Relationship between Gb3 Expression and Cytotoxicity of Shiga-like Toxin I

Suk-Hwan Lim, M.D., Gi-Young Kim, Ph.D.*, Hyung-Chun Kim, M.D., Ph.D.*
Young-Hee Kim, Yong-Hae Son, Yang-Hyo Oh, M.D., Ph.D*
and Yeong-Min Park, M.D., Ph.D*, †

Department of Pediatrics, Chonnam National University Medical School, Kwangju
Department of Microbiology and Immunology, Pusan National University College of Medicine, Pusan Medical Research Center†, Dong-A University College of Medicine, Pusan, Korea*

Purpose : Infection with Shiga-like toxin (SLT)-producing *Escherichia coli*, an emerging human pathogen found particularly in young children under 5 years of age, causes a spectrum of illnesses with high morbidity and mortality, ranging from diarrhea to hemorrhagic colitis and hemolytic uremic syndrome. Host mediators play an important role in the pathogenesis of SLT-I toxicity. The experiments described here were designed to investigate the effect of SLT-I on TNF-α production and to understand the effect of TNF-α on GB3 expression. We also further examine the relationship between the Gb3 level and the differential susceptibility of cells to the cytotoxic action of SLT-I.

Methods : The effect of purified SLT-1 from *E. coli* O157 : H7 (ATCC 43890) on tumor necrosis factor-α (TNF-α) production in Raw264.7 cells was investigated. Many mediators regulate endothelial cell membrane expression of the glycolipid globotriaosyleramide (Gb3), which serves as the toxin receptor, suggesting that the host response to the toxin or other bacterial products may contribute to pathogenesis by regulating target cell sensitivity to the toxins. Therefore, the relationships between Gb3 expression and cytotoxicity against SLT-I on three types of cells were evaluated.

Results : Detectable levels of TNF-α were produced as early as six hours after induction and continued to increase during 48 hours by SLT-I. It was also found that Vero cells and dendritic cells (DC2.4 cells) expressed high levels of Gb3, 83% and 68%, respectively, and that Raw264.7 cells had a low level of Gb3 (29%) and appeared refractory to cytotoxicity against SLT-I. Vero cells and DC2.4 cells expressing high levels of Gb3 were highly susceptible to SLT-I. Furthermore, macrophages showed a resistance to SLT-I cytotoxicity, despite the fact that Gb3 expression was enhanced.

Conclusion : These results strongly suggest that the expression of Gb3 is necessary but not sufficient to confer sensitivity of macrophages to SLT-I and further underpin the important role of SLT-I and its Gb3 receptors in the pathogenesis of *E. coli* O157 infection. **(J Korean Pediatr Soc 2003;46:143-153)**

Key Words : *E. coli* O157, Shiga-like toxin, tumor necrosis factor-α, glycolipid globotriaosyleramide

INTRODUCTION

The family of Shiga-like toxins (SLTs) produced by *Escherichia coli* strains represents potent biological cytotoxins, which enter the cytosol of the target cell, completely truncate protein synthesis, and thereby induce the death of the cell[1]. Infection with SLT-producing *E. coli* (STEC, mainly strain O157 : H7) is associated with hemorrhagic colitis and systemic complications, such as hemolytic uremic syndrome (HUS) in humans[2] and edema disease (ED) in piglets[3]. HUS is the most frequent cause of acute renal failure in children[4]. SLT similar to the Shiga toxin was first identified as a cytotoxin for Vero cells[5, 6]. Subsequent studies have demonstrated that SLTs are capable of inducing cellular death in various types of cells[7]. In contrast, the cytotoxic effect of SLT-I on monocytes and

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책임저자 : 박영민, 부산대학교 의과대학 미생물학교실
Tel : 82-51-240-7557 Fax : 82-51-243-2259
E-mail : immunpym@pusan.ac.kr

macrophages has been found to be negligible. Recent studies have shown that SLT-I is capable of stimulating macrophages or monocytes to produce various cytokines[8]. Increased production of proinflammatory cytokines may contribute to the development of inflammatory reactions in STEC-associated diseases.

Though there are abundant evidences of platelet-microvascular endothelial interaction, perturbation of the fibrinolytic cascade, and arachidonic acid metabolism[9, 10], the precise disease mechanisms remain enigmatic[11]. SLTs bind with high affinity to the membrane-anchored glycosphingolipids (Gb3) and globotetraosylceramide (Gb4). Glycolipid binding is mediated by the lectin-like, pentameric B subunit of SLTs[12]. Retrograde intracellular transport of endocytosed SLTs to the endoplasmic reticulum via the Golgi apparatus[13] is a prerequisite for the protein-synthesis-inhibiting action of the toxins[14]. In the case of SLT-I, the 32 kD (293 amino acids) A subunit is inserted via its C-terminal domain into a central pore created by the pentameric arrangement of five identical 7.7 kD B subunits (69 amino acids)[15]. The A subunit of SLT-I possesses intrinsic N-glycosidase activity. In cell-free systems they both hydrolyze a specific adenine residue at position 4324 of the 28S ribosomal RNA of the 60S mammalian ribosomal subunit. This post-transcriptional modification leaves the phosphodiester bond intact, but effectively interferes with the binding and coordinated function of eukaryotic elongation factors EF-1 and -2[16].

Despite conspicuous evidences for the involvement of SLTs in the pathogenic processes, little is known about the mechanism by which they exert their damage. Accumulating data indicates that host mediators may play an important role in the toxicity of SLTs. Based on histological studies it has been suggested that direct cytotoxic damage to endothelial and colonic cells enables the influx of macrophages and neutrophils to the site of the injured tissue. The local release of inflammatory factors stimulates the inflammatory reaction and intensifies the tissue damage. Consistent with this assumption is the observed correlation between increased production of TNF-α, interleukin-6 (IL-6) and IL-1β. The mechanism of cytokine-mediated toxin sensitization was involved in the increased synthesis and membrane expression of Gb3[17]. Barrett et al.[18], demonstrated that TNF activity could be elicited from murine macrophages by treatment with purified SLT-II. On the basis of the collective results of these *in vitro*, it was hypothesized that an immunologic component may be operative in the pathogenesis of HUS associated with infection with SLT-I-producing bacteria[19].

