Low-density lipoprotein protects *Vibrio vulnificus*-induced lethality through blocking lipopolysaccharide action

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Accepted 10 September 2007

Abbreviations: VVC, *Vibrio vulnificus* cytotoxin

Abstract

Lipoprotein plays a role in the host defense against bacterial infection, and its serum level has been demonstrated to be an important prognosis factor of survival. We have previously demonstrated that LDL directly inactivates the hemolytic activity of *Vibrio vulnificus* cytotoxin (VVC) *in vitro*. The object of this study was therefore to examine whether the LDL-mediated inactivation of VVC leads to protection against lethal infection of *V. vulnificus* *in vivo*, using wild and VVC-deficient *V. vulnificus* strains. Unexpectedly, we found that LDL protects mouse lethality induced by VVC-deficient as well as wild

V. vulnificus strain. We also demonstrated that LDL blocks *V. vulnificus* LPS-induced lethality in mice. These results suggest that LDL preferentially act on endotoxin rather than exotoxin in the protection against *V. vulnificus*-induced mice lethality.

Keywords: endotoxemia; lipopolysaccharides; lipoproteins, LDL; *Vibrio vulnificus*; vvP protein, *Vibrio vulnificus*

Introduction

Bacterial constituents within human blood are involved in the generation of bacterial sepsis and septic shock, including exotoxin, endotoxin (LPS), peptidoglycans and lipoteichoic acid. Thus, scavengers of bacterial products are expected to protect against bacterial infection and sepsis, such as plasma lipoproteins (Canturk *et al.*, 2002; Delgado-Rodriguez *et al.*, 2002). Indeed, lipoproteins protect against the infection and inflammation by blocking endotoxin activity (Pajkrt *et al.*, 1996; Netea *et al.*, 1998). Furthermore, recently it has been known that total cholesterol, HDL and LDL levels on admission are inversely associated with the disease severity (Wu *et al.*, 2004, Chien *et al.*, 2005, Vermont *et al.*, 2005). These findings suggest that lipoproteins play roles in the host defense against the bacterial infection, and its serum level is an important prognosis factor of survival. However, effect of lipoprotein on *V. vulnificus* infection is not clear.

V. vulnificus is known to be a life-threatening gram-negative pathogen that causes serious septicemia in human through the consumption of contaminated seafood such as oysters. *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 (Canturk *et al.*, 2002). *V. vulnificus* cytotoxin (VVC) has been incriminated as a virulence factors in *V. vulnificus* infection (Pajkrt *et al.*, 1996). Indeed, antibodies against the cytotoxin were detected in the blood of *V. vulnificus* infected mice or a human that survive *V. vulnificus* disease (Gray and Kreger, 1986). These results suggest that VVC might be importantly involved in the pathogenesis of *V. vulnificus* infection. Thus, inactivation of VVC could be impor-

tant to survival of *V. vulnificus*-infected host.

LDL induces the inactivation of *Staphylococcus aureus* α -toxin (Bhakdi *et al.*, 1983). Furthermore, we also found that LDL inactivates VVC hemolytic activity *in vitro* (Kim and Kim, 2002, Park *et al.*, 2005). These findings suggest that LDL could prevent the toxicity of VVC and lead to survival of mice infected by *V. vulnificus*. Therefore, we aim to examine whether the LDL-mediated inactivation of VVC leads to protection against the lethal infection of *V. vulnificus* *in vivo*, using wild and VVC-deficient *V. vulnificus* strains.

Materials and Methods

Mice

Female ICR mice, 25 to 28 g weights, 6 to 7 weeks old, were obtained from the Orient Bio Inc. (Korea), were acclimated to the Chonbuk National University Medical School Facility for 5 days and fed *libitum* with regular chow. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University. Mice were intraperitoneally injected with cultured *V. vulnificus* (2×10^8 colony forming unit (CFU)/mouse, 0.1 ml) as described by Lee *et al.* (2004). Mice were pre-injected with an 0.1 ml of saline or LDL-saline solution 2 h before the bacterial injection and monitored for 72 h for their mortality.

Bacterial strain and culture

Two strains of *V. vulnificus*, MO6-24/O (wild strain) and CVD 707 (VVC-deficient strain) were kindly supplied by the University of Maryland School of Medicine (Wright and Morris, 1991). Both strains were cultured in brain heart infusion (BHI) broth as described by Kreger *et al.* (1988). The bacteria were precultured at 37°C for 4 h in BHI broth (BD, Franklin Lakes, NJ) by picking one colony from the bacteria-cultivated TCBS agar plate (BD, Franklin Lakes, NJ). The stabilized bacteria were transferred to ten volume of new BHI broth, then further cultured with shaking at 37°C for 4 h. The bacteria numbers were calculated by measurement of turbidity with a spectrophotometer (DU530, Beckman, Fullerton, CA).

