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Involvement of intracellular signaling cascades in inflammatory responses in human intestinal epithelial cells following *Vibrio cholerae* infection

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ABSTRACT

Vibrio cholerae, the etiological agent of cholera, leads to the induction of host cell nuclear responses and the activation of proinflammatory cytokines in the cultured intestinal epithelial cells. However, the host cell signaling pathway leading to proinflammatory response is not explored. In this study, we demonstrated that *V. cholerae* infection on intestinal epithelial cells results in the activation of extracellular signal-regulated kinases1/2(ERK1/2) and p38 of the mitogen activated protein kinase (MAPK) family. *V. cholerae* induced intracellular pathways in Int407 cells leading to the activation of protein kinase A (PKA) and protein tyrosin kinase (PTK) in upstream of MAPK and nuclear factor-kappaB (NF-κB) pathway. Inhibitor study of Ca^{2+} and phospholipase-gamma (PLC-γ) pathway suggested the possible involvement of Ca^{2+} signaling in the *V. cholerae* pathogenesis. *V. cholerae* culture supernatants as also insertional mutants of *ctxA*, *toxR* and *toxT* genes modulate the activation of MAPK and NF-κB signaling pathways. MAPK and NF-κB signaling pathway activation were also modulated by adherence and motility of *V. cholerae*. Studies with inhibitor of NF-κB, MAPK, PTK, PKA, PKC, Ca^{2+} and PLC pathways showed differential cytokine secretion in Int407 following *V. cholerae* infection. Therefore *V. cholerae* mediated induction of nuclear responses through signal transduction pathway and subsequent activation of proinflammatory cytokines in Int407 modulated by *V. cholerae* secretory factors, virulence, adhesion/motility which might explain some of its reactogenic mechanisms.

1. Introduction

The etiologic agent of the cholera is a highly motile non-invasive Gram-negative organism *Vibrio cholerae*, which colonizes the small intestine and produces a potent enterotoxin called cholera toxin (CT)—a major virulence determinant that is primarily responsible for the diarrheal syndrome (Kaper et al., 1995). At the molecular level, the pathogenesis of cholera is a multifactorial process and involves several genes encoding virulence factors that aid the pathogen in its colonization, coordinated expression of virulence factors, and toxin action. *V. cholerae* ToxR, the master regulator controlling the expression of several virulence factors, acts synergistically with TcpP on the ToxT promoter which then activates the genes encoding CT, TCP and accessory colonization factors (Childers and Klose, 2007). Several studies have established association of motility and adhesion with virulence in *V. cholerae* (Gardel and Mekalanos, 1996; Postnova et al., 1996).

A characteristic feature of *V. cholerae* induced pathology is the induction of an early inflammatory response in the intestinal epithelial cells which could be evidenced by the infiltration of polymorphonuclear leukocytes (PMN) in the lamina propria (Qadri et al., 2002), lymphocytes (Pastore et al., 1976), mononuclear cells (Gangarosa et al., 1960), as well as elevated level of LTB_4 and myeloperoxidase (Silva et al., 1996). The major enterotoxin CT has been demonstrated to strongly promote the production of IL-6 by rat IEC-6 epithelial cells (McGee et al., 1993). In the search for effective vaccines, adaptive immunity against *V. cholerae* and CT has been investigated intensely (Flach et al., 2005). Much less is known about the innate defense mechanisms during cholera that may be involved in the early defense against the infection and also in initiation of the adaptive immune response. Cholera has long been considered a classic paradigm of non-inflammatory toxigenic diarrhoea (Farthing, 1997) until observations showed that *V. cholerae* infection induces an increase in inflammatory components (Saha et al., 2000). Fullner et al. (2002) showed evidence of inflammation including infiltration of PMN, tissue damage, localized release of tumor necrosis factor (TNF-α), high serum titers of interlukin (IL)-6 and the neutrophil chemoattractant protein macropahge inhibitory protein (MIP)-2 by accessory toxins of *V. cholerae* in a mouse pulmonary model of infection. A few recent reports have also documented the release of IL-8 upon *V. cholerae* infection in the intestinal epithelial cells (Rodriguez et al., 2001; Sarkar and Chaudhuri, 2004; Zhou et al., 2004). Besides IL-8, our recent