The experiment described here was designed to investigate the effect of SLT-I on TNF-α production and to understand the effect of TNF-α on GB3 expression. We also further examine the relationship between the Gb3 level and the differential susceptibility of cells to the cytotoxic action of SLT-I.

MATERIALS AND METHODS

1. Bacterial strain and culture

E. coli O157 (ATCC 43890, serotype O157 : H7) was provided by the Korean National Institute of Health (KNIH, Seoul, Korea). This strain was grown on the modified syncase medium described by O'Brien et al. (1982)[20] consisting of 10 g casimino acid (Difco), 1.17 g NH_4PO_4, 0.05 g $MgSO_4$, 0.005 g $MnCl_2$, 2 g glucose, 0.04 g L-tryptophan and 0.02 g nicotinic acid in 1 liter of distilled water (pH 8.0), and which was used for the bacterial culture after its treatment with Chelex 100. Cells were cultured at 37℃ for 48 h with vigorous shaking.

2. Purification of SLT-I

E. coli O157 was resuspended in 100 mL of 50 mM sodium phosphate (pH 7.4) containing 140 mM NaCl (Buffer A) and 200 mg polymyxin B sulfate at 7730 USP units/mg (Sigma Chemical Co., St. Louis, MO). The supernatant was collected and was subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added at 0℃ to 80% saturation (523 g/L) and stirred until completely dissolved. The resulting precipitate was collected by centrifugation at 16,000 g for 20 min at 4℃. The pellet was redissolved in 9 mL of buffer A and dialyzed against 4 liters of 10 mM sodium phosphate buffer (pH 7.4; buffer B) for 16 h at 4℃. The dialysate was used immediately or stored at -20℃.

The dialysate was applied to a Matrex Gel Green A agarose (Amicon Corp., Lexington, MA) column (1.6×43 cm) equilibrated with 10 mM sodium phosphate (pH 7.4). The periplasmic proteins were allowed to bind for 90 min at room temperature. The column was then washed with 200 mL of buffer B, containing 150 mM of NaCl, and the chromatogram was developed with a 500 mL, 0.15 to 0.6 M NaCl gradient in buffer B at a flow rate of 0.5 mL/min. Five-milliliter fractions were collected.

3. Purification of SLT-I B subunit (SLT-I B)

E. coli JM105 containing plasmid pSBC32 (provided by Calderwood SB at Massachusetts General Hospital, Boston) was used for the purification of SLT-I B. This plasmid expresses the non-toxic SLT-I B from *E. coli* under control of the *trc* promoter. The crude total protein with SLT-I B from 12-liters of culture supernatant of *E. coli* JM105 was applied to a Q-Sepharose XL column (Phamacia, Sweden : 1.6×30 cm) equilibrated with 0.05 M of Tris-HCl buffer and then with 3,000 mL of a linear gradient of NaCl (0 to 1.0 M) in Tris-HCl buffer (pH 8.6). The preparation from the gel filtration column (Sephacryl S-100; Pharmacia, Sweden) was dialyzed against 50 mM of Tris-HCl (pH 8.0) containing 10 mM of NaCl. Fractions with B subunit were collected and concentrated to a small volume by ulrafiltration with an Amicon PM-10 membrane. Protein was determined by the Bradford assay[21] with bovine serum albumin (Sigma) as a standard. SDS-polyacrylamide gel electrophoresis (PAGE) with Commassie blue staining and Western blots (immunoblots) was performed on 1.0-mm, 15 % gels according to Laemmli[22].

4. Cell culture and stimulation

BALB/c-derived macrophages (Raw264.7 cells), Vero cells and DC2.4 cells were grown in RPMI1640 containing heat- inactivated 10% fetal bovine serum (FBS, Gibco). All media were supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B (medium and supplements were purchased from Sigma, St. Louis, MO). Cells were seeded in 96-well microtitration plates at a concentration of 10^5 cells/well in RPMI1640 (Gibco, Grand Island, NY) with heat inactivated 0.5% FBS. After 1 day, the medium was replaced with fresh medium and SLT-I or LPS was added as indicated in the text. Thioglycolate-elicited peritoneal exudates cells (PEC) were obtained from ICR mice by intra-peritoneal injection of 3 mL of 0.405 g/100 mL Brewer-thioglycolated broth (Difco Labs, Detroit, MI) and lavage of the peritoneal cavity with 5 mL of medium 3 days later. PEC were washed twice and resuspended in RPMI 1640 containing 10% heat-inactivated FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Macrophages were isolated from PEC as described by Klimetzek and Remold[23]. Briefly, PEC were seeded at a density of 1×10^5 cells/mL on petri dishes and the macrophages were allowed to adhere for 3 h at 37℃ in 5% CO_2 humidified atmosphere. The nonadherent cells were removed and cold PBS (15 mL) containing 1.5% FBS was added, followed by 0.3 mL of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were harvested. The purity of the PEC was assessed by flow cytometry and the percentage of Mac 3 (PharMingen)-positive cells was approximately 93.0%.

