CXC Chemokine Responses of Intestinal Epithelial Cells to Shiga-toxigenic *Escherichia coli*

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Abstract

Since Shiga-toxigenic *Escherichia coli* (STEC) strains are not considered to be entero-invasive, the mechanism(s) by which Shiga toxin (Stx) gains access to the circulation and to target tissues expressing its target receptor Gb_3 is crucial to the disease process. There is increasing evidence that by facilitating translocation of Stx across the intestinal epithelium and by transporting bound toxin to remote sites such as the renal endothelium, polymorphonuclear leucocytes (PMNs) play a key role in the pathogenesis of serious STEC disease. Plasma levels of PMN-attracting CXC chemokines such as IL-8 also appear to correlate in humans with the severity of disease. Thus, the capacity of STEC strains to elicit CXC chemokine responses in intestinal epithelial cells may be a crucial step in pathogenesis.

In order to determine which STEC factor(s) are responsible for the induction of CXC chemokine responses by intestinal epithelial (HCT-8) cells, a real-time reverse transcription PCR assay was developed to quantitatively measure relative expression of chemokine mRNA for IL-8, ENA-78, GCP-2, MGSA, MIP-2α and MIP-2β. Similarly, a commercially available sandwich ELISA was used to measure levels of IL-8 protein secreted by HCT-8 cells in response to infection with STEC. When HCT-8 cells were infected with the well-characterised locus of enterocyte effacement (LEE)-negative O113:H21 strain 98NK2 or the LEE-positive STEC strain EDL933, there were significant differences in the levels of chemokine mRNA and IL-8 protein expression. In particular, the LEE-negative strain 98NK2 induced significantly higher and earlier levels of chemokine mRNAs, including IL-8, MIP-2α and MIP-2β at 1 and 4 h, and ENA-78 at 4 h. However, EDL933 elicited no significant upregulation of any of the chemokine mRNAs at 1 h, and only modest increases in IL-8, MIP-2α and MIP-2β by 4 h, post-infection. These results were confirmed by IL-8 ELISA which showed that 98NK2 elicited significant levels of IL-8 protein by 2 h post-infection, and remained high until 4 h post-infection. In comparison, EDL933 did not elicit significant IL-8 induction over that of control cells, even at 4 h post-infection.

When a range of STEC isolates from clinical samples were tested for their capacity to induce chemokine production in HCT-8 cells, highly significant differences were observed between the strains. Infection of HCT-8 cells with a range of LEE-negative STEC strains isolated from patients with severe STEC disease resulted in significantly higher and earlier upregulation of IL-8 and MIP-2α mRNA than that elicited by several LEE-positive STEC strains. Similarly, levels of IL-8 protein in LEE-negative STEC-infected HCT-8 culture supernatants were significantly higher than in LEE-positive STEC-infected culture supernatants. Only one LEE-positive strain, an O26 strain 95ZG1, was capable of inducing chemokine responses comparable to that induced by infection with the LEE-negative STEC strains. These results were also shown not to be attributable to differences in the adherence, initial doses or growth of the strains during the assay, or to a loss of viability of the HCT-8 cells. These results, therefore, suggest that there may be interesting differences in the ability of STEC strains to induce chemokine production in intestinal epithelial cells.

The factor(s) that contribute to chemokine induction by epithelial cells in response to STEC were then examined. The difference in responses could not be attributed to the expression or non-expression of LEE genes, the presence or absence of an STEC megaplasmid or to differences in O serogroup. Although purified Stx1 and Stx2 were able to induce IL-8 and MIP-2α mRNA, and IL-8 protein, the levels of chemokine induction in response to wild-type STEC did not correlate with the type or amount of Stx produced by these strains *in vitro*. Similarly, deletion of the single stx_2 gene from 98NK2 had no significant effect on chemokine induction compared to wild-type 98NK2-infected HCT-8 cells. Interestingly, several of the LEE-negative STEC strains eliciting the strongest chemokine responses belonged to flagellar serotype H21. Incubation of HCT-8 cells with purified H21 flagella elicited IL-8 and MIP-2α mRNA responses similar to those seen in the presence of the most potent LEE-negative STEC strains. Deletion of the *fliC* gene largely abolished the capacity of 98NK2 to elicit IL-8 and MIP-2α mRNA and IL-8 protein responses in HCT-8 cells. Similarly, deletion of both stx_2 and *fliC* from 98NK2 elicited a response similar to that observed with deletion of *fliC* alone.