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reports have shown the induction of proinflammatory cytokines IL-1α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α and epithelial neutrophil-activating peptide-78 (ENA-78) with concomitant reduction of anti-inflammatory cytokines transforming growth factor-beta (TGF-β) in intestinal epithelial cells upon *V. cholerae* infection (Bandyopadhaya et al., 2007b). Recently Holmgren et al. established the involvement of innate defense molecules against *V. cholerae* infection (Flach et al., 2007). *V. cholerae* activates the transcription factor NF-κB and subsequently upregulate the transcription of genes encoding proinflammatory cytokines/chemokines in intestinal epithelial cells (Bandyopadhaya et al., 2007a,b). Indeed, the adherence and motility of *V. cholerae* induces the translocation of NF-κB (Bandyopadhaya et al., 2007a) and the transcription of target genes in Int407 following Toll-like receptor (TLR) engagement (Bandyopadhaya et al., 2008) as well as activation of IL-1β, IL-6 and TNF-α was attenuated in TLR4$^{-/-}$ mice (Haines et al., 2005). The extracellular signal-regulated kinases (ERK1/2 or p44/42 mitogen-activated protein kinases (MAPK)), the c-Jun NH_2-terminal kinases/stress activated protein kinase (SAPK/JNKs) and p38 MAPK are extremely conserved signaling components involved in the response to stress and immunity also in lower organisms as well as macrophages and neutrophil activation (Caffrey et al., 1999) and recently we have shown that *V. cholerae* flagellin could activate ERK1/2 and p38 MAPK in intestinal epithelial cells (Bandyopadhaya et al., 2008). However, the signaling cascade as well as the specific *V. cholerae* components involved in the induction and regulatory mechanisms of mucosal inflammatory responses to infection by *V. cholerae* are still largely unknown.

Therefore we attempted to investigate the mechanisms by which *V. cholerae* induces nuclear responses and produce proinflammatory cytokines in human intestinal epithelial cells. We could demonstrate that activation of MAPK and NF-κB mediated signaling pathways in *V. cholerae* infected intestinal epithelial cells required viable pathogen which results in the activation of the transcription factors. We speculate these transcription factors mediate the production of proinflammatory cytokines which may be important in inducing *V. cholerae* induced pathology. By inhibitor study, we were able to identify the upstream protein kinases of the MAPK and NF-κB pathway involved in the inflammatory response in human intestinal epithelial cells following *V. cholerae* infection. This is, to our knowledge, the first report providing an initial insight into the events to determine whether the host intracellular signaling pathways are modulated by secreted factors, cell surface components, virulence or the adhesion process itself and further delineates the role of signaling pathways in the *V. cholerae* mediated cytokine induction.

2. Experimental procedures

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All *V. cholerae* O395 and *Escherichia coli* strains were maintained at −70 °C in Luria–Bertani (LB) medium containing 20% (v/v) glycerol. *E. coli* and *V. cholerae* cells were grown in LB medium. Streptomycin and ampicillin concentration were 1 mg/ml and 15 μg/ml respectively for *V. cholerae* wherever appropriate. *V. cholerae* gene-specific insertional mutant O395Y3N was constructed as described previously (Sarkar et al., 2005).

