Vibrio vulnificus cytolysin induces hyperadhesiveness of pulmonary endothelial cells for neutrophils through endothelial P-selectin: a mechanism for pulmonary damage by *Vibrio vulnificus* cytolysin

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Abstracts

Vibrio vulnificus **cytolysin forms transmembrane pores that are permeable to calcium ions in pulmonary endothelial cells, and has been suggested as an important virulence factor that sequestrate neutrophils primarily in the lung. To elucidate the mechanism we investigated whether the cytolysin affect the expression of endothelial P-selectin and adhesiveness of pulmonary endothelial cells for neutrophils. The cytolysin increased the adhesiveness of CPAE cell, a pulmonary endothelial cell line, for neutrophils in a concentration- and time-dependent manner. The increase of adhesiveness occurred within several minutes after the cytolysin exposure, persisted up to 90 min, and was not affected by cycloheximide. Furthermore, flow cytometric analyses showed that cytolysin enhanced the level of P-selectin on CPAE cell surface. Therefore, these results suggest that the cytolysin-induced hyperadhesiveness of pulmonary endothelial cells for neutrophils is mediated by the mobilization of endothelial P-selectin to the cell surface.**

Keywords: *Vibrio vulnificus* cytolysin, P-selectin, endothelial cells, neutrophils

Introduction

Vascular endothelial cells respond to a variety of inflammatory mediators including hormones, cytokines, oxidants, bacterial toxins, and various physiologically active compounds (Zimmerman *et al.*, 1985; Schleimer and Rutledge, 1986; Patel *et al.*, 1991; Weller *et al.*, 1992; Palmblad *et al.*, 1994; Krüll *et al.*, 1996). Endothelial cells, by being a first and only available cells in the vasculature, are ready target cells leading to the pathogenesis of human inflammatory disorders. Among the many roles, endothelial cells participate in the neutrophil infiltration to inflammatory sites by providing adhesive molecules for the docking of circulating leukocytes. Endothelial cells are known to express various adhesion molecules, which may regulate neutrophil adhesion such as selectins [E-selectin, endothelialleukocyte adhesion molecule-1 (ELAM-1) or P-selectin, granule membrane protein-140 (GMP-140)], vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (Bevilacqua *et al.*, 1989; Bonfanti *et al.*, 1989; Lorant *et al.*, 1991; Larsen *et al.*, 1992; Bevilacqua and Nelson, 1993; Calos and Harlan, 1994; Boyce *et al.*, 2002). Recent studies show that vascular endothelial cells actively participate in the genesis of inflammatory and systemic alterations occurring in sepsis sustained by gram-negative bacteria. These results suggest that alteration of endothelial cell functions may be primarily associated with the pathogenesis of sepsis in gram-negative bacterial infection.

Vibrio vulnificus is known to be a life-threatening pathogen that causes septicemia and serious wound infection in human. *V. vulnificus* infection is characterized by the high fatality rates of 70% and the primary attack against people who are immunocompromised or have underlying diseases such as liver cirrhosis (Blakes *et al.*, 1979; Park *et al.*, 1991). Kreger and Lockwood (1981) demonstrated that the cytolysin present in culture medium of *V. vulnificus* showed hemolytic activity and cytotoxicity for mammalian cells in culture, and acted as a vascular permeability factor. Even submicrogram amount of the cytolysin is fatal to mice when injected intravenously (Gray and Kreger, 1985). Furthermore, recent data show that *V. vulnificus* cytolysin activates mammalian cells through the stimulation of nitric oxide synthase (NOS) as well as guanlyate cyclase (Kook *et al.*, 1996; Kang *et al.*, 2002). These reports suggest that the cytolysin is the major factor in the pathogenesis of *V. vulnificus* infections. However, the exact pathogenic mechanism of the cytolysin in the progress of the

disease is still not known.

The cytolysin-induced acute lung injury, was characterized by extensive perivascular edema and leukocyte infiltration in perivascular space (Song *et al.,* 1998). The cytolysin increase vascular permeability and sequestrate neutrophil in the lung of mice (Park *et al.*, 1996) where the cytolysin bound to pulmonary endothelial cells induced the cell death through the formation of transmembrane pores on plasma membrane (Kim, 1997). These findings suggest that pulmonary endothelial cells might be important target cells of the cytolysin in the pathogenesis of *V. vulnificus* infection.

