Shiga Toxin Kills Epithelial Cells Isolated from Distal but not Proximal Part of Mouse Colon

Mari KASHIWAMURA,^a Kohta KUROHANE,^a Takashi TANIKAWA,^a Aya DEGUCHI,^a Daisei MIYAMOTO,^b and Yasuyuki IMAI^{*,a}

^aLaboratory of Microbiology and Immunology and the Global COE Program, University of Shizuoka School of Pharmaceutical Sciences; and ^bDepartment of Biochemistry, University of Shizuoka School of Pharmaceutical Sciences; 52–1 Yada, Suruga-ku, Shizuoka, Shizuoka 422–8526, Japan.

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Shiga toxins (Stxs) are major virulence factors produced by enterohemorrhagic *Escherichia coli* O157:H7 colonizing the human and cattle intestines. We previously demonstrated that recombinant binding subunits (Stx1B) bound to the mucosal epithelium of the distal but not that of the proximal part of the mouse colon. Here we developed a method for isolating colon epithelial cells from the proximal and distal parts separately. Enrichment of epithelial cells was confirmed by the expression of cytokeratin. There was no difference in the epithelial cell purity between the proximal and distal colon preparations. The isolated epithelial cells from the distal colon were found to display binding sites for recombinant Stx1B whereas those from the proximal colon were not. Taking advantage of this single cell isolation, we examined the effect of Stx1 holotoxin on the epithelial cells. Consistent with the expression of the binding sites, Stx1 induced apoptosis of the epithelial cells from the distal but not those from the proximal colon. The results provide direct evidence that mouse colon epithelial cells are susceptible to Stx1 toxicity corresponding to the expression of binding sites for toxins.

Key words Shiga toxin; apoptosis; epithelial cell; distal colon; *Escherichia coli* O157:H7

Shiga toxins (Stxs) are virulence factors of enterohemorrhagic *Escherichia coli* (EHEC) strains such as serotype O157:H7.¹⁾ There are two types of Stx, Stx1 and Stx2, which are associated with human diseases with life-threatening complications, including hemolytic-uremic syndrome (HUS) and central nervous system involvement. Stx holotoxin (70 kDa) consists of a 32 kDa cytotoxic subunit (A subunit) and a pentamer of 7.7 kDa binding subunits (B subunit).^{2,3)} The B subunits of Stx1 and Stx2 both bind to carbohydrate ligand globotriaosylceramide (Gb₃, Gal α 1 $\rightarrow$ 4Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer), also known as CD77, on human germinal center B cells and Burkitt's lymphoma cells.^{4,5)}

In children with HUS, the most severe and earliest damage is found in the left and transverse portions of the colon (distal parts of the colon).⁶⁾ In mouse system, we have previously demonstrated that the recombinant B subunit of Stx1 (Stx1B) avidly binds to the epithelium of distal but not proximal parts of the colon by means of immunohistochemical methods.⁷⁾ Recent study also demonstrated a restricted binding of Stx2 to the distal colonic epithelium in rats.⁸⁾ However, it is not clear whether the binding of Stx holotoxin provokes epithelial cell damage. In this study, we isolated viable epithelial cells from the mouse proximal and distal colon separately. We compared the binding of Stx1B and susceptibility to Stx1 holotoxin using isolated epithelial cells from different parts of the colon.

MATERIALS AND METHODS

Mice Specific-pathogen-free female CD-1 (ICR) mice were obtained from Japan SLC Inc. (Shizuoka, Japan), and were used between 6 and 8 weeks of age. Animal care and experiments were undertaken in compliance with the ethical guidelines for the animal facilities of the University of Shizuoka.

