Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by non-toxic mutant Stx1 which lacks *N*-glycosidase activity

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Abstract Stx1 and Stx2 produced by Shiga toxin-producing *Escherichia coli* are cytotoxic due to their *N*-glycosidase activity on 28S rRNA. In this study, we have shown that proinflammatory cytokine mRNAs, especially IL-8, were induced by Stx1 and Stx2 in Caco-2 cells. A non-toxic mutant of Stx1 which lacks *N*-glycosidase activity did not induce cytokine mRNAs. IL-8 production at the protein level was enhanced by Stx1 and Stx2, but not by the mutant Stx1. These results demonstrate that Shiga toxins induce expression and synthesis of cytokines in Caco-2 cells and their *N*-glycosidase activity is essential for the induction.

Key words: Shiga toxin; Cytokine; Interleukin-8; RNA *N*-glycosidase; Colon epithelial cell; Shiga toxin-producing *Escherichia coli*

1. Introduction

Shiga toxin 1 (Stx1) and Stx2 produced by Shiga toxin-producing *Escherichia coli* (STEC) are implicated in bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in STEC-infected patients [1]. Shiga toxins share AB_5 holotoxin molecular structure [2,3] and the holotoxin binds via the B subunit pentamer to neutral glycolipid globotriaosylceramide (Gb_3) on the target cell surface. Shiga toxins are incorporated by endocytosis and transported to endoplasmic reticulum by retrograde transport system [4] and the A subunit hydrolyzes the *N*-glycoside bond of the adenosine at position 4324 of 28S rRNA [5–7], resulting in inhibition of cellular protein synthesis and eventual cell death.

Although it is widely accepted that the cytotoxicity of Shiga toxins is directly responsible for the vascular injury observed in hemorrhagic colitis and hemolytic uremic syndrome [8], a possible role of cytokines in the pathogenesis of the disease has also been suggested [9]. Recently, it was reported that Stx1 induces cytokine production in monocytes and macrophages in culture [9,10]. Stx1 and Stx2 also enhanced interleukin-8 (IL-8) production in T84 cells, a human colon epithelial cell line [11]. However, the involvement of the A subunit of the toxin in cytokine induction still remains controversial; the Stx1 B subunit alone has enhanced cytokine production in T84 cells [11], but not in monocytes and macrophages [10]. The importance of the enzymatic activity of the A subunit of Shiga toxins in the induction of cytokine has not been assessed.

We have previously reported the development of a non-toxic mutant of Stx1 in which two amino acids of the A subunit were replaced, and have shown that the cytotoxicity of the mutant toxin is 1/300 000 that of the wild-type due to the lack of *N*-glycosidase activity [12]. Since the mutant Stx1 differs from wild-type Stx1 only in the potential active site of *N*-glycosidase, it is a good tool to assess the importance of the enzyme activity in cytokine induction by Shiga toxins. In this report, we examined the effects of the mutant Stx1, in comparison with Stx1 and Stx2, on cytokine production and cell viability of Caco-2 cells, a human colon epithelial cell line.

2. Materials and methods

2.1. Toxins, serum and reagents

Recombinant Stx1, Stx2 and the mutant Stx1 in which glutamic acid at position 167 and arginine at position 170 of the A subunit were replaced by glutamine and leucine, respectively (Stx1/E167Q-R170L), were prepared as described previously [12–14]. The mutant Stx1 exhibited similar profiles of HPLC on a TSK gel G-2000 SW, polyacrylamide gel disc electrophoresis and SDS-polyacrylamide gel slab electrophoresis to those exhibited by wild-type Stx1 [14]. Endotoxin content of the Shiga toxin preparations was less than 0.016 EU per mg as determined by the HS Limulus Test (Wako Pure Industries Ltd., Osaka, Japan). Rabbit anti-Stx1 antiserum was prepared as described previously [13]. Hybridoma-13C4, which produces a monoclonal antibody against B subunit of Stx1, was obtained from the American Type Culture Collection (Rockville, MD) and its culture supernatant was used as a source of the monoclonal antibody. Human recombinant IL-1β was a gift from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Lipopolysaccharide (LPS) derived from *E. coli* O26:B6 was purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified essential medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Intergen Inc., Purchase, NY), with 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (all from Gibco BRL, Gaithersburg, MD). Confluent Caco-2 cells were treated with 2 mM butyric acid sodium salt (Wako) for 4 days and used for all experiments, since the treatment of Caco-2 cells had been reported to increase the content of Gb_3, binding of the Stx1 B subunit and sensitivity to Stx1 [15].