In this study, we investigated the effects of *V. vulnificus* cytolysin on functions of pulmonary endothelial cells such as endothelial adhesivity toward neutrophils. Here, we present the first evidence that *V. vulnificus* cytolysin induces hyperadhesiveness of pulmonary endothelial cells for neutrophils through endothelial P-selectin, causing pulmonary damage by *V. vulnificus* cytolysin.

Materials and Methods

Materials

BSA, hydrogen peroxide (H_2O_2), cyclohexhaximide, dodecyltrimethyl-ammonium bromide, dianisidine dihydrochlroride, Dulbecco's phosphate buffered saline (PBS), fluorescein isothiocyanatelabeled antimouse IgG, and Hank's balanced salt solution (HBSS) were purchased from Sigma (St. Louis. MO.). Anti-P-selectin monoclonal antibody (CTB201) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA.). Percoll was from Pharmacia (Mil-waukee, WI.), Fura-2/acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Eugene, OR.), heart infusion broth and RPMI 1640 was from Gibco (Grand Island, NY.). All other reagents were of the highest purity grade available.

Endothelial cell culture

The pulmonary endothelial cell line, CPAE (ATCC CCL 209) was obtained from Korea Cell Bank (College of Medicine, Seoul National University, Korea). CPAE cells were cultured in culture media [RPMI 1640 supplemented with 15% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and ampotericin B (0.25 mg/ml)] in a humidified atmosphere of 5% CO_2. When CPAE cells (30-32 passages) had grown to confluence, adherent cells were removed under sterile conditions from the culture flask by gentle trypsinization (0.05% trpysin, 0.02% EDTA). The cell suspensions were immediately used for various experiments.

Purification of *V. vulnificus* cytolysin

A virulent strain of *V. vulnificus* E4125 was kindly supplied by Dr. M. H. Kothary (Department of Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Ad-ministration, Washington D. C.). The strain was cultured in the heart infusion diffusate broth as described by Kreger *et al.* (1988). Cytolysin was purified to homogeneity from the culture supernatant by a modification of the method developed by Kim *et al.* (1992).

Isolation of neutrophils

Neutrophils suspensions were prepared from EDTA venous blood of healthy rats using the modification of the method of Wright *et al.* (1988). The neutrophils were isolated by discontinuous plasma Percoll gradients using lipopolysaccharide-free reagents [55%/70% Percoll (v/v) in 10 mM Hepes-buffered HBSS (pH 7.3) without Ca^{2+} or Mg^{2+}].

Neutrophil-CPAE cell adherence assay

The adherence of neutrophils to endothelial cells was determined with the cytolysin-treated CPAE cell monolayers in 24-well culture plate. Confluent CPAE cells were washed twice with 10 mM Hepes-buffered HBSS (pH 7.3) containing 5 mM glucose. The cytolysin added to CPAE cell monolayers of each wells and incubated at 37°C. At the end of each incubation period, endothelial cells were fixed with 1% paraformaldehyde/PBS at room temperature for 15 min. After washing of CPAE cells three times with 10 mM Hepes-buffered HBSS (pH 7.3) containing 5 mM glucose, neutrophil suspensions (1 x 10^6 cells/well) were added to each well. The cell mixture was incubated at 37°C for 60 min. CPAE cell monolayers were then washed three times with warm PBS containing 5 mM glucose to remove nonadherent neutrophils. The number of neutrophils bound to CPAE cell monolayers was determined by a modified myeloperoxidase assay of Park *et al.* (19). Briefly, 0.5 ml of dodecyltrimethylammonium bromide [0.5% (w/v)] in modified PBS (PBS without Ca^{2+} and Mg^{2+}, pH 6.0) was added to the wells for 30 min at room temperature to lyse all cells, which caused the release of myeloperoxidase from the neutrophils adherent to endothelial cells. After adding dianisidine dihydrochlroride (0.2 mg/ml) and H_2O_2 (0.4 mM) in modified PBS to a total volume 1 ml of the reaction mixture for 20 min at room temperature, absorbances was read at 450 nm in spectrophotomer (Beckman, DU-65). Absorbance in the lysate reflects the number of endothelial cell-bound neutrophils. Percentage of neutrophil adhesion was calculated as myeloperoxidase enzyme activities in the lysate in relation to their total activities added. Heated cytolysin (toxin kept at 37°C overnight) was designed as control toxin, because its hemolytic activity (HU) was lost rapidly at 37°C.