5. TNF-α determination

Cytokine levels in culture supernatants were determined by a standard sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, Nunc MaxiSorp (Nalge Nunc International, Roskilde, Denmark) plates were coated for 24 h at 4℃ with rat anti-murine TNF-α mAb (R&D Systems, Minneapolis, MN). The plates were blocked, supernatant samples were added, and the plates were incubated for one hour at 4℃. The plates were then washed and incubated for 60 min at room temperature with biotin-conjugated rat anti-murine TNF-α monoclonal antibodies followed by an alkaline phosphatase-conjugated goat anti-biotin monoclonal antibody (R&D Systems, Minneapolis, MN). The fluorescent substrate for alkaline phosphatase (R&D Systems, Minneapolis, MN) was used to develop the assay, and fluorescence was measured with a microtiter plate reader (Dynex, Chantilly, VA), using excitation and emission wavelengths at 450 nm. To quantify the amount of cytokine present in test samples, values were extrapolated from standard curves established by analyzing different dilutions of recombinant murine TNF-α.

6. Cytotoxicity assay

The cytotoxicity of SLT-I for Vero cells, DC2.4 cells, and Raw264.7 cells was assessed by an MTT cytotoxicity assay[24]. After treatment with the purified SLT-I or LPS for 48 h on a 96 well-culture plate, the cells were washed twice with PBS and incubated with 110 μL of 0.5 mg/mL MTT for 2 h at 37℃. The medium was discarded and 100 μL of acidic isopropanol (0.04 N HCl) was added, and then, after 30 min incubation. The absorbance at 570 nm was read by using a microplate reader.

7. FITC-conjugated B subunit (SLT-I B-FITC) preparation and Immunophenotyping of Gb3

The pure SLT-I B subunit was dialyzed in a 0.1 M sodium carobnater buffer (pH 7.4) overnight. It added flu-ororescein isothiocyanate (FITC; Molecular Probes) to 50 μg per mg of B subunit. The complexes were incubated at

room termperature for 1 h. The unreacted FITC was removed by a process of gel filtration (Sephadex G25). After 24 h, the culture in RPMI 1640 containing 0.5% FBS, Vero cells, DC2.4 cells, and Raw264.7 cells were labeled with SLT-1 B-FITC. Briefly, different cell types were incubated with 0.5 μL of SLT-I B-FITC (1 mg/mL) on ice for 20 min. Cells were then washed twice with PBS/10% BSA and centrifuged at 200 g for 5 min. Binding of SLT-1 B-FITC was measured using a flow cytometer (Faxcalibur, BD).

8. Statistical analysis

Unless otherwise indicated, the results were expressed as the mean±SEM of data obtained from triplicate experiments. Statistical analysis was performed by a paired Student t-test. Differences at $P<0.05$ or $P<0.01$ were considered statistically significant.

RESULTS

1. Preparations of SLT-I, SLT-I B and anti-SLT-I antibody

Periplasmic proteins of *E. coli* were released with polymyxin B sulfate, a well-established technique for release of SLTs[2]. The proteins were precipitated with ammonium sulfate at 80% saturation and dialyzed, and the dialysate was applied to a Matrex Gel Green A agarose column. SLT-I was eluted as a single peak with 0.35 M of NaCl. All toxin activity was detected in column fractions 30 through 40. These fractions were pooled and used for protein characterization. The results of SDS-PAGE and immunoblotting assay of the purified SLT-I are shown in Fig. 1A. SLT-I gave two bands staining for protein corresponding to the A and B subunit. The molecular weights of the A and B subunits were estimated to be about 33 kD and 6.3 kD, respectively. Also, SLT-I B consistently appeared as a diffuse band and the molecular weights of the B subunits were smaller than 7.7 kD as shown previously[16]. Mulvey et al[25] observed this characteristic band morphology.

For anti-SLT-I monoclonal antibody preparation, the ammonium precipitated protein from the culture media of 13C4 cells was applied to a column (1.6×30 cm) of Q-Sepharose ion-exchange chromatography equilibrated with 50 mM of Tris-HCl (pH 7.4). Elution was then carried out with a linear gradient of NaCl, from 0 to 1 M, at the flow rate of 1 mL/min. The elution profile is shown in Fig. 1A. The

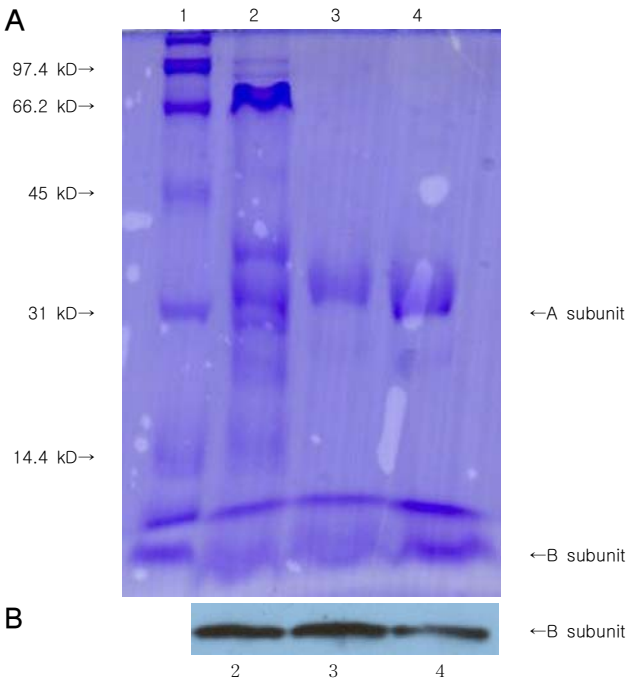

Fig. 1. (A) SDS-PAGE analysis of SLT-I from *E. coli* O157 (ATCC 43890). The samples were analyzed on 15% polyacrylamide gels. The arrows on the right hand side of the figure indicate the location of the A and B subunit bands. **(B)** An immunoblotting assay of purified SLT-I was conducted using anti-B mAb as the secondary antibody. The positions of the standard proteins used to calibrate the gels are indicated by their molecular weights on the left hand side of the Fig. 1 : molecular size marker, 2 : ammonium sulfate precipitated proteins, 3 : Matrix gel green A pool of fractions, 4 : purified SLT-I.