Reagents Preparation of recombinant binding subunits

of Stx1 (Stx1B) and digoxigenin-labeled Stx1B (DIG-Stx1B) were performed as described previously.⁹⁾ Stx1 holotoxin was kindly provided by Dr. Shinji Yamazaki (Osaka Prefecture University, Osaka, Japan).³⁾ Fluorescein isothiocyanate-labeled sheep anti-digoxigenin Fab fragments (FITC-anti-DIG), rhodamine-labeled sheep anti-digoxigenin Fab fragments (rhodamine-anti-DIG), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and an *in situ* cell death detection kit (fluorescein) were purchased from Roche Diagnostics (Tokyo, Japan). Polyclonal rabbit anti-cow cytokeratin (wide spectrum screening) was purchased from Dakocytomation (Glostrup, Denmark), and Texas Red-goat anti-rabbit immunoglobulin G (IgG) (H+L) from Vector (Burlingame, California, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan), and fetal bovine serum (FBS) from HyClone (South Logan, Utah, U.S.A.). Penicillin G and L-glutamine were purchased from MP Biomedicals (Solon, Ohio, U.S.A.), Triton X-100 from Tokyo Chemical Industries (Tokyo, Japan), and streptomycin sulfate and gentamicin from Wako Pure Chemicals (Osaka, Japan). Percoll was purchased from GE Healthcare Biosciences (Uppsala, Sweden), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Nacalai Tesque (Kyoto, Japan), and 2-mercaptoethanol (2-ME) and bovine serum albumin (BSA) from Sigma (St. Louis, Missouri, U.S.A.). Recombinant human epidermal growth factor (EGF) and insulin-transferrin-selenium supplement (ITS) were purchased from Invitrogen (Carlsbad, California, U.S.A.), and mouse collagen type IV and Matrigel from Japan Becton Dickinson (Tokyo, Japan).

Isolation and Purification of Epithelial Cells Mice were killed under ether anesthesia and then their colons were removed. Each colon was divided into proximal (<3 cm below the cecum) and distal (>5 cm below the cecum) parts. The colon segments were flushed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin G, 100 μ g/ml

* To whom correspondence should be addressed. e-mail: imai@u-shizuoka-ken.ac.jp

streptomycin sulfate and 25 $\mu\text{g/ml}$ gentamicin to remove feces. The colon segments were then everted, ligated at one end, and inflated by injecting PBS according to the published method.^{10,11} The opposite end was then ligated. The segments were agitated in 30 ml of PBS containing 0.02% ethylenediaminetetraacetic acid (EDTA), 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin sulfate and 25 $\mu\text{g/ml}$ gentamicin in 50-ml tubes using a vortex mixer, with 10 cycles of mixing for 15 s each. The cells released from the colon segments were transferred to 15-ml tubes, and then centrifuged for 10 min at $200\times g$ at 4°C. Each cell pellet was suspended in DMEM containing 5 mM HEPES (pH 7.4). The cell suspension was then centrifuged for 30 min through a 5%, 15%, 40% discontinuous Percoll gradient in 5 mM HEPES-DMEM for 30 min at $560\times g$ at 4°C. Epithelial cells were recovered from the 5% and 15% Percoll interface, and then were washed by centrifugation for 10 min at $200\times g$ in DMEM at 4°C.

Immunofluorescence The purified cells were suspended in PBS containing 0.02% EDTA, and then were forced to become attached to MAS-coated glass slides (Matsunami Glass, Osaka, Japan) by means of a cytocentrifuge. The cells were fixed in acetone for 30 s at room temperature and then air-dried. Only in the case of cytokeratin detection was involved, cells were permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature, and then washed three times with PBS. Nonspecific binding sites were blocked with 3% BSA in PBS for 10 min at room temperature. The cells were incubated with either rabbit anti-cow cytokeratin (undiluted) or DIG-Stx1B (5 $\mu\text{g/ml}$ in 3% BSA-PBS) for 30 min at room temperature. After washing three times with PBS, each sample was incubated with 100 μl of the respective secondary reagent diluted in 3% BSA-PBS, Texas Red-goat anti-rabbit IgG (5 $\mu\text{g/ml}$) containing DAPI (0.01 $\mu\text{g/ml}$), or rhodamine anti-DIG (2 $\mu\text{g/ml}$) containing DAPI for 30 min at room temperature. After washing three times with PBS, the cells were mounted with Gel/Mount (Biomedex, Foster City, California, U.S.A.). Cell-associated fluorescence was observed under a fluorescence microscope (BX-51; Olympus, Tokyo, Japan). The numbers of cytokeratin-positive cells, cells positive for Stx1B binding, and total cells (nuclear staining with DAPI) were determined using an objective lens of $20\times$ magnification. At least 100 cells per field and three fields for slide were counted.