Flagella were then purified from the high chemokine-inducing STEC strains 95HE4 (O91:H7) and 95ZG1 (O26:H11). Purified H7 and H11 flagella were similarly able to induce

both IL-8 and MIP-2α mRNA, and IL-8 protein, in HCT-8 cells at levels similar to their respective wild-type strains. Deletion of *fliC* from two other STEC strains, 97MW1 (O113:H21) and 86-24 (O157:H7), confirmed that flagellin was responsible for the majority of chemokine responses in these wild-type strains. However, an inability of EDL933 to induce these responses was unexpected and later found to be due to a lack of expression of H7 flagella by this strain. Purified H21 FliC (His_6-FliC) alone was able to induce chemokine production (including IL-8, MIP-2α and MIP-2β at 1 and 4 h, and ENA-78 at 4 h) by HCT-8 cells at similar levels to that observed for 98NK2. Taken together, these data suggest that although Stx is capable of inducing CXC chemokine responses, the elevated responses observed in cells infected with certain STEC strains are largely attributable to the production of flagellin.

Purified His_6-H21 flagellin was also able to induce p38 MAPK activation *in vitro* and IL-8 and MIP-2α mRNA were superinduced in the presence of both Stx2 and H21 flagellin. Blockade of the p38 pathway with SB203580 resulted in a down-regulation of IL-8 protein levels (by up to 61%) in response to H21 flagellin, but not IL-8 mRNA, suggesting that this inhibition may occur post-transcriptionally. Blocking the ERK and JNK pathways similarly decreased IL-8 secretion in response to H21 flagellin, suggesting that all three MAPK pathways are involved in this response. Indeed, concurrent inhibition of all three pathways resulted in virtually complete inhibition of IL-8 protein production (98%). Transfected HeLa and MDCK cells stably expressing TLR5 activated p38 in the presence of purified H21 flagellin, whereas dominant-negative (DN) TLR5-expressing cells did not, supporting previous studies that show that flagellin acts via TLR5. These data suggest that TLR5 and the p38, ERK and JNK MAPK pathways all play an important role in the response of intestinal epithelial cells to H21 flagellin from STEC, and that the combined effects of Stx and flagellin on host intestinal epithelial cells may result in an augmented inflammatory response.

A role for flagellin in virulence was then investigated. BALB/c mice were orally inoculated with wild-type 98NK2 or 98NK2Δ*fliC*. Of the 16 mice challenged with the wild-type strain 98NK2, 9 (56%) died during the experiment (median survival time 7.6 days). However, only 3 of 16 mice (19%) challenged with 98NK2Δ*fliC* died (median survival time > 14 days). The difference in survival rate was statistically significant. No significant

differences in the level of intestinal colonisation of 98NK2 or 98NK2Δ*fliC* were observed. Thus, flagellin directly contributes to the virulence of STEC in streptomycin-treated mice. Since the streptomycin-treated mouse is a model for systemic Stx-mediated pathology, these results suggest that the pro-inflammatory effects of flagellin play an important role in the pathogenesis of Stx-mediated STEC disease *in vivo*.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Trisha Jayne Rogers, 14 September 2004.

Dedication

I would like to dedicate this thesis to my grandfather,

William Dudley Cottle,

who passed away during its creation.

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List of Abbreviations

Abbreviations that are acceptable to the Journal of Bacteriology are used in this thesis without definition in the text. Additional abbreviations are defined when first used in the text and are listed below.