active fractions were Nos. 28-33. A fraction of NaCl (about 0.4 M) gradient elution containing the protein-bound polysaccharide has an obvious peak when using the Bradford method. Each fraction was loaded at 15% SDS-PAGE (Fig. 1B). The crude SLT-I fractions (Nos. 28-33) were dialyzed against 10 mM of NaCl containing 50 mM of Tris-HCl (pH 7.4). The concentrated SLT-I solution was then applied to the Sephacryl S-100 (1.5×100 cm) and eluted with the same buffer solution at the flow rate of 1 mL/min. The profile of gel filtration chromatography is shown in Fig. 2A. The active fractions (Nos. 28-36) loaded at 15% SDS-PAGE (Fig. 2B). The SLT-I B migrated faster than previously known SLT-I B. The discrepancy was that SLT-I B appeared as diffuse bands on Commassie blue-stained SDS polyacrlyamide gels (Fig. 1B and 2B). However, a more densely stained, sharply defined zone always appeared at the tailing (upper) edge of these SLT-I B bands.

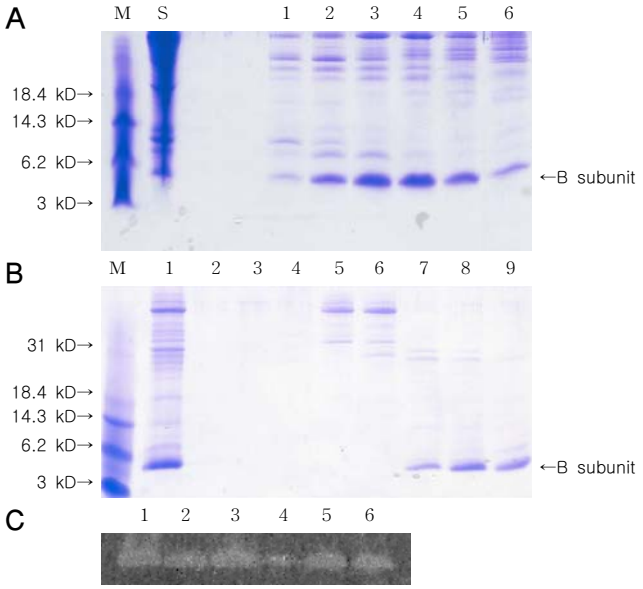

Fig. 2. (A) Anion exchange chromatography SLT-I B from 12-liters of culture supernatant of *E. coli* JM105 on Q-Sepharose column (1.6×30 cm). The column was eluted with 50 mM of Tris-HCl (pH 7.4). An SDS-PAGE analysis of aninon exchange-purified SLT-I B was conducted. M : molecular weight marker, S : crude sample, 1 : fraction no 28, 2 : fraction no. 29, 3 : fraction no. 30, 4 : fraction no. 31, 5 : fraction no. 32, 6 : fraction no. 33. **(B)** Gel filtration (a) of SLT-I B from 12-liters of culture supernatant of *E. coli* JM105 on Sephacryl S-200 column (1.5×100 cm). The column was eluted with 10 mM of NaCl containing 50 mM of Tris-HCl (pH 7.4). An SDS-PAGE analysis of gel filtration-purified SLT-I B was conducted. The samples were analyzed on 15% polyacrlyamide gels. The arrows on the right-hand side of the figure indicate the location of the SLT-I B bands. The positions of the standard proteins used to calibrate the gels are indicated by their molecular weights on the left hand side of the figure. M : molecular weight marker, S : crude sample, 1 : fraction no 28, 2 : fraction no. 29, 3 : fraction no. 30, 4 : fraction no. 31, 5 : fraction no. 32, 6 : fraction no. 33, 7 : fraction no. 28, 8 : fraction no. 34, 9 : fraction no. 35. **(C)** SLT-I B was represented by immunoblotting assay after gel filtration and ion exchange chromatography. 1 : fraction no. 29, 2 : fraction no. 30, 3 : fraction no. 31 after gel filtration chromatography, 7 : fraction no. 28, 8 : fraction no. 34, 9: fraction no. 35 after ion-exchange chromatography.

2. Macrophages are refractory to the SLT-I cytotoxicity

Therefore, this study was carried out to examine whether the quantitative expression of Gb3 receptors was related to cytotoxicity in sensitive Vero cells and DC2.4 cells and macrophages. As shown in Fig. 3 and 4, Vero cells and DC2.4 cells expressed high levels of Gb3, 83% and 68%, respectively. Vero cells and DC2.4 cells having high levels of Gb3 were highly susceptible to SLT-I. Raw264.7 cells still had 29% of Gb3, but were refractory against SLT-I cytotoxicity. Raw264.7 cells were generally refractory to

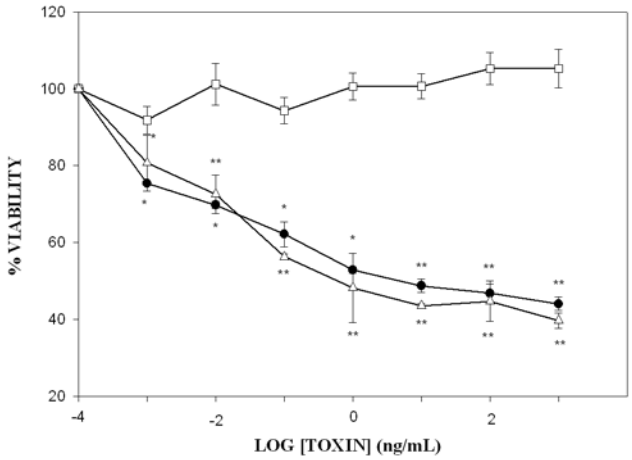

Fig. 3. Comparative cytotoxicities of SLT-I for Raw264.7 cells, DC2.4 cells and Vero cells. Cells were incubated with 10-fold dilutions of the toxins in 96-wells for 48 h at 37℃ in humidified 5% CO_2. Viability was measured by the uptake of MTT. Untreated cells were used to determine percent viability (□ : Raw264.7 cells, ● : DC2.4 cells, △ : Vero cells). The data shown is the averages of two separate experiments. Statistical significance : $^{*}P<0.05$ and $^{**}P<0.01$ vs. non-treatment values. Values are mean±S.D., n=6.