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Abbreviations: CD_{50}, 50% cytotoxic dose; Gb_3, globotriaosylceramide; IL-8, interleukin-8; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; RT-PCR, reverse transcription polymerase chain reaction; Stx, Shiga toxin; STEC, Shiga toxin-producing *Escherichia coli*; TNF-α, tumor necrosis factor-α

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2.3. Detection of cytokine mRNAs by reverse transcription-PCR (RT-PCR)

Cells were treated for 6 h with 1, 10 or 100 ng/ml of the different Shiga toxin preparations, 10 ng/ml of IL-1β or 1 μg/ml of LPS. In some experiments, Stx1 was preincubated with rabbit anti-Stx1 serum (1:500) or normal rabbit serum (1:500) at 37°C for 2 h prior to the treatment. Total RNA fraction of the cells was prepared by the AGPC method [16] and RT-PCR was performed as described previously [17]. Primers, protocols and the size of products for PCR are listed in Table 1. The aliquots of PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

2.4. Detection of cytokine proteins by ELISA

Cells were treated for 48 h with various concentrations of the different Shiga toxin preparations, 10 ng/ml of IL-1β or 1 μg/ml of LPS. The culture supernatants were collected by centrifugation and analyzed immediately or stored at −40°C until use.

ELISA for IL-8 was performed as described previously [18], using monoclonal mouse anti-human IL-8 antibody (PetroTech EC Ltd., London, UK), polyclonal rabbit anti-human IL-8 antibody (Genzyme, Cambridge, MA) and horseradish peroxidase-labeled goat anti-rabbit IgG (Southern Biotechnology Associates Inc., Birmingham, AL). The minimum detection level by this method was 39.4 pg/ml. ELISA for TNF-α, MCP-1 or MIP-1α was also performed using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. Assay for cytotoxicity

Cells were treated with various concentrations of the different Shiga toxin preparations, 10 ng/ml of IL-1β or 1 μg/ml of LPS for 72 h. Relative number of cells was determined using the WST-1 Cell Counting Kit (Wako).

2.6. Inhibition of protein synthesis

Cells cultured in 96-well plates of the multiScreen Assay system (Millipore, Bedford, MA) were treated for 2 h with various concentrations of Shiga toxins. After removal of the toxin-containing medium, cells were incubated with 2 μCi/well of [^{3}H]leucine (DuPont New England Nuclear, Boston, MA) in leucine-free DMEM at 37°C for 90 min, washed and treated with 10% TCA solution at 4°C for 15 min. The wells were washed with 5% TCA solution three times, dried completely, and radioactivity of the remaining [^{3}H]leucine in each well was counted by Top Count Microplate Scintillation Counter (Packard, Meriden, CT) in Microscint 20 Scintillation Cocktail (Packard).

2.7. Assay for bindings of the toxins to Gb_3

Indicated amounts of Gb_3 isolated from porcine erythrocytes (Wako) were applied to HPTLC plates (Whatman, Clifton, NJ) and developed in chloroform/methanol/water, 60:35:8 (v/v). After blocking with blocking buffer (1% bovine serum albumin, 0.15 M Tris-HCl, pH 7.6), plates were incubated with wild-type or mutant Stx1 in blocking buffer. Bound toxins were detected by hybridoma-13C4 culture supernatant, biotinylated horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) and streptavidin peroxidase (Sigma), and visualized with peroxidase substrate, 3,3′-diaminobenzidine tetrahydrochlorine (Wako). Gb_4 isolated from human erythrocytes (Sigma) was used as a control to confirm the specificity of the binding. Monoclonal antibody against Gb_3 (anti-CD77 clone 38-13; Cosmo Bio Co. Ltd., Tokyo, Japan) and biotinylated rabbit anti-rat IgM antibody (Zymed Laboratories, San Francisco, CA) were used to detect Gb_3 on HPTLC plates.