Flow cytomatric analysis for P-selectin on CPAE cell

Confluent CPAE cells were washed twice with 10 mM
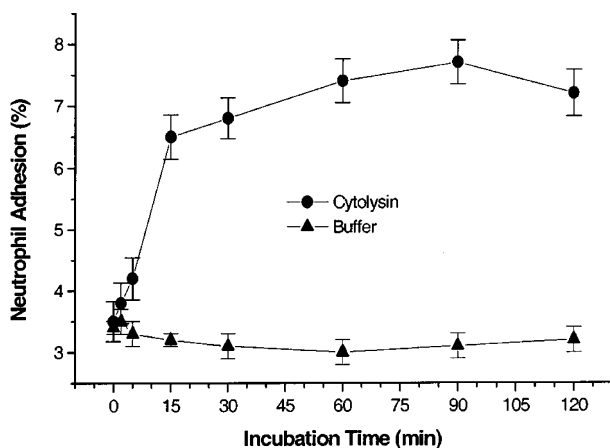
Figure 1. Kinectics of hyperadhesiveness of endothelial cells for neutrophils by *V. vulnificus* cytolysin. Endothelial cells were incubated with 1 HU/ml of the cytolysin at 37°C for the indicated times. The cells were fixed with 1% paraformaldehyde and neutrophils were added to endothelial cells. Percentage of neutrophil adhesion was determined as described in Materials and Methods. Each value depicts the mean ± SE obtained from five separate experiments.
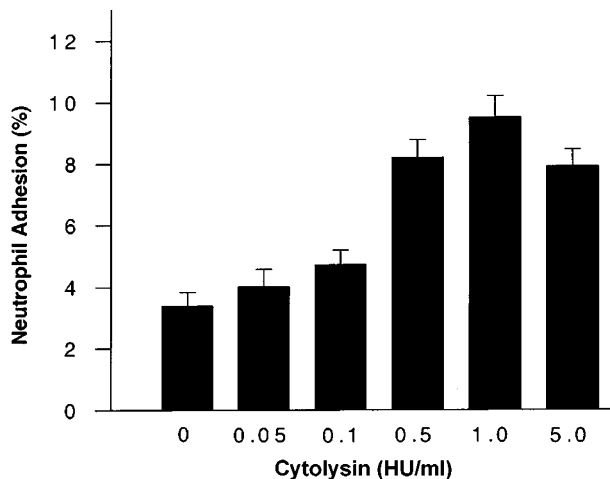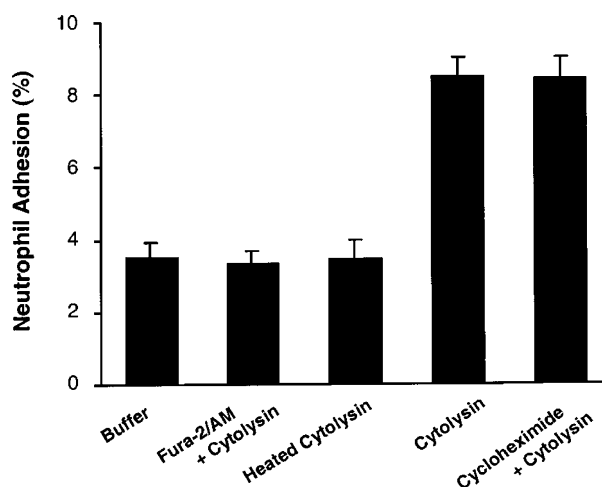

Figure 2. Hyperadhesiveness of endothelial cells for neutrophils by *V. vulnificus* cytolysin at the various concentrations. Endothelial cells were incubated with the cytolysin at 37°C for 90 min. The cells were fixed with 1% paraformaldehyde and neutrophils were added to endothelial cells. Percentage of neutrophil adhesion was determined as described in Materials and Methods. Each value depicts the mean ± SE obtained from five separate experiments.