Flow Cytometry Purified epithelial cells (5×10^5) suspended in 100 μl of PBS containing 0.1% NaN_3 and 0.1% BSA (PBS-BSA) were incubated with DIG-Stx1B (10 $\mu\text{g/ml}$) for 30 min at 4°C. After washing three times by centrifugation in PBS-BSA for 5 min at 4°C, the cells were incubated in 100 μl of FITC-anti-DIG (0.4 $\mu\text{g/ml}$ in PBS-BSA). After 30 min incubation at 4°C, the cells were washed twice in PBS-BSA. Cells were passed through a piece of nylon mesh and then analyzed with a flow cytometer (EPICS XL; Beckman Coulter, Fullerton, California, U.S.A.). The results were analyzed using FlowJo software (version 8.0.3; Tree Star, Ashland, Oregon, U.S.A.).

TUNEL Assay Epithelial cells were suspended in DMEM supplemented with 10% FBS, ITS (150 ng/ml insulin, 82 ng/ml transferrin, and 0.1 ng/ml sodium selenite), 10 ng/ml EGF, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 10 mM HEPES (pH 7.4), 50 μM 2-ME and 2 mM L-glutamine.

Cells were added to 16-well chamber slides (Nalge Nunc, Rochester, New York, U.S.A.) that had been coated with either mouse collagen type IV or Matrigel. The cells were cultured for 16 h at 37°C under a humidified atmosphere of 5% CO_2 /95% air. The cells were then cultured in the presence or absence of 0.1 ng/ml of Stx1 for an additional 24 h at 37°C. Stx1-treated or control cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature, washed once with PBS, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer (pH 6.9) for 2 min at 4°C. After washing twice with PBS, the cells were incubated with 3% BSA-PBS for 15 min at room temperature. DNA fragmentation was detected by means of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction using the *in situ* cell death detection kit (fluorescein) according to the manufacturer's instructions. After washing twice with PBS, the cells were incubated with rabbit anti-cow cytokeratin (undiluted) to counterstain epithelial cells for 30 min at room temperature. After washing twice with PBS, the cells were incubated with 5 $\mu\text{g/ml}$ Texas Red-goat anti-rabbit IgG for 30 min at room temperature. After washing three times with PBS, the cells were mounted using Gel/Mount. We determined whether each cytokeratin-positive cell exhibited a TUNEL signal under a fluorescence microscope. We counted more than 100 cytokeratin-positive cells in each well (triplicate culture) using an objective lens of $20\times$ magnification. The apoptosis index is expressed as the percentage of the TUNEL-positive cells per number of cytokeratin-positive cells examined.

Statistical Analysis Data are expressed as means $\pm$ standard deviation (S.D.). Statistical significance was analyzed by means of Student's *t*-test. *p* values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

We separately prepared epithelial cells from the proximal and distal colon by Percoll density gradient centrifugation. Their viability was more than 80% (trypan blue dye exclusion). When cells were stained with anti-cytokeratin (a marker for epithelial cells) followed by counterstaining with DAPI (nuclear staining), the majority of cells have cytokeratin in the cytoplasm regardless of the proximal or distal colon (Figs. 1A, B). Cytokeratin was expressed in $87\pm 3\%$ (mean $\pm$ S.D.) of cells from the proximal colon and $91\pm 2\%$ of cells from the distal colon, respectively, in three independent experiments. The results indicated successful isolation of epithelial cells from mouse colon.

The epithelial cells isolated from the proximal or distal colon were incubated with DIG-Stx1B followed by incubation with rhodamine anti-DIG antibodies. DAPI was used for nuclear staining. Epithelial cells isolated from the proximal colon did not (Fig. 1C), whereas ones from the distal colon did reveal binding sites for DIG-Stx1B (Fig. 1D). DIG-Stx1B binding was observed in $3.1\pm 2.9\%$ (mean $\pm$ S.D., $n=6$) of cells from the proximal colon and $73.3\pm 5.5\%$ of cells from the distal colon, respectively. This is consistent with our previous results using an immunohistochemical method involving frozen sections.⁷⁾