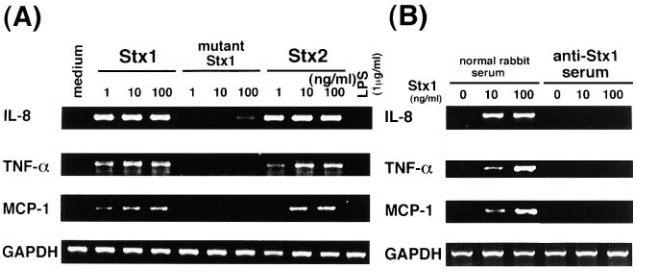

Fig. 1. Induction of cytokine mRNAs by Shiga toxins in colon epithelial cell line. A: Expression of cytokine mRNAs detected by RT-PCR, using total RNA extracted from Caco-2 cells treated with Shiga toxins for 6 h. B: Specific inhibition of Stx1-induced cytokine mRNAs by anti-Stx1 serum. Cytokine mRNA expression detected by RT-PCR, using total RNA extracted from Caco-2 cells treated for 6 h after preincubation with serum for 2 h.

3. Results

3.1. Induction of cytokine mRNAs in Caco-2 cells by Shiga toxins

The expression of cytokine mRNAs in Caco-2 cells was analyzed by RT-PCR. The expression of mRNAs of IL-8, TNF-α and MCP-1 was induced by 1–100 ng/ml of Stx1 and Stx2 (Fig. 1A). The induction was completely inhibited by preincubation of Stx1 with anti-Stx1 antiserum but not with normal serum (Fig. 1B). No induction of cytokine mRNA was observed by 1 μg/ml of LPS of *E. coli* O26:B6 (Fig. 1A). These results indicate that the induction of cytokine mRNAs could be solely attributed to Stx1 but not to LPS or to any other bacterial components. Stx1 and Stx2 also induced mRNA expression of MIP-1α and MIP-1β, but not IL-1β, an inducer of IL-8 in Caco-2 cells [19] (data not shown).

No induction of cytokine mRNAs of IL-8, TNF-α and MCP-1 (Fig. 1A) and MIP-1α, MIP-1β and IL-1β (data not shown) was observed by the mutant Stx1, except for a slight induction of IL-8 mRNA when treated with 100 ng/ml, as discussed below.

3.2. Enhancement of IL-8 production by Shiga toxin

The production of the cytokine protein was determined by ELISA. As shown in Fig. 2, enhancement of IL-8 production by Stx1 and Stx2 was also observed at the protein level. Treatment with 10 pg/ml of Stx1 and Stx2 enhanced IL-8 production, and the enhancement reached its peak at 100 pg/ml of the toxins. The enhancement decreased at higher doses of the

Table 1
Primers and conditions for RT-PCR

	5′ primers (5′-3′)	3′ primers (5′-3′)	PCR cycles	Size of PCR product (bp)
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC	27–35[a]	292
TNF-α	GGACGTGGAGCTGGCCGAGGAG	CACCAGCTGGTTATCTCTCAGCTC	48–54[b]	352
MCP-1	ACTGAAACTCGCACTCTCGCCTC	TGTCTGGGGAAAGCTAGGGGAAAAT	29–35[a]	389
MIP-1α	TCCTTTCTTGGCTCTGCTGACACTC	CTCAGGCACTCAGCTCTAGGTCG	39–45[b]	335
MIP-1β	TGTCCTGTCTCTCCTCATGCTAGTA	GCTCAGTTCAGTTCCAGGTCATACA	39–45[b]	264
IL-1β	AAACAGATGAAGTGCTCCTTCCAGG	TGGAGAACACCACTTGTTGCTCCA	27–35[a]	391
GAPDH	GGGAGCCAAAAGGGTCATCATCTC	CCATGCCAGTGAGCTTCCCGTTC	11–17[a]	353

[a]Cycles of 94°C for 1 min, 62°C for 2 min, and 72°C for 2 min.
[b]Cycles of 94°C for 40 s, 62°C for 1 min, and 72°C for 90 s.

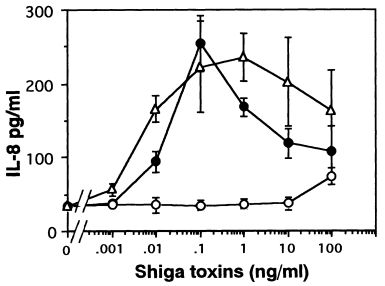

Fig. 2. Enhancement of IL-8 protein by Shiga toxins in human colon epithelial cell line. IL-8 concentration measured by ELISA in culture supernatant of Caco-2 cells treated for 48 h by Stx1 (●), the mutant Stx1 (○) or Stx2 (Δ). Means ± S.D. ($n = 3$) are shown.

toxins, but was still observed by treatment with up to 100 ng/ml of both toxins.