Hepes-buffered HBSS (pH 7.3) containing 5 mM glucose, trypsinized and incubated with 1 U/ml of cytolysin at 37°C for 1-2 h. The cells were washed and incubated with anti-P-selectin monoclonal antibodies (10 μg/ml) for 30 min in ice. After washing with phosphate-buffered saline, the cells were stained with fluorescein isothiocyanate-labeled anti-mouse IgG and subjected to a flow cytometric analysis.

Results and Discussion

Previous results had demonstrated that *V. vulnificus*

Figure 3. Effect of cycloheximide and heating on hyperadhesiveness of endothelial cells for neutrophils by *V. vulnificus* cytolysin. Endothelial cells were preincubated with 10 μg/ml cycloheximide or 3 μM Fura-2/AM at 37°C for 15 min, and the cells were treated with 1 HU/ml cytolysin in the presence of cycloheximide or Fura-2/AM at 37°C for 90 min. After treatment, neutrophils were added to endothelial cells without paraformaldehyde fixation. Adhesion study for the heated cytolysin was performed by the same method except for the preincubation of cycloheximide. Percentage of neutrophil adhesion was determined as described in Materials and Methods. Each value depicts the mean ± SE obtained from five separate experiments.

cytolysin induced the elevation of intracellular $[Ca^{2+}]_i$ in pulmonary endothelial cells (Rho *et al.*, 2002). Ca^{2+} is known to play an essential role in the stimulation of endothelial cell function, such as hyperadhesion activity for neutrophils, secretion of adhesion molecules, and production of eicosanoids (Grimminger *et al.*, 1990; Zimmerman *et al.*, 1990; Sugama *et al.*, 1992; Palmblad *et al.*, 1994). Therefore, cytolysin-induced increase of $[Ca^{2+}]_i$ could likely affect the function of endothelial cells. In this study pulmonary endothelial cell adhesiveness for neutrophils was studied using endothelial cell monolayer.

The cytolysin increased endothelial adhesiveness for neutrophils in a time-dependent manner (Figure 1). The endothelial adhesivity increased rapidly at 15 min after the cytolysin exposure, and the response was sustained up to 90 min. When endothelial cell monolayers were treated with various concentrations of cytolysin, endothelial hyperadhesiveness was induced in a dose-dependent manner, with a maximal value at 1 HU/ml of cytolysin (Figure 2). At these experimental conditions, there was no endothelial cell lysis in a view of LDH leakadege from endothelial monolayer (data not shown). These results indicate that the cytolysin causes the expression of adhesion molecules from pulmonary endothelial cells. Possible effect of contaminating endotoxin that are known to induce the adhesion of neutrophils to endothelial cells was ruled out. The cytolysin preparation was heat treated to inactivate cytolysin but endotoxin. As shown at Figure 3, the heated cytolysin had no effect on the adhesion of neutrophils. This result clearly suggests that the hypera-

dhesiveness of endothelial cells for neutrophils was evoked by the cytolysin peptide itself and not by contaminating endotoxin.

The adhesion molecules expressed by the cytolysin can be either newly synthesized or secreted from intracellular stores. In order to determine whether the hyperadhesiveness of the cytolysin-treated endothelial cells toward neutrophils is dependent on protein synthesis, the influence of cyclohexaximide on adhesive effect of the cytolysin was studied. Treatment of endothelial cells with cycloheximide did not prevent neutrophil adhesion to the cytolysin-challenged endothelial cells (Figure 3). This finding suggests that neutrophil adhesion mediated by the cytolysin is not dependent on protein synthesis. It usually takes hours rather than minutes to synthesize the new adhesion molecules in target cells after stimuli challenge. Thus, this result is closely correlated with the finding that the endothelial adhesivity induced rapidly within 15 min after the cytolysin challenge.