the cytotoxic activity of the toxins for 48 h with the highest dose (1 μg/mL). SLT-I B, nonenzymatic subunit, had no cytotoxicity against 3 types of cells having sensitivity of SLT-I (data not shown). These results strongly suggest that the expression of Gb3 is related to the cytotoxicity of the cell, but not linear.

3. Macrophages show an enhanced TNF-α production in response to SLT-I

To evaluate the effect of SLT-I on TNF-α production by Raw264.7 cells, the Raw264.7 cells were incubated with purified SLT-I and LPS (Fig. 5). TNF-α production was induced more slowly than that from Raw264.7 cells treated with LPS. Next, the time- and dose-dependences of TNF-α production induced by SLT-I-treated Raw264.7 cells were examined. Raw264.7 cells were cultured with 200 ng/mL of SLT-I or 100 ng/mL of LPS at 37℃ from 0 to 48 h, and then these cytokines' activity in the culture supernatant was monitored. A significant level was found at 6 h after the addition of SLT-I or LPS, and maintained for at least 48 h. TNF-α was increased time- and dose-dependently. No differences between 1,000 ng/mL of SLT-I and 100 ng/mL of LPS were observed with respect to the level of TNF-α released in the cultures.

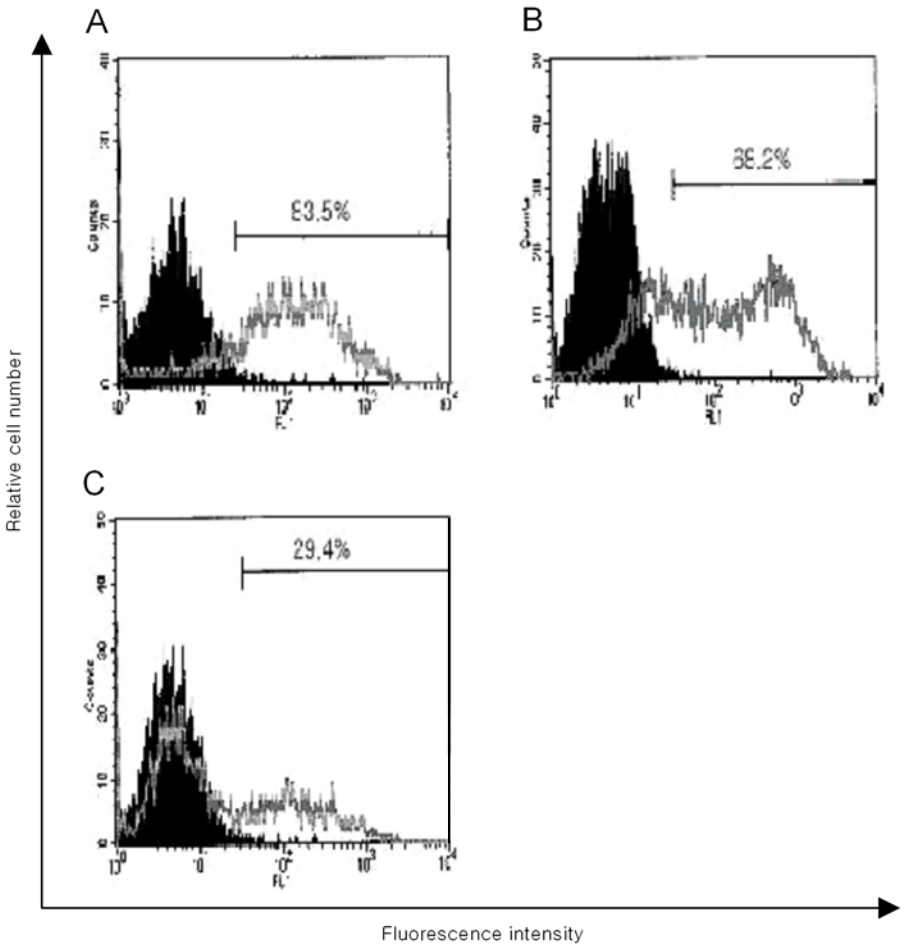

Fig. 4. Representative flow cytometric histograms illustrating the expression of Gb3 in Vero cells **(A)**, DC2.4 cells **(B)** and Raw264.7 cells **(C)**. Cells were incubated for 24 h at 37℃ in the culture medium containing 0.5% FBS. After incubation, the SLT-I B-FITC labeled cells. Data is representative of two experiments.

4. TNF-α enhances the Gb3 expression in macrophages

Endothelial cell damage by SLT-I in vitro is potentiated by the additional exposure of inflammatory mediators, such as TNF-α, LPS, and IL-I[26]. TNF-α makes the endothelial cells sensitive to SLT-I by the induction of Gb3 on the surface[26]. To evaluate whether the cytotoxic resistance of macrophages caused by SLT-I was due to a low level in Gb3 expression on the cell surface, experiments were performed.