$A_{260, 405, 570, 600}$	Absorbance at 260, 405, 570 or 600 nm, respectively
α-DIG	Anti-Digoxigenin-Fab fragment
A/E	Attaching and effacing
α-Hly	*E. coli* α-haemolysin
AIEC	Adherent-invasive *E. coli*
Amp	Ampicillin
AP	Alkaline phosphatase
AP-1	Activator protein-1
astA	EAggEC heat-stable enterotoxin (EAST1) gene
BCIP	5-Bromo-4-chloro-3-indoyl-phosphate (or X-phosphate)
Bfp	Bundle forming pili
BL-3	Bovine lymphoma cells
BSA	Bovine serum albumin
Caco-2	Human colonic epithelial cells
cat	Chloramphenicol acetyl transferase gene cassette
CDEC	Cell-detaching *E. coli*
Ces	Chaperone for *E. coli* secreted proteins
CFU	Colony-forming unit
CHO	Chinese hamster ovary
Cml	Chloramphenicol
C-terminus	Carboxy terminus
Cu	Plasmid-cured
CXC	Cysteine (C)-X-C motif
CXCR1/2	CXC receptor 1/2 (or IL-8RA/B)
DAEC	Diffusely-adherent *E. coli*
Daudi	Human lymphoma cells
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
DTT	Dithiothreitol
eae	*E. coli* attaching and effacing (intimin) gene
Eaf	EPEC adherence factor
EAggEC	Enteroaggregative *E. coli*
EAST1	EAggEC heat-stable enterotoxin 1
Efa1	EHEC factor for adherence
EHEC	Enterohaemorrhagic *E. coli*

EHEC-Hly	EHEC-enterohaemolysin (or Ehx)
Ehx	EHEC-enterohaemolysin (or EHEC-Hly)
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamine (E)-leucine (L)-arginine (R) motif
ENA-78	Epithelial-derived neutrophil activating peptide-78
EpeA	EHEC plasmid-encoded autotransporter
EPEC	Enteropathogenic *E. coli*
ERK	Extracellular regulated kinases
Esc	*E. coli* secretion system protein
Esp	*E. coli* secreted protein
EspI	*E. coli* secreted protease, island encoded
EspP	Extracellular serine protease, plasmid encoded
ETEC	Enterotoxigenic *E. coli* (ETEC)
Etp	EHEC type II secretion pathway protein
ExPEC	Extraintestinal pathogenic *E. coli*
F-actin	Filamentous actin
FCS	Foetal calf (bovine) serum
FRT	FLP recombinase recognition target site
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gb_3	Globotriaosylceramide (Galα[1→4]Galβ[1→4]Glc-ceramide)
Gb_4	Globotetraosylceramide (GalNAcβ[1→3]Galα[1→4]Galβ[1→4]-Glc-ceramide)
GCP-2	Granulocyte chemotactic protein-2
G-CSF	Granulocyte colony stimulating factor
GMVEC	Glomerular microvascular endothelial cells
Gro	Growth related oncogene
h	Hour(s)
HBSS	Hanks' balanced salt solution
HC	Haemorrhagic colitis
HCA-7	Human colon carcinoma cells
HCT-8	Human colonic epithelial cells
HeLa	Human cervical epithelial cells
Henle 407	Human intestinal epithelial cells
HEp-2	Human laryngeal epithelial cells
HepA3	Hepoxilin A3
HI	Heat-inactivated
HIMEC	Human intestinal microvascular endothelial cells
HRMEC	Human renal glomerular microvascular endothelial cells
HRP	Horseradish peroxidase
HRTEC	Human renal tubular epithelial cells
HT-29	Human colonic epithelial cells
HUS	Haemolytic uraemic syndrome
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular cell adhesion molecule type 1
Ig	Immunoglobulin
Iha	IrgA homologue adhesin
IκB-α	Inhibitory subunit of κB
IL	Interleukin
IL-8RA/B	Interleukin-8 receptor A/B (or CXCR1/2)
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside

