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Research Article

Shiga toxin 1 interaction with enterocytes causes apical protein mistargeting through the depletion of intracellular galectin-3

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ABSTRACT

Shiga toxins (Stx) 1 and 2 are responsible for intestinal and systemic sequelae of infection by enterohemorrhagic *Escherichia coli* (EHEC). However, the mechanisms through which enterocytes are damaged remain unclear. While secondary damage from ischemia and inflammation are postulated mechanisms for all intestinal effects, little evidence excludes roles for more primary toxin effects on intestinal epithelial cells. We now document direct pathologic effects of Stx on intestinal epithelial cells. We study a well-characterized rabbit model of EHEC infection, intestinal tissue and stool samples from EHEC-infected patients, and T84 intestinal epithelial cells treated with Stx1. Toxin uptake by intestinal epithelial cells *in vitro* and *in vivo* causes galectin-3 depletion from enterocytes by increasing the apical galectin-3 secretion. This Shiga toxin-mediated galectin-3 depletion impairs trafficking of several brush border structural proteins and transporters, including villin, dipeptidyl peptidase IV, and the sodium-proton exchanger 2, a major colonic sodium absorptive protein. The mistargeting of proteins responsible for the absorptive function might be a key event in Stx1-induced diarrhea. These observations provide new evidence that human enterocytes are directly damaged by Stx1. Conceivably, depletion of galectin-3 from enterocytes and subsequent apical protein mistargeting might even provide a means whereby other pathogens might alter intestinal epithelial absorption and produce diarrhea.

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Abbreviations: Stx, Shiga toxin; Stx1B, B-subunit of Shiga toxin 1; EHEC, enterohemorrhagic Escherichia coli; Gb3, globotriaosylceramide; TNF-α, tumor necrosis factor α; CTB, B-subunit of cholera toxin; HRP, horse radish peroxidase; DPPIV, dipeptidylpeptidase IV; RT-PCR, reverse-transcription polymerase chain reaction; IEC, intestinal epithelial cells

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Introduction

Shiga toxin-producing EHEC is one of the major foodborne pathogens by virtue of the frequency in the USA and the severity of the associated illnesses [1]. EHEC infections cause illness in approximately 75 000 people per year in the United States [2]. Recent epidemics such as those related to contaminated leafy green vegetables have increased these totals [2]. While most EHEC-infected individuals develop watery and bloody diarrhea and recover, up to 20% of these patients, mostly children or elderly, develop life-threatening systemic complications, including hemolytic uremic syndrome (HUS) [3,4]. A prospective cohort study [5] provided evidence that antibiotic use significantly increased risks of developing HUS. Improved understanding of Stx-induced intestinal pathophysiology and progression to HUS is necessary to provide the basis for new treatment strategies. Both hemorrhagic colitis and HUS are believed to be thrombotic complications of Stx-induced microvascular injury, since Stx can target globotriaosylceramide (Gb3) receptors on microvascular endothelial cells in gut, kidney, and brain [6].

It has also been assumed that primary damage to enterocytes in EHEC infection results from bacterial attaching and effacing lesions, and that interaction between intestinal epithelial cells (IEC) and Stx are less important. Although there are only a few studies that address the questions of primary damage of IEC by Stx, the majority of them have been done using Gb3-positive IEC models. The absence of detectable amounts of Gb3 in normal human enterocytes [7–9] adds an additional complexity to the problem and prompts the development of new approaches to determine the consequences of the interaction between Stx and enterocytes.