5. Macrophages show a resistance to SLT-I cytotoxicity, despite the fact that Gb3 expression is enhanced

The expression levels of Gb3 were detected in peritoneal macrophages, which were cultured with TNF-α. We found that TNF-α augmented Gb3 expression compared with the untreated negative control in peritoneal macrophages (Fig. 6). The population of Gb3 expression in peritoneal macrophages was strongly increased at 6 h after treatment of TNF-α and then slowly reduced to the control level at 36 h. Also, we investigated the cytotoxicity of 100 ng/mL of SLT-I in peritoneal macrophages and DC2.4 cells after TNF-α treatment for 12 h. Peritoneal macrophages and Raw264.7 cells, with the exception of DC2.4 cells, did not show cytotoxic changes (Fig. 7).

DISCUSSION

Studies on the role of SLTs and Shiga toxin in pathogenesis have focused primarily on the potent cytotoxic activity of the toxins, and indeed, direct toxin-mediated

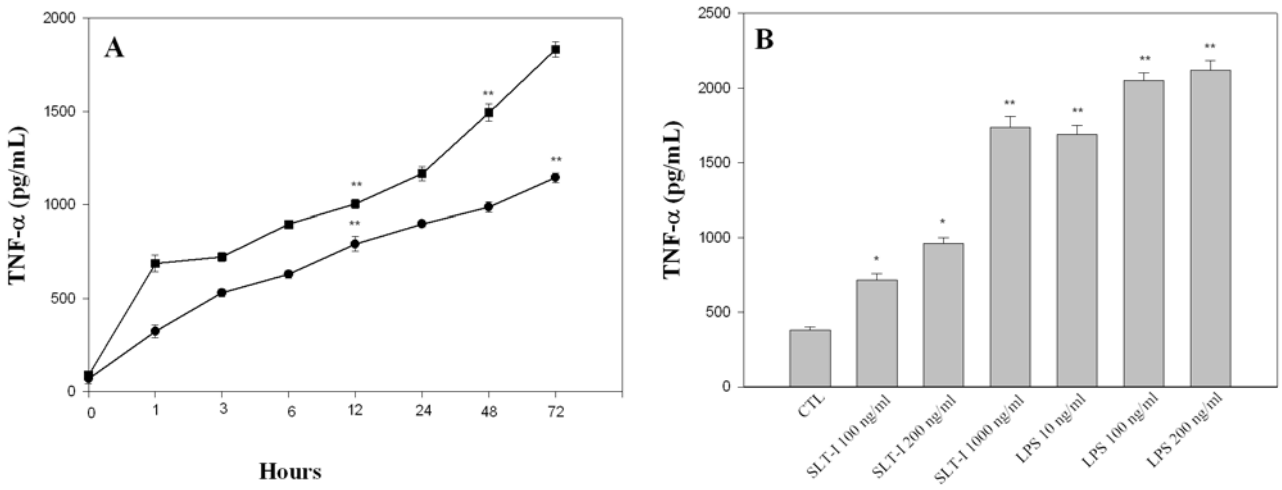

Fig. 5. Time- **(A)** and dose-dependent **(B)** TNF-α production by Raw264.7 cells stimulated by SLT-I and LPS. Raw264.7 cells were stimulated by 200 ng/mL of SLT-I (■) and 100 ng/mL of LPS (●) for 72 h at 37℃ in a humidified 5% CO_2 incubator **(A)**. To evaluate dose-dependent TNF-α production, Raw264.7 cells were stimulated by SLT-I (100, 200, and 1,000 ng/mL) or LPS (10, 100, and 200 ng/mL) for 24 h. TNF-α activities in the culture supernatants from Raw264.7 cells were measured as described in Materials and Methods. Significance was determined using the Student *t*-test versus the control group ($^{*}P<0.05$ and $^{**}P<0.01$).

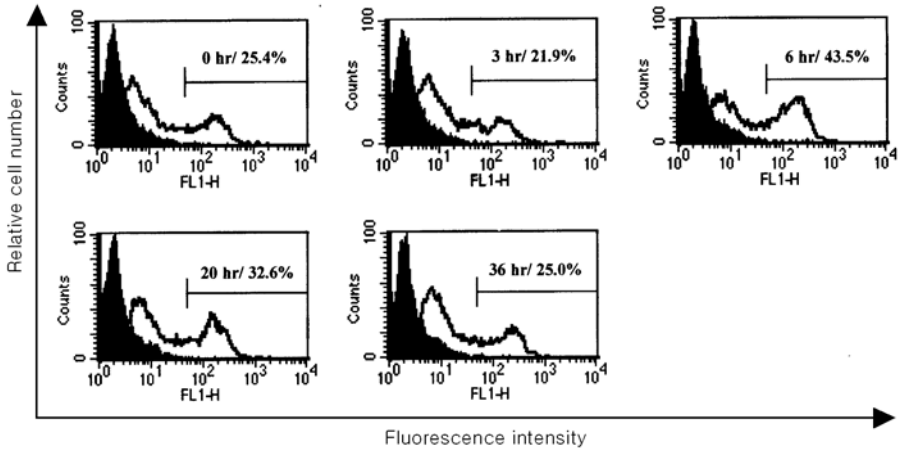

Fig. 6. Time-dependent Gb3 expression from peritoneal macrophages with treatment of TNF-α (50 ng/mL). Cells were incubated with 50 ng/mL of TNF-α in 96-wells for 24 h at 37℃ in humidified 5% CO_2. After incubation with TNF-α, cells were stained using SLT-I B-FITC.

cytotoxicity may be an important component in colonic ulceration and the subsequent vascular damage in the central nervous system and kidneys characteristic of human infection with SLT-producing bacteria. The binding specificity of SLT-I B for Galα1-4 Gal residues of glycosphingolipids has been extensively investigated[27] and there are many discrepant reports between Gb3 expression and cytotoxicity against SLTs. Binding of SLT-I to the glycolipid Gb3 receptors results in receptor-mediated endocytosis, intracellular trafficking of the toxin to the Golgi network and endoplasmic reticulum, ultimately, the translocation of the enzymatic A subunit into the cytosol where ribosome inactivation occurs.