IRAK	IL-1 receptor-associated kinase
IrgA	Iron regulated gene A
JNK	c-Jun N-terminal kinases
Kan	Kanamycin
KatP	Catalase-peroxidase, plasmid encoded
LB	Luria-Bertani
LCT	Large clostridial toxins
LD_{50}	50% lethal dose
LEE	Locus for enterocyte effacement
Ler	LEE-encoded regulator
lifA	Lymphostatin gene
LIM	Locus for improving microcolony formation
LIX	LPS-induced CXC chemokine
LPA	Locus of proteolysis activity
LPF	Long polar fimbriae
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen activated protein kinase
mCXCR2	Murine CXCR2 homologue
MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
MEK	Mitogen-activated protein kinase kinase
MEKK-1	Mitogen-activated protein kinase kinase kinase 1
MGSA	Melanocyte growth stimulating activity (or Gro-α)
MHA-T	Microangiopathic haemolytic anaemia and thrombocytopenia
mIL-8Rh	Murine IL-8 receptor homologue
MIP-2α	Macrophage inflammatory protein-2α (or Gro-β)
MIP-2β	Macrophage inflammatory protein-2β (or Gro-γ)
MNEC	Meningitis (neonatal)/sepsis-associated *E. coli*
MOPS	3-(N-morpholino)-propanesulphonic acid
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene 88
NBT	4-Nitroblue tetrazolium chloride
NF-κB	Nuclear factor-κB
Ni-NTA	Nickel-nitrilotriacetic acid
nt	Nucleotide(s)
NTEC	Necrotoxic *E. coli*
N-terminus	Amino terminus
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
Pap	P fimbrial proteins
PBS	Phosphate buffered saline
PD	PD98059
PEEC	Pathogen-elicited epithelial chemoattractant
Per	Plasmid-encoded regulator
Pil	Type IV pilus biosynthesis locus
PMN	Polymorphonuclear leucocyte
PMSF	Phenylmethylsulfonyl fluoride
pO113	Megaplasmid of STEC O113:H21 strain 98NK2
pO157	Megaplasmid of O157:H7 STEC
PRR	Pattern recognition receptor
pSFO157	Megaplasmid of sorbitol fermenting STEC O157:H$^-$

PssA	Protease secreted by STEC
^{R}	Resistant
Raji	Human lymphoma cells
RDEC	Rabbit diarrhoeagenic *E. coli*
REPEC	Rabbit enteropathogenic *E. coli*
RT-PCR	Reverse-transcription polymerase chain reaction
RTX	Repeats in toxin
^{S}	Sensitive
Saa	STEC autoagglutinating adhesin
SAPK	Stress activated protein kinases
SB	SB203580
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the means
Sep	Secretion of *E. coli* proteins
Sfp	Sorbitol-fermenting EHEC O157 fimbriae, plasmid-encoded
SLT	Shiga-like toxin
SLTEC	Shiga-like-toxin producing *E. coli*
SP	SP600125
STa	Heat-stable enterotoxin a
STEC	Shiga-toxigenic *E. coli* or Shiga toxin-producing *E. coli*
Str	Streptomycin
Stx	Shiga toxin
T84	Human colonic epithelial cells
TAI	Tellurite resistance- and adherence-conferring island
TAK-1	Transforming growth factor-β-activated kinase-1
TBE	Tris-borate-EDTA buffer
TCP	Toxin coregulated pilus
TE	Tris-EDTA buffer
TEER	Trans-epithelial electrical resistance
TEMED	N,N,N'N'-tetramethyl-ethylene-diamine
THP-1	Human monocytic cells
Tir	Translocated intimin receptor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
ToxB	Toxin B
Tra	Conjugal transfer region
TRAF6	TNF-receptor-associated factor 6
TTBS	Tween-Tris buffered saline
TTP	Thrombotic thrombocytopenic purpura
UPEC	Uropathogenic *E. coli*
VCAM-1	Vascular cell adhesion molecule type 1
Vero	African green monkey kidney epithelial cells
VT	Verocytotoxin
VTEC	Verocytotoxin-producing *E. coli*
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside
X-pho	5-Bromo-4-chloro-3-indoyl-phosphate (or BCIP)