IL-8 production was not enhanced by the mutant Stx1 at concentrations up to 10 ng/ml, and detectable enhancement was observed at 100 ng/ml. There was no difference in IL-8 production when treated with 1 μg/ml LPS of *E. coli* O26:B6 compared to control (LPS-treated, 43.7 ± 8.4 pg/ml vs. control, 42.7 ± 3.2 pg/ml; $P > 0.1$). Treatment with 10 ng/ml of IL-1β, a positive control, enhanced IL-8 production to 2019.5 ± 96.2 pg/ml.

Determinations of protein levels of TNF-α, MCP-1 and MIP-1α by ELISA were also performed, but the levels of these cytokines in culture medium of Caco-2 cells were less than the corresponding minimal detection levels, even after the treatment with various concentrations of Shiga toxins or LPS (data not shown).

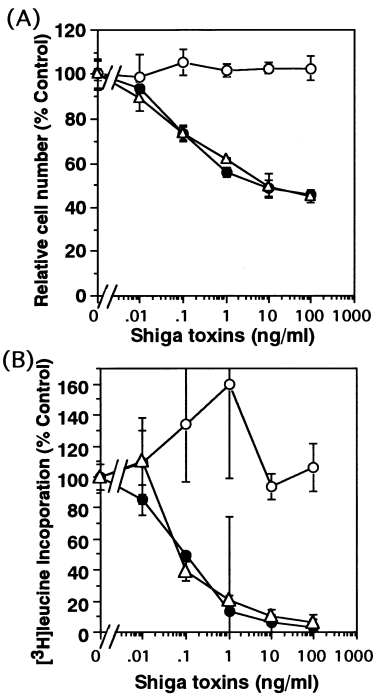

Fig. 3. Cytotoxicity and inhibitory effect in cellular protein synthesis of Shiga toxins in human colon epithelial cell line. Relative cell number (A) and relative incorporation of [^{3}H]leucine (B) of Caco-2 cells treated for 72 h and 2 h, respectively, with Stx1 (●), the mutant Stx1 (○) or Stx2 (Δ) are shown. Means ± S.D. ($n = 4$) are shown.

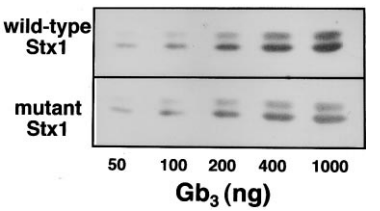

Fig. 4. Binding activity of wild-type and mutant Stx1 to the receptor Gb_3. Binding of wild-type or the mutant Stx1 with the indicated amount of purified Gb_3 on HPTLC are visualized.

3.3. Cytotoxicity of Shiga toxins

Comparable cytotoxicity was observed by Stx1 and Stx2 to Caco-2 cells (Fig. 3). At 10 pg/ml, neither Stx1 nor Stx2 reduced the cell number, but at 100 pg/ml or higher concentrations, both toxins showed cytotoxicity. Cytotoxicity of Shiga toxins for Caco-2 cells was much lower than for Vero cells, currently known to be one of the most sensitive cells to Shiga toxins [20]. The mutant Stx1 (Fig. 3), 10 ng/ml of IL-1β or 1 μg/ml of LPS of *E. coli* O26:B6 (data not shown) did not show any cytotoxicity to Caco-2 cells.

3.4. Effect of Shiga toxins on cellular protein synthesis

Comparable inhibition on cellular protein synthesis was observed by Stx1 and Stx2 in Caco-2 cells (Fig. 3B). The mutant Stx1 (Fig. 3B), 10 ng/ml of IL-1β or 1 μg/ml of LPS of *E. coli* O26:B6 (data not shown) did not cause any effect on cellular protein synthesis in Caco-2 cells.

3.5. Binding of the mutant toxin on Shiga toxin receptor, Gb_3

To exclude the possibility that the replacement of amino acids of A subunit of Stx1 causes any change in binding property of the toxin to its receptor, the binding of the mutant Stx1 to Gb_3 was assessed on HPTLC. As shown in Fig. 4, both wild-type and the mutant Stx1 showed similar binding pattern to purified Gb_3. Double bonds detected on HPTLC were confirmed to be Gb_3 by anti-Gb_3 staining (data not shown). No binding of Stx1 to Gb_4 was detected (data not shown). These results indicated that the B subunit of the mutant Stx1 retains the similar binding ability to the receptor.