LDL preparation

LDL was isolated from human plasma by the method of Koo *et al.* (2000). Briefly, serum was obtained from bloods of normal healthy adults by a centrifugation at $1,200 \times g$ for 10 min. Lipoproteins

were purified by sequential preparative ultra centrifugations of serum in the presence of EDTA and protease inhibitors as preservatives. After a centrifugation of serum at $100,000 \times g$ for 20 h at 15°C in swing bucket rotor (SW 41Ti, Beckman, Fullerton, CA), the top layer of VLDL was removed with a Pasteur pipette. The pellets in the infranant were resuspended and adjusted to the density of 1.063 g/ml by NaBr. The LDL fraction was obtained by a centrifugation of infranant at $100,000 \times g$ for 24 h at 15°C. The LDL was extensively dialyzed against PBS.

V. vulnificus LPS preparation

V. vulnificus was grown in BHI broth containing 1% NaCl at 37°C and harvested by a centrifugation at $10,000 \times g$ for 30 min. The cells were washed with deionized water three times and soaked overnight in acetone. The cells were then centrifuged, washed twice with acetone, and dried at room temperature. LPS was prepared from the acetone-dried cells by the method of Bahrani and Oliver (1991). Briefly, 5 g of acetone-dried cells were suspended in equal volumes of preheated (69°C) water and 90% phenol and the mixture was stirred for 15 min. After cooling on ice, the mixture was centrifuged at $10,000 \times g$. The aqueous phase was collected and extracted three times. The aqueous phases were pooled and dialyzed against deionized water for 3 to 5 days and then lyophilized. The fluffy powder, dissolved in 50 mM Tris-HCl, pH 7.0, was treated with bovine pancreatic RNase (40 μ g/ml) and DNase I (100 μ g/ml) at 37°C for 1 h. Then, proteinase K (10 μ g/ml) was added and the reaction mixture was incubated at 60°C for 1 h. The mixture was repeatedly ultracentrifuged at $105,000 \times g$ for 2 h until the optical density of the supernatant was less than 0.01 at 260 and 280 nm. The purified LPS was dissolved in water and lyophilized. LPS was dissolved in a saline solution and dispersed by brief sonication.

Serological analysis

Blood clinical data were obtained from the Chonbuk National University Hospital. The informed consent was obtained. The blood samples were analyzed for total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides using an automatic blood analyzer (Hitachi, Tokyo, Japan).

Statistical Analysis

All experimental data are mean $\pm$ SD. Statistical analysis was performed using ANOVA test, Rank

Table 1. Lipid profiles in patients with *V. vulnificus* infection.

	Lived	Dead	<i>P</i> value
Number	4	11	
Sex (Male/Female)	4/0	7/4	
Age	55.0 ± 16.3	59.2 ± 15.1	0.65
Total cholesterol (< 200 mg/dl)	190.8 ± 40.0	71.7 ± 15.7	0.0001
Triglyceride (< 200 mg/dl)	124.7 ± 63.3	64.8 ± 76.7	0.26
HDL-cholesterol (48.9-73.5 mg/dl)	26.3 ± 13.6	20.4 ± 8.12	0.38
LDL-cholesterol (< 130 mg/dl)	155.3 ± 40.7	35.6 ± 13.9	0.0001

Data were presented as mean ± SD.

and Student's *t* test, and *P* < 0.05 was considered to be significant.

Results

We first analyzed relationship between the severity of *V. vulnificus*-infected patients and serum levels of LDL cholesterol (LDLC). To assess initial serum levels of LDLC and their correlations with the clinical outcome for patients with *V. vulnificus* infection, we performed a retrospective study against patients (*n* = 16) diagnosed as *V. vulnificus* septicemia in the Chonbuk National University Hospital (Table 1). Death cases (*n* = 12) showed lower serum levels of LDLC compared to normal patients (35.6 ± 13.9 ; *P* < 0.0001, normal, < 130 mg/dl), but survival cases (*n* = 4) showed higher levels compared to death cases and similar serum levels to normal patients (155.3 ± 40.7 ; *P* < 0.0001). Moreover, total cholesterol of death cases (71.7 ± 15.7 ; *P* < 0.0001, normal, < 200 mg/dl) was also lower than those of survival cases (190.8 ± 40.0). These results suggest that the fatality of human *V. vulnificus* infection is reciprocally correlated with serum total cholesterol and LDLC.

Many studies suggested that VVC might be a virulence factor for *V. vulnificus* infection. We demonstrated that LDL inactivates the hemolytic activity of VVC *in vitro*. Thus, we hypothesized that the lower survival in *V. vulnificus* infected patients with lower LDLC is due to lower LDL-mediated inactivation of VVC. To demonstrate this, we examined the protective effect of LDL preinjection against the lethality of mice infected with wild and VVC-deficient *V. vulnificus* strains. We first confirmed that VVC-deficient *V. vulnificus* could not produce VVC in the culture supernatant by western blotting (Figure 1). We also examined whether exogenous LDL injection increases LDL serum level. A single peritoneal injection of LDL increased serum LDL level 1.5 times at a dose of 10 mg/kg LDL and two times at a dose of 50 mg/kg LDL 2 h after the injection compared to an injection of

