Cell cycle arrest and apoptosis induced by enteroaggregative 
Escherichia coli in cultured human intestinal epithelial cells

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Abstract

Purpose. Enteroaggregative Escherichia coli (EAEC) is an emerging enteric pathogen causing diarrhoeal diseases in multiple epidemiological and clinical settings. However, understanding of the pathogenesis of the disease caused by this organism is still suboptimal. Studies have indicated that enteric bacteria induced cell cycle arrest and apoptosis in host intestinal epithelial cells might play a vital role in the pathogenesis caused by these organisms. In this study an attempt was made to assess EAEC-induced apoptosis and cell cycle modulation in human intestinal epithelial cell lines.

Methodology. INT-407 and HCT-15 cells were infected with EAEC-T8 (clinical isolate) as well as plasmid cured variant of EAEC-T8 (EAEC-pT8). Propidium iodide staining was done to select the time of infection and the incubation period of the infected culture. Apoptosis was further assessed in EAEC infected both the cell lines by annexin-V-FLUOS & propidium iodide, cell death detection ELISA, DNA strand breaks and microscopic analysis. Further, the DNA content of the EAEC-infected cells at different phases of cell cycle was also monitored.

Results. We have found that EAEC could induce apoptosis in human small intestinal as well as colonic epithelial cell lines, which was assessed by the expression of phosphatidylserine on host cell surface, internucleosomal cleavage of host cell DNA and microscopic analysis of the characteristic apoptotic features of these cells. EAEC was also found to arrest cells at S phase and G2-M phase of the cell cycle.

Conclusions. EAEC-T8 could induce maximum apoptosis and cell cycle modulation in both small intestinal and colonic epithelial cells. Further, we have observed that the plasmid of this organism had maximum contribution to these processes. The outcome of this study has undoubtedly led to a better understanding of the basic mechanism of pathogenesis caused by EAEC.

INTRODUCTION

Enteroaggregative Escherichia coli (EAEC) is being recognized as an important etiological agent of endemic and epidemic diarrhoea [1–3]. Although the mechanism of EAEC infection is not completely understood, a three-stage model has been suggested based on various studies [4]. It involves initial mucosal aggregative adherence conferred by several fimbrial and afimbrial adhesins at stage I; biofilm formation at enterocyte surface in stages II and III involves an inflammatory response and mucosal toxicity.

Understanding of EAEC pathogenesis is challenging due to heterogeneity among the strains [5] and thus expression of various virulence factors. These include adhesins, toxins, AggR (a master regulator to control the expression of the adhesins), dispersin and an anti-aggregation protein transporter. The genes of most of these virulence factors are present on a megaplasmid (60–65 MDa), associated with the aggregative phenotype of the organism, thereby implicating the importance of the plasmid in the disease process [6, 7].

The enteric bacteria may develop specialized strategies to disrupt key eukaryotic cell functions including cell cycle alteration in order to establish persistent colonization [8]. Studies have indicated that enteric pathogens could induce apoptosis in host cells, which may allow bacteria to inhibit cell turnover, ensure their survival, evade immune cells and gain nutrients [9–12]. The most obvious scenario where killing of the host cells would be beneficial to the pathogens is the induction of apoptosis in professional phagocytes like macrophages [13]. Except enterotoxigenic
E. coli strain, all other six types of diarrheagenic E. coli including EAEC were shown to induce apoptosis in murine macrophage cell lines [14, 15]. It has been reported that enteric pathogens could induce apoptosis, a programmed cell death in host epithelial cells, which might play a vital role in the pathogenesis caused by these organisms [8, 16]. Recently, Serapio-Palacios et al. [17] have demonstrated that enteropathogenic E. coli (EPEC) could induce epithelial cell death through caspase and calpain activation [17]. However, Wong et al. [18] documented that some strains of EPEC could block both the process of inflammation and programmed cell death by injecting multiple virulence effector proteins directly into the infected cells [18]. EAEC was shown to induce intestinal mucosal damage in human and animal studies [19–21]. However, the nature of EAEC-induced intestinal epithelial cell death has not been reported until now. Thus, in the present study, an attempt has been made to assess cell cycle alteration and apoptosis in EAEC-infected human intestinal epithelial cell lines.

METHODS

Bacterial strains and cell lines
EAEC-T8 [a clinical isolate harbouring aggregative adherence fimbriae (AAF) II] was obtained from the National Institute of Cholera and Enteric Diseases (Kolkata, India). The strain was checked for the presence of mega plasmid, EAEC-specific PCR product [22] and the characteristic aggregative adherence pattern to the HEp-2 cells [23]. The plasmid cured strain (EAEC-pT8) was derived from EAEC-T8 strain by repeated passage in increasing concentrations of acriflavine [24] and confirmed by the absence of plasmid as well as the EAEC-specific PCR product.