2.2. Construction of V. cholerae mutant strains

Internal fragments of the gene of interest (*cheY-3*) *V. cholerae* O395 were amplified from genomic DNA using the oligonucleotide pairs cheY-3F5′-CTTCGAGATCTGGGGTTCAA-3′ and *cheY*-3R 5′-GGCAGGTGCTTCAGTTCTTC-3′. The fragments were then cloned into a T-vector-pTZ57R/T of InsT/Aclone™ PCR Product Cloning Kit (MBI-Fermentus, Hanover, MD, USA) to obtain the recombinant plasmids, which were then restriction digested with appropriate enzymes and the inserts cloned into pGP704 to get the respective recombinant plasmid. These recombinant plasmids were then transformed into a λ pir lysogen of *E. coli* SM10 (Miller and Mekalanos, 1988). Ampicillin-resistant transformants containing recombinant plasmids were selected and were then conjugally transferred from *E. coli* SM10 to *V. cholerae* O395 (Sm^r). Transconjugants resistant to both ampicillin and streptomycin were selected. The mutant was verified either by PCR or Southern blotting (data not shown).

Table 1
Bacterial strains used in this study.

Strains	Relevant genotype or phenotype	Source/reference
Vibrio cholerae		
O395	O1 serotype Ogawa, biotype classical, streptomycin resistant, CT+	Laboratory collection
O395Y3N	O395 insertion *cheY-3* gene	Present study
O395Y4N/O395YN	O395 insertion in *che*Y-4 gene	Sarkar et al. (2005)
O395FLAN	O395 insertion in *flaA* gene	Sarkar et al. (2005)
O395OMPUN	O395 insertion in *ompU* gene	Do
O395TOXTN	O395 insertion in *toxT* gene	Do
O395TOXRN	O395 insertion in *toxR* gene	Do
O395CTXAN	O395 insertion in *ctxA* gene	Do
Escherichia coli		
DH5α	F$^-$ f80d/*lacZ* DM15 D(*lacZYA arg* F) U169 *rec* A1 *end* A1 *hsd*R17(r_k,m_k^-) *sup*E441- thi-1 *gyr*A *rel*A1	Bethesda research laboratories USA
SM10	*thi-1 thr,leu,tonA,LacY,SupE,recA*::RP4-2-Tc::Mu,λ-pir	Miller and Mekalanos (1988)
Plasmids		
pTAdv	3.9 kb, Km^r,Amp^r, with 3′T overhang	Clontech
pTZ57R/T	2.886 kb, Amp^r, with 3′T overhang	MBI Fermentas
pGP704	Amp^r *oriR6K mob RP4* MCS of M13 tg 131	Miller and Mekalanos (1988)

2.3. Cell culture, infection and stimulation

Human intestinal epithelial cell line Int407 from National Center for Cell Sciences, Pune, India, was grown and maintained in minimal essential medium (MEM, GIBCO-BRL, Gaithersburg, MD, USA) while T84 cell line (a gift from Dr. S Visyeswariah, IISc, Bangalore) was grown in Dulbecco Modified Eagle medium and Ham's F-12 medium (DMEM/F-12, GIBCO-BRL) at pH 7.4, supplemented with 10% fetal bovine serum (GIBCO-BRL) containing penicillin/streptomycin and gentamycin in the presence of 5% CO_2 at 37 °C. Cells were seeded in T-75 tissue culture flasks (Falcon, USA). Bacteria from overnight culture suspended in fresh MEM medium without antibiotic were added to each flask at an MOI ∼ 100. The infected and non-infected T-75 flasks were incubated for different time periods at 37 °C under 5% CO_2.

For stimulation by supernatant, bacterial culture supernatants (equivalent to an MOI of 100 bacteria/cell and 5 × 100 MOI) were centrifuged; filter (0.2 μm) sterilized, added to Int407 cells and incubated at 37 °C under 5% CO_2 for 3.5 h. In some experiments, supernatant was heat-treated (30 min, 95 °C), trypsin treated (2 h, 40 μg/ml, Invitrogen, Life Technologies, USA) or proteinaseK treated (2 h, 200 μg/ml, Invitrogen) before incubation. Stimulation with commercial CT (Sigma–Aldrich, USA) was done at concentrations of 4.5 ng/ml (equivalent to that obtained in the culture supernatant