It has also been shown that SLT-I B resembles the extracellular domain of the 63 kD subunit of the IFN-α receptor in its amino acid sequences[28]. This provided a molecular explanation for the earlier observation that cells that were Gb3-deficient were resistant to both IFN-α-induced growth inhibition and SLT-I cytotoxicity[29, 30]. There was also evidence that endothelial cells treated with inflammatory mediators may become more susceptible to injury. Human umbilical and adult vein endothelial cells

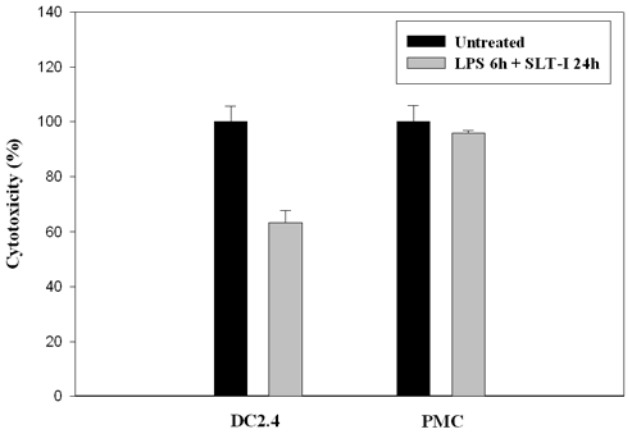

Fig. 7. Comparative cytotoxicities of SLT-I for peritoneal macrophages and DC2.4 cells. After treatment of 50 ng/mL of TNF-α for 6 h, cells were incubated with 10-fold dilutions of the toxins in 96-wells for 24 h at 37℃ in humidified 5% CO_2. Viability was measured by the uptake of MTT. Untreated cells were used to determine percent viability. The data shown is the averages of two separate experiments.

preincubated with inflammatory mediators TNF-α and IL-1 were found to be more sensitive to the toxic effect of SLT-I[31] by induction of the Gb3 on their surface[32].

This further favors the suggestion that inflammatory mediators may play an important role in the pathogenesis of HUS. The culprits of this local release may be monocytes and macrophages, which upon activation are able to produce and release a number of inflammatory mediators including TNF-α and IL-1[33]. Therefore, it was necessary to investigate if SLT-I itself was able to interact with macrophages and to induce the release of cytokine from these cells.

In this study, we purposed to investigate the effect of SLT-I on cytotoxicity and TNF-α production and to understand the effect of TNF-α on GB3 expression. We also further examined the relationship between Gb3 level and the differential susceptibility of cells to the cytotoxic action of SLT-I.

The Gb3 expression and cytotoxicity of SLT-I against 3 types of cells were evaluated. It was found that Vero cells and DC2.4 cells expressed high levels, 83% and 68%, of Gb3, respectively, and that Raw264.7 cells had a low level of Gb3 (29%) (Fig. 4) and appeared refractory to cytotoxicity against SLT-I. While Vero cells and DC2.4 cells having high levels of Gb3 were also found to be the highly susceptible to SLT-I. In accordance with earlier reports [34, 35], Vero cells and dendritic cells expressing high levels of Gb3 were sensitive to the cytotoxic action of SLT-I (Fig. 3)[36]. In contrast, Raw264.7 cells were generally refractory to the cytotoxic activity of the toxins for 48 h with the highest dose (1 μg/mL). These results strongly suggest that the expression of Gb3 is necessary, but not linear, for the SLT-I cytotoxicity.

SLT-I induces mouse peritoneal macrophages to synthesize TNF-α in a time- and concentration-dependent manner. This observation closely agrees with an observation of Tesh et al[37], who reported that mouse peritoneal macrophages express low amounts of Gb3 and that interaction of SLT-I with these macrophages induces the production of IL-1, IL-6, and TNF-α. Our observation that SLT-I induces the release of TNF-α also agrees with in vivo data of van Setten et al.[38]. TNF-α stimulates Gb3 expression on macrophages (Fig. 6). The amount of Gb3 expression in peritoneal macrophages was strongly increased at 6 h after treatment of TNF-α and then slowly reduced to the control level at 36 h. The mean fluorescent intensity of TNF-α-treated cells did not differing from that of TNF-α-untreated cells (data not shown).

To clarify the relationship between the Gb3 level and cytotoxicity of SLT-I in macrophages, we assessed the cytotoxic activity of SLT-I on the TNF-α-treated macrophages. Peritoneal macrophages, except DC2.4 cells, did not show cytotoxic changes (Fig. 7). The exact nature of the receptor involved in SLT-I binding to mouse macrophages remains to be clarified. Interestingly, interaction of SLT-I with the receptor on macrophages did not affect the viability of these cells, despite the fact that the cells expressing Gb3 were almost doubling at 36 h after TNF-α treatment. However, macrophages do respond to SLT-I by releasing TNF-α. Therefore, it is likely that macrophages are less susceptible to the toxic effect of SLT-I, although it cannot be excluded that the lower number of SLT-I receptors on macrophages partly contributes to the lack of sensitivity for SLT-I cytotoxicity.

There are tenable explanations for the nonlinear relation between Gb3 and toxin sensitivity. For example, SLT-I B aggregates as a pentamer, as shown by chemical cross-linking[19] and confirmed by radiographic crystallography[20]. Multivalent binding of toxin to Gb3 via the B pentamer may be facilitated as the density of surface Gb3 increases. This would be particularly efficient if Gb3 were to concentrate in the clathrin-coated regions of membrane where Shiga toxin uptake occurs[5, 6]. In this way, small changes in the total receptor content on macrophages might mediate

the greater observed changes in the biologic effects of the toxin. Alternatively, if there is a separate translocation mechanism with which Gb3-bound toxin must interact, increased membrane Gb3 content could also enhance the chance of this occurring, again resulting in a sharp increase in toxicity as receptor content slowly increased. Thus, when the content of Gb3 on macrophages is low and dispersed, much of the surface Gb3 may be unable to function as a receptor.