Figure 1. No production of *V. vulnificus* cytotoxin in VVC knockout *V. vulnificus* (CVD707). Wild type *V. vulnificus* (MO6-24/O) (A) and VVC knockout *V. vulnificus* (CVD707) (B) were cultured for 4 h in brain heart infusion broth as described in Material and Methods. The culture supernatant was concentrated with ammonium sulfate and analyzed by western blot with anti-VVC antibody.

saline (Figure 2A). Wild *V. vulnificus* infection by a single injection of 2×10^8 CFU induced death of all mice within 60 h from the injection. A single injection of LDL (10 and 50 mg/kg) before the infection significantly increased survival rate in a dose dependent manner. Especially, more than 80% mice survived the infection in 50 mg/kg LDL-pretreated group (*n* = 5) (Figure 2B). The difference of virulence of WT and VVC mutant was not obvious in the presence of LDL (Figure 2C). This finding is consistent with a previous report showing that VVC expression from *V. vulnificus* is not correlated to the lethality of infected mice (Wright and Morris, 1991), suggesting that LDL-mediated inactivation of VVC is not a cause for the protective effect of LDL against *V. vulnificus* infection.

We next focused on which virulent constituent is blocked by LDL. LPS is a substance of outer-

Figure 2. Effect of LDL preinjection on the lethality induced by wild (B) and VVC deficient (C) *V. vulnificus* strains. LDL (10 and 50 mg/kg) was intraperitoneally injected to mice. Two h later, serum LDL levels was measured as described in Materials and Methods (A). Error bars, s.d.; $n = 5$ in each group. * $P < 0.002$, ** $P < 0.005$, compared with saline group. Two h after LDL injection, wild type (MO6-24/O) and VVC knockout *V. vulnificus* (CVD707) (2×10^6 CFU in 0.1 ml) was intravenously injected. Survival was determined during the 3 day period after injection, after which there was no further loss of animal life. The survival rate of the group treated with LDL is significantly different from the survival rate of the control group ($P < 0.001$ by the log rank test).

membrane in gram-negative bacterium and can induce endotoxemia (Parker *et al.*, 1995). Thus, we purified LPS from *V. vulnificus* and examined whether LDL pretreatment protects *V. vulnificus* LPS-induced lethality. A single injection of purified LPS (20 mg/kg) from *V. vulnificus* induced 100% lethality within 50 h (12 mice died out of a total of 12). A single injection of LDL (10 and 50 mg/kg) before LPS injection significantly delayed *V. vulnificus* LPS-induced lethality in a dose dependent manner (Figure 3). These data suggest that LDL prevents the toxicity of *V. vulnificus* LPS and inhibits the lethality induced by *V. vulnificus* LPS.

Discussion

It has been known that serum levels of lipid,

lipoproteins, and lipoprotein-associated proteins are significantly changed in patients with sepsis (Barlage *et al.*, 2001; Pussinen *et al.*, 2001; Carpentier and Scrue, 2002; Khovidhunkit *et al.*, 2004). Total cholesterol, HDL, and LDL are reduced in serum of patients with sepsis and in contrast, plasma triglyceride is elevated (Fraunberger *et al.*, 1999; Pussinen *et al.*, 2001; Kitchens *et al.*, 2003; van Leeuwen *et al.*, 2003; Khovidhunkit *et al.*, 2004). In this study, we observed that levels of total cholesterol, triglyceride and LDL is reduced but level of HDL is relatively unchanged in patient with *V. vulnificus* sepsis, which shows a different serum profile of lipid from other sepsis. Especially, LDL level is remarkably reduced in serum of patient with *V. vulnificus* sepsis.

We also observed a positive effect of LDL supplement at septicemia induced by *V. vulnificus*

Figure 3. Effect of LDL preinjection on lethality induced by purified *V. vulnificus* LPS. LDL (10 and 50 mg/kg) or saline was injected to mice, intraperitoneally. Two h after, LPS (20 mg/kg) was intraperitoneally infused. Survival was determined during indicated time period after injection. The survival rates of groups treated with 10 and 50 mg/kg of LDL are significantly different from the survival rate of the control group ($P < 0.05$ and $P < 0.01$, respectively, by log rank test).

infection in an animal model. This finding suggests that LDLC has a defense mechanism in host with *V. vulnificus* infection. This is consistent with a previous report that the improvements in morbidity and mortality obtained by intensive insulin therapy in critically ill patients are partially attributable to increases in the serum levels of LDL and HDL lipoproteins. The changes in septic patient lipid profile is mediated by peroxisome proliferators-activated receptors (PPARs) (Beigneux *et al.*, 2000; Vantinen *et al.*, 2005). Indeed, a PPAR agonist improved survival in an animal model of enotoxemia (Liu *et al.*, 2005). Thus PPAR agonists seem to be an attractive therapeutic tool for *V. vulnificus* sepsis, which is needed for further study.

Acknowledgements

This work was supported by grants of the Korea Science and Engineering Foundation (M10528010003-05N2801-00310 and R11-2002-100-02007-0) and the grant of Post-Doc. Program, Chonbuk National University (EK Song).

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