For neutrophil adhesion to activated endothelial cells, cell-cell adhesion molecules on endothelial cell surfaces are P-selectin, E-selectin, and ICAM-1 (Bevilacqua and Nelson, 1993; Carlos and Harlan, 1994; Boyce *et al.*, 2002). E-selectin is cytokine-inducible adhesion molecules expressed in endothelial cells through *de novo* protein synthesis, which is induced by endotoxin or inflam-matory cytokine interleukin-1 (IL-1) or tumor necrosis factor (TNF). This expression peaks in 4-6 h, declines to basal levels by 24-48 h, and requires *de novo* RNA and protein synthesis (Lorant *et al.*, 1991). Unlike E-selectin, P-selectin is synthesized constitutively and stored intracellularly in endothelial cells. Thus, P-selectin (granule membrane protein-140, GMP-140) of en-dothelial cell secretory granules is rapidly redistributed to the plasma membrane during cellular activation and degranulation, which requires extracellular calcium ions (Geng *et al.,* 1990). In our experimental conditions, buffer change in $[Ca^{2+}]_i$ with Fura-2/AM abrogated neutrophil hyperadhesion mediated by the cytolysin (Figure 3), suggesting that the hyperadhesive effect of cytolysin on endothelial cells is associated with calciumdependent redistribution of P-selectin on endothelial cell surface. A variety of mediators, thrombin and histamine have been shown to induce rapid surface expression of P-selectin, and the expression of P-selectin at the cell surface is short lived, declining substantially within several minutes (Bonfanti *et al.*, 1989; Sugama *et al.*, 1992). However, there are reports that new P-selectin synthesis may be induced by cytokines such as IL-1 and TNF in a manner similar to that of E-selectin (Palmblad *et al.*, 1994). Induction of ICAM-1 by the cytokines (IL-1 and TNF) as well as endotoxin requires *de novo* protein synthesis secondary to the transcription of the ICAM-1 mRNA as E-selectin. In this study, endothelial adhesivity increased within
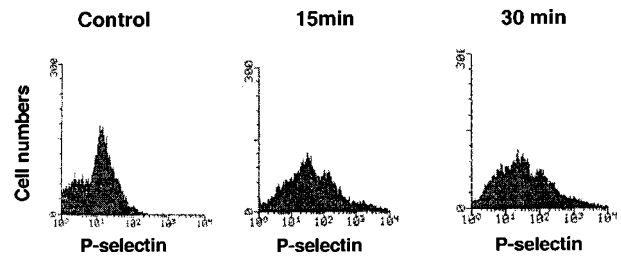
Figure 4. Up-regulation of P-selectin on endothelial cells by *V. vulnificus* cytolysin. Endothelial cells were trypsinized and incubated with 1 HU/ml of cytolysin. After indicated time, the cells were washed, stained with anti-p-selectin antibody and analyzed with flow cytometry.

several minutes after the cytolysin exposure, and the response was sustained up to 90 min. The cytolysin-induced neutrophil adhesion was protein synthesis-independent since the cytolysin-induced adhesion was not inhibited by cycloheximide pretreatment. Thus, the previous results strongly indicate that the cytolysin might induce the expression of adhesion molecules, such as P-selectin from storage granules of endothelial cells. We next examined whether P-selectin is associated with the cytolysin-induced hyperadhesiveness of endothelial cells for neutrophils. Flow cytometric analyses showed up-regulation of P-selectin on surface of endothelial cells by the cytolysin (Figure 4). These results suggest that the cytolysin can induce mobilization of endothelial P-selectin to the endothelial cell surface.

In conclusion, the results of this study indicate that *V. vulnificus* cytolysin induces the hyperadhesiveness of pulmonary endothelial cells for neutrophils, which is mediated by the cytolysin-induced mobilization of en-dothelial P-selectin to the cell surface. The hyperadhesive effects of the cytolysin appear to be largely mediated by an interaction between P-selectin on endothelial cells and a ligand on neutrophils.

Acknowledgments

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