4. Discussion

This study demonstrated that the *N*-glycosidase activity of the A subunit of Shiga toxins is essential to induce cytokines in a human colon epithelial cell line. Expression of mRNAs of IL-8, TNF-α, MCP-1, MIP-1α and MIP-1β was induced by Stx1 and Stx2, but not by the mutant Stx1 which lacked *N*-glycosidase activity. IL-8 production was enhanced by Stx1 and Stx2, and the involvement of *N*-glycosidase activity was confirmed also at the protein level. The reduced level of IL-8 induction at both mRNA and protein levels by the highest concentration of the mutant Stx1 seems to be comparable to its reduced activity (1/300 000) [12] as compared with wild-type Stx1, suggesting that the cytokine-inducing activity is directly correlated with the enzyme activity. It is interesting to note that the enzymatic activity is implicated in apparently opposite activities: inhibition of cellular protein synthesis and enhancement of the certain cytokine production.

In our preliminary investigations, the increase of IL-8 mRNA by Shiga toxins was more than 100-fold when measured by competitive RT-PCR, which was comparable to that

by IL-1β. Compared with the 6–8-fold enhancement of IL-8 production by Shiga toxins at the protein level shown in this study, the increase of IL-8 mRNA by Shiga toxins was much greater. This can be partly explained by their inhibitory effect on cellular protein synthesis and resulting cytotoxicity to Caco-2 cells. The highest dose of Stx1 caused persistent inhibition of protein synthesis in Caco-2 cells, but IL-8 production was still much more abundant than in control; i.e. the enhancement of IL-8 synthesis occurs under the condition in which synthesis of most proteins is inhibited. Under this condition, the induction of IL-8 mRNA may overcome the inhibition of protein synthesis. The overall effect of the toxins on protein synthesis may cause the characteristic pattern of dose dependence of their cytokine-inducing activity.

Recently, many reports have shown that bacterial cytotoxins have activities that modify cellular signaling events. For example, some bacterial cytotoxins have specific activity on the cellular signaling molecule, Rho: ADP-ribosylation by *Clostridium botulinum* toxin C1, C3 and D [21–23] and *O*-glucosylation by *Clostridium difficile* toxin A and B [24,25]. Some other bacterial cytotoxins also have enzymatic activities in different events: ADP-ribosyltransferase activity of cholera toxin produced by *Vibrio cholerae* activate Gs constitutively, eventually increasing the cAMP level [26]; phospholipase C activity of α toxins of *Clostridium perfringens* induces expression and synthesis of intracellular leukocyte adherence molecule-1 and IL-8 [27]. We propose that Shiga toxins have an activity other than inhibition of cellular protein synthesis, and this study suggests that RNA *N*-glycosidase activity of Shiga toxins stimulates the cellular signaling pathway to induce cytokines.

This study has also demonstrated that 10 pg/ml of Stx1 or Stx2 is sufficient to induce cytokine production in a human colon epithelial cell line. It has been reported that the minimum effective dose of Stx1 or Stx2 required for cytokine induction was 1 μg/ml of Stx1 in monocytes or macrophages [9,10] and 10 ng/ml of Stx1 and Stx2 in T84 cells [11], which is 10^6- and 10^4-fold of the CD_{50} of Shiga toxins to Vero cells, respectively. The observation that the minimal dose of Shiga toxins for IL-8 induction is comparable to their cytotoxic dose to highly sensitive cells such as Vero cells [12] suggests that similar levels of Shiga toxins could exist in the circulation or in some regions of STEC-infected patients and show the activities of cytotoxicity and cytokine induction.

IL-8 is known to be a chemokine which mainly activates and chemoattracts neutrophils [28,29]. It has been reported that neutrophil migration to the intestinal epithelium is observed in STEC infection [30]. Elevated levels of IL-8 in plasma and polymorphoneutrophil activation have been reported in HUS patients in childhood relative to that of patients only with diarrhea [31,32]. Activated neutrophil-dependent injury of the vascular endothelial cells in HUS patients has also been reported [33]. The human colon epithelial cells are supposed to be the first target cells of Shiga toxin, when STEC colonizes and produces Shiga toxins in the human colon. The cytokine-inducing activity of Shiga toxins might be relevant to the pathological and clinical observations in these patients.

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