃음으로 격려해주시고 관심을 갖고 충고도 아끼지 않으셨던 함경수 교수님께도 감사드립니다. 그리고 졸업하기까지 깊은 관심을 갖고 지도해주신 생물신소재학과 유호진 교수님, 박일선 교수님, 신송엽 교수님께도 감사드립니다. 그리고 심사해주시고 여러가지 각도에서 생각하는 방법을 가르쳐주신 정혜광 교수님, 성노현 교수님, 박종군 교수님, 김재일 교수님께도 감사드립니다. 또한 지도를 아끼지 않으시고 심사해 주신 생명공학과 박열 교수님, 양영기 교수님, 김성준 교수님, 미국학회에서 따뜻하게 챙겨주셨던 정현숙 교수님, 학생들 입장에서 여러 가지로 배려해주시는 전홍성 교수님, 박윤경 교수님께도 진심으로 감사드립니다.

졸업을 하기까지 가족보다 더 오랜 시간을 부딪기며, 서로 잘 견디고 잘 따라준 사랑하는 실험실 후배들에게 (부지런하고 성실해서 항상 든든했던 종우, 씩씩하고 똘망똘망한 행동으로 항상 자극제가 되어준 은희, 묵묵히 언니 마음을 많이 헤아려주었던 으리, 힘든시기를 잘 견뎌준 재영, 지금은 함께하지 못하지만 꼼꼼한 성격으로 부족한 부분을 채워줬던 남희, 많은 웃음을 선사했던 지호) 진심으로 고마운 마음을 전합니다. 또 후배지만 친구처럼 수다도 떨어주고 샌드백처럼 스트레스도 풀어줬던 홍석이, 가끔 만나서 많이 웃게해주는 상큼한 민영이, 함께 연차보고서 쓰며 허무개그로 웃음을 주며 힘든 일 함께해준 봉석이와 재영오빠, 누리 일로 정신없어도 잘 배려해 주었던 으뜸이와 정애, 모든걸 초월한 듯한 표정으로 여유를 갖게 해준 인덕오빠와 홍범오빠에게도 고마

움을 전합니다. 지금은 다른 곳에서 일하고 있지만 호탕한 웃음으로 스트레스 날려줬던 성진오빠, 잊지 않고 들러서 열심히 하라며 든든하게 격려해주었던 종준오빠, 가끔 만나서 옛날 얘기하며 서로 정말 잘되길 바라는 선배 영란언니, 정아언니, 재성오빠, 상록오빠, 성민오빠에게도 고마움을 전합니다. 나중에 알게 되었지만 항상 만나면 즐겁고 많은 위로가 되었던 동갑쟁이 태원이, 조금 많이 엉뚱해서 만나면 즐거운 현기씨, 선배로 만났으면 더 좋았을 것 같은 정원교 교수님에게도 고마움을 전합니다.

피곤하다는 핑계로 집안일 미루고 친구처럼 많이 얘기도 들어주지 못했던 사랑하는 동생 효은이, 군대에 있지만 학생누나 입장 많이 헤아려 주어 생각만 해도 든든한 막둥이 철이, 6년이라는 시간동안 묵묵하게 지켜봐주고 많이 아껴주고 배려해준 사랑하는 재원이에게도 미안하다는 말과 고맙다는 말을 전합니다. 학생이라는 핑계로 제대로 용돈한번 드리지 못했던 하늘나라에 계시는 할아버지와 외할머니께 손녀의 안타까운 마음과 감사의 마음을 전합니다. 마지막으로 퇴직하시고 여러 가지로 힘드신 상황에서도 마음 편하게 공부할 수 있도록 뒷바라지 해주신 아빠, 행여나 건강 헤칠까 전전 긍긍하셨던 엄마, 손녀가 졸업하기만을 손꼽아 기다리셨던 할머니, 그리고 저를 사랑하고 아껴주신 모든 분 들게 감사드리며 씩씩하고 행복하게 열심히 사는 모습을 보여드릴 것을 약속드립니다.

저작물 이용 허락서

학 과	생물신소재	학 번	20057516	과 정	박사
성 명	한글: 박 정 은 한문 : 朴 貞 恩 영문 : Park Jung Eun				
주 소	광주광역시 동구 서석동 조선대학교 단백질소재연구센터				
연락처	062-230-6768		E-MAIL	ccoppi@lycos.co.kr	
논문제목	한글 : 분비성 세균 단백질분해효소에 의한 톨-유사 수용체 활성화 및 염증반응 유도기작				
	영어 : Activation of Toll-like receptors by a secretory bacterial protease to induce an inflammatory response				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2008년 10월 15일

저작자 : 박 정 은 (서명 또는 인)

조선대학교 총장 귀하