In the current study, we explore direct Stx1 effects on enterocytes. We demonstrate that Stx1 uptake by Gb3-negative IEC causes galectin-3 secretion from their apical domains. This secretion significantly depletes intracellular galectin-3 levels in these cells, studied *in vivo* and *in vitro*. Galectin-3 is a β-galactoside-specific lectin implicated in diverse cellular and extracellular signal transduction pathways [10,11]. It is highly expressed in human small intestinal and colonic epithelial cells [12,13]. Decreased galectin-3 levels in human colonic epithelial cells have been previously described in other diarrheal diseases, most notably Crohn's disease [14,15]. We show here that Stx1 interactions with IEC significantly increase apical galectin-3 secretion *in vitro* and *in vivo*. This Stx1-induced galectin-3 depletion from enterocytes leads to the mistargeting of several key structural and absorptive brush border membrane proteins, including dipeptidyl-peptidase IV (DPPIV), villin, and sodium/proton exchanger isoform 2 (NHE2).

Overall, these data demonstrate that apical secretion of galectin-3 by IEC as a result of the direct impact of Stx1 (and also Stx2) on enterocytes might contribute to the development of toxin-induced diarrhea.

Materials and methods

Cell culture

Human colon cancer polarized epithelial T84 cells (A.T.C.C., Manassas, VA) were grown and maintained in DMEM (Dulbecco's modified Eagle's medium) F-12 medium (1:1), supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Monolayers (passage 31–45) were grown on polycarbonate inserts with 0.4-μm pore size (Costar, Cambridge, MA) or on glass coverslips for 7–10 days. Experiments were performed when monolayers achieved a transepithelial electrical resistance TER > 1500 $\Omega \cdot cm^2$.

Materials

Stx1 and recombinant B-subunit of Stx1 (Stx1B) were provided by Dr. A. Kane (Tufts New England Medical Center, Boston, MA). Purified B-subunit of Cholera toxin (CTB) was from List Biological Laboratories, Inc (Campbell, CA). We used the following mouse monoclonal antibodies (mAbs): anti-galectin-3 from BD Pharmingen (for immunoblotting) (San Diego, CA) and from Affinity BioReagents (for immunofluorescence) (Golden, CO), anti-villin (a gift from Dr. M. Arpin, Curie Institute, Paris and BD Transduction Laboratories), anti-DPPIV was a kind gift from Dr. A. Hubbard (Johns Hopkins University, Baltimore, MD), anti-GAPDH from US Biological (Swampscott, MA), anti-EGFR from Upstate (Lake Placid, NY). Secondary fluorescent antibodies and wheat germ agglutinin (WGA) conjugated to the Alexa Fluor 568 were from Invitrogen (Carslbad, CA) or from Rockland Immunochemical Inc. (Gilbertsville, PA). Stx1, Horseradish peroxidase (HRP) and CTB were conjugated with Alexa fluor 680 reactive dye according to the manufacturer's protocols (Invitrogen). Polyclonal antibodies: anti-NHE2 was a kind gift from Dr. M. Tse (Johns Hopkins University, Baltimore, MD); anti-NHE3 was from Santa Cruz Biotechnology, Inc. (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Detection of intracellular Stx

Confluent T84 cells were incubated for varying times with Stx1 conjugated to Alexa 680 fluorescent dye (Stx1-680) in serum free DMEM/F12. Cells were then washed three times with cold PBS to remove extracellular toxin. The fluorescence intensity of Stx1-680, which corresponds to the amount of intracellular Stx1, was measured in total cell lysates (supplemental Material and Methods) by fluorescence plate reader and normalized to the autofluorescence of total cell lysate from untreated cells.

Human tissue specimens

The human tissue samples of EHEC-infected patients collected during the 1993 *Escherichia coli* O157:H7 epidemic in the Western USA [16] were obtained from the tissue bank of Seattle Children's Hospital via a Johns Hopkins sponsored material transfer agreement. The samples were coded, so no patient identifiers were linked to the specimens. These studies were approved by the Institutional Review Boards (or equivalent committees) of the Children's Hospital and Regional Medical Center, Seattle, Washington.

Detection of secreted galectin-3 in cell conditioned media and in stool samples

Following treatments of T84 monolayers with Stx1, B-subunit of cholera toxin (CTB), or Horseradish peroxidase (HRP) for varying