Distinctive results were obtained with Chinese hamster ovary (CHO)-KI cells, which normally lack Gb3 and do not increase Gb3 content in the presence of 1,5-dideoxy-1,5-imino-D-galactitol (DIG) and thus can be considered to be intrinsically resistant to Shiga toxin[39]. When liposomal Gb3 was introduced into these cells by the identical fusion protocol used for HeLa cells and Vero cells, there was no change in the sensitivity of CHO cells, even though increased Shiga toxin binding was readily demonstrated. This raises the possibility that macrophages may lack either the mechanism for translocation of Gb3-bound toxin from the cell surface to the cytoplasm or a second protein or glycoprotein receptor needed to interact with the uptake mechanism and/or direct the complex to the trans-Golgi region of the cell[25] and beyond to the endoplasmic reticulum [34], where shiga toxin exerts its biochemical action. Although we have not directly tested the effect of Shiga toxin on isolated macrophages' ribosomes, it is also remotely possible that macrophages' ribosomes are not susceptible to the action of Shiga toxin. This hypothesis explains the results of Boyd et al.[11], who found that some piget tissues able to bind toxin in vivo are not damaged.

Kiarash et al[40] have reported that SLT-I/Gb3 binding in a lipid matrix is affected by heterogeneity in the ceramide fatty acid chain length. The short chain fatty acid containing-Gb3 isoforms allow SLT-I-mediated cytotoxicity; on the other hand, the long chain fatty acid containing-Gb3 isoforms show IFN-α-mediated antiviral activity. These results suggested overlapping carbohydrate epitopes on the Gb3 molecule, which are differentially available in these Gb3 fatty acid homologues in a lipid environment. Raw264.7 cells also have low levels of Gb3 receptor and no cytotoxicity. It is suggested that isoforms of Gb3 have a low affinity and endocytosis maybe did not occur in Raw264.7 cells. These isoforms may be related to signal transduction in macrophages. Our report extends this observation to mouse peritoneal macrophages for which Gb3 expression can be induced by TNF-α. Furthermore, an increased expression of Gb3 on macrophages of SLT-1 did not change the cytotoxicity. Taken together, these results further underpin the important role of SLT-I and its Gb3 receptors in the pathogenesis of *E. coli* O157 infection.

국문 요약

Shiga-like Toxin I의 세포독성과 수용체 Gb3 발현과의 관계

전남대학교 의과대학 소아과학교실,
부산대학교 의과대학 미생물학교실*,
동아대학교 의과대학 의학연구소†

임석환 · 김기영* · 김형천* · 김영희*
손용해* · 오양효* · 박영민*, †

목 적 : Shiga-like toxin (SLT)을 생산하는 *Esherichia coli*에 의한 감염은 설사, 출혈성 대장염(hemorrhagic colitis) 및 용혈성 요독 증후군(hemolytic uremic syndrome)을 특징으로 하며, 특히 5세 이하의 소아에게서 심각한 결과를 초래한다. SLT-I의 병인으로는 다양한 숙주 매개인자들이 알려져 있다. 본 연구에서는 *E. coli* 0157 : H7 (ATCC 43890)로부터 정제한 SLT-I이 포유동물 세포들에 대한 세포독성과 종양괴사인자(tumor necrosis factor-α; TNF-α)의 생산에 미치는 효과를 측정하였으며, SLT-I의 수용체인 glycolipid globotriaosylceramide (Gb3)의 발현과 SLT-I의 세포독성의 관계를 규명하고자 하였다.

방 법 : SLT-I과 SLT-I B를 순수분리 정제하고 SLT-I B-FLTC 접합체를 제조하여 vero 세포, 대식세포 및 수지상세포를 대상으로 세포독성능을 측정하고 세포독성능의 차이가 SLT-I의 수용체인 Gb3의 발현과 상관관계가 있는지를 Flow cyotmetry로 분석하였다. 또한 대식세포의 종양괴사인자 생산능은 ELISA법으로 시행하였다.

결 과 : SLT-I은 대식세포(Raw264.7)로부터 TNF-α의 생산을 증가시켰다. 연구 대상 세포 중 SLT-I에 감수성을 나타낸 Vero 세포와 수지상세포(dendritic cells)는 Gb3 발현이 각각 83%와 68%로 높았으며, 29%의 낮은 Gb3 발현을 보인 Raw264.7 세포는 감수성을 보이지 않았다. 따라서 위의 결과로부터 SLT-I에 감수성을 보이지 않은 Raw264.7 세포를 대상으로 Gb3 발현 정도와 SLT-I의 세포독성의 관계를 규명하고자 Gb3의 발현을 증가 시킨 후 SLT-I의 세포독성을 재차 평가하였다. 이 결과 TNF-α의 처리에 의하여 6 h에 Gb3의 발현이 정점(43.5%)에 이르렀으며 36 h에 정상 수준(25.0%)으로 환원되었다. 그러나, Gb3의 발현이 증가함에도 불구하고 SLT-I의 세포독성에는 변화가 관찰되지 않았다. 따라서, SLT-I에 의한 세포독성은 세포의 종류에 따라서 다르며 또한, Gb3의 발현정도

에만 의존적이지는 않을 것으로 생각된다.

결 론 : 이와 같은 결과는 E. coli 0157의 감염증 병인 연구에 있어 SLT-I과 Gb3의 발현의 상관관계에 대한 보다 심도 있는 연구가 필요함을 시사한다.

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