We also compared the binding of DIG-Stx1B to unfixed cells by means of flow cytometry. Cells were incubated with

Cytokeratin

StxB1

Stx1 holotoxin (TUNEL)

Fig. 1. Representative Fluorescent Micrographs of Epithelial Cells Isolated from the Proximal and Distal Mouse Colon

Cells enriched by Percoll density gradient centrifugation from proximal (<3 cm below the cecum; panels A, C and E) and distal (>5 cm below the cecum; panels B, D and F) parts of the mouse colon were examined as follows: (A, B) Cells were stained with anti-cytokeratin (red) and then counter-stained with DAPI (blue). The majority of cells expressed cytokeratin regardless of the part of the colon. (C, D) Cells were incubated with DIG-Stx1B plus rhodamine-anti-DIG (red) and then counter-stained with DAPI (blue). Cells isolated from the distal (D), but not the proximal part, of the colon (C) carried binding sites for Stx1B. (E, F) Cells were cultured on collagen type IV-coated slides and then treated with Stx1 holotoxin (0.1 ng/ml) for 24 h at 37°C. The TUNEL method (green) was employed for the detection of apoptotic cells and then the cells were counterstained with anti-cytokeratin (red). Nuclei with fragmented DNA were more abundant in the cells from the distal colon (F) after exposure to Stx1. Arrows indicate examples of apoptotic nuclei (F). An objective lens of 40× magnification was used and bars represent 50 μ m.

DIG-Stx1B followed by FITC-anti-DIG antibodies, and fluorescence signals above nonspecific ones (FITC-anti-DIG alone) as well as background ones (no DIG-Stx1B and no FITC-anti-DIG) were evaluated. In the case of cells from the proximal colon, only a slight shift in the fluorescence intensity was observed above the nonspecific level and the background (Fig. 2A). In contrast, a marked shift was observed for cells from the distal colon (Fig. 2B). Similar results were consistently obtained from three independent experiments.

Viable cell isolation from mouse colon allowed us to examine whether Stx1 holotoxin exhibits selective cytotoxicity toward epithelial cells from the distal colon. After exposure to Stx1 holotoxin (0.1 ng/ml), apoptosis was assayed by the TUNEL method (Figs. 1E, F). In the case of epithelial cells from the distal colon, the number of nuclei reflecting DNA fragmentation was significantly higher ($p < 0.0005$) upon Stx1 treatment compared with in cells without Stx1 treatment (Fig. 2C). In contrast, there was no difference in the number of apoptotic cells among epithelial cells from the proximal colon between with and without the Stx1 treatment ($p > 0.05$). The viability of primary-isolated epithelial cells was somewhat low after 2-d-culture, however, the background was similar for cells from the proximal and distal colon. When cells were cultured on Matrigel-coated slides, Stx1-induced apoptosis was also selectively observed for epithelial cells from the distal colon (data not shown).

In conclusion, we found that epithelial cells isolated from the mouse distal, but not proximal, colon carried binding sites for Stx1B. Stx1 holotoxin was found to induce apoptosis of the epithelial cells of the distal, but not the proximal, colon. The results provide direct evidence that the binding of Stx to colon epithelial cells can result in cell damage.

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Fig. 2. Selective Binding of Stx1B and Selective Toxicity of Stx1 Holotoxin to Viable Epithelial Cells of the Distal Colon

(A, B) Unfixed epithelial cells were incubated with or without DIG-Stx1B followed by FITC-anti-DIG, and then analyzed with a flow cytometer. The abscissa of histograms represents the fluorescence intensity on a log scale. Histograms of cells incubated with DIG-Stx1B plus FITC-anti-DIG (thick lines), FITC-anti-DIG alone (thin lines), and nothing (thin lines with shaded areas) are presented for cells from the proximal (A) and distal (B) colon, respectively. (C) Epithelial cells were cultured in the presence or absence of Stx1 (0.1 ng/ml) for 24 h at 37°C. DNA fragmentation was detected by the TUNEL method, and epithelial cells were identified with anti-cytokeratin. Apoptosis index (ordinate) is the percentage (means $\pm$ S.D. for six independent experiments) of TUNEL-positive cells out of all the cytokeratin-positive cells.

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