INT-407 (an epithelial cell line derived from human embryo small intestine) and HCT-15 (a colon carcinoma cell line) procured from the National Centre for Cell Science (Pune, India) were used in this study. These cell lines were grown in minimum essential media and Roswell Park Memorial Institute media (Gibco-BRL; Invitrogen), respectively, at 37 °C in a humidified CO₂ incubator (Eppendorf) maintaining 5% CO₂–95% air.

Infection protocol
INT-407 and HCT-15 cells were seeded into 24-well cell culture plates (5×10⁴ cells well⁻¹) separately. After 2 h starvation in serum and antibiotic-free media, cells were infected with the overnight grown culture of EAEC-T8 and EAEC-pT8 (1:100 multiplicity of infection) separately for 3 h at 37 °C for maximum adherence [25]. After 3 h of infection, bacteria were washed out with PBS, and the cells were incubated further for 2 h with fresh media containing gentamicin (50 µg ml⁻¹) to kill the remaining bacteria.

Propidium iodide staining
INT-407 cells were infected with EAEC-T8 and EAEC-pT8 separately under the same conditions (as mentioned in the infection protocol) and further cultured in serum-free media for 12 to 36 h. The cells were detached by trypsinization, washed and fixed with 70% chilled ethanol (1 h, 4 °C), and propidium iodide (PI) staining was performed [26]. The cells were acquired by a flow cytometer (FACS Canto II; Becton Dickinson) and analysed using ‘FACS Diva’ software (Becton Dickinson). The mean fluorescence intensity of the labelled cells in hypodiploid region was directly correlated to the percentage of dead cells [27]. In another set of experiments, EAEC-infected HCT-15 cells were cultured for required period (as obtained from previous set of
experiments), and the cells were processed as described earlier. Cells cultured in absence of bacteria served as negative controls in all sets of experiments.

**Evaluation of apoptosis**

EAEC-induced apoptosis in both the cell lines was assessed by annexin-V-FLUOS/PI staining, cell death detection ELISA (CDD-ELISA), measurement of DNA strand breaks and microscopic analysis (fluorescence microscopy and transmission electron microscopy). Uninfected cells cultured under the same conditions were taken as controls in all experiments.

### Annexin-V-FLUOS and PI staining

For annexin-V-FLUOS and PI staining, the cells were suspended in 10 mM HEPES (pH 7.4)/0.14 M NaCl/2.5 mM CaCl$_2$ containing annexin-V-FLUOS (2 µg ml$^{-1}$) and PI (2.5 µg ml$^{-1}$), incubated (15 min) in dark and analysed on FACS Canto II [28].

### CDD-ELISA

For this assay, the cells were lysed and centrifuged (9500 g, 15 min), and the supernatant was subjected to photometric quantitative sandwich-enzyme immunoassay using CDD ELISA kit (Roche) according to the manufacturers’ instructions.

**Fig. 2.** (a) Representative dot plots indicating the gated cells and the FACS analysis for the assessment of EAEC-induced apoptosis in INT-407 and HCT-15 cells by annexin-V-FLUOS and PI staining after 24 h incubation with different strains of EAEC. (b) Bar diagrams indicate the apoptotic cells (%) in INT-407 and HCT-15 cells under different conditions. Each bar is the mean±SD of values obtained from three independent experiments of each set, performed in triplicates. One-way ANOVA; ***P<0.001 (cells+EAEC-T8/EAEC-pT8 vs cells only); **P<0.01 (cells+EAEC-T8 vs cells+EAEC-pT8).
instructions. The extent of apoptosis in the cells was expressed as apoptotic index [29].

**Measurement of DNA strand breaks**

DNA strand breaks were measured in the cells by the terminal deoxynucleotidyl transferase (TdT)–deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) assay using APO-Direct kit (Becton Dickinson Pharmingen). Briefly, the cells were washed, fixed and stained with FITC-labelled dUTP in presence of TdT according to the manufacturers’ instructions. The cells were counterstained with PI and analysed by flow cytometer using Cell Quest software [28].

**Microscopic analysis**

For fluorescence microscopy, INT-407 cells were fixed and incubated (1 h, 37 °C) with the staining solution containing reaction buffer, TdT enzyme and FITC-dUTP. After washing with the rinse buffer, the cells were incubated with PI/RNase staining buffer. All the buffers were provided in the Apo-Direct kit. Furthermore, 10 µl of cell suspension from each set of the experiments was applied on a slide, and the cells were assessed by fluorescence microscopy.

For transmission electron microscopy, the cells (HCT-15) were processed according to the method of Dytoc et al. [30] and examined for the presence of apoptotic cells under transmission electron microscope (H-7500; Hitachi) at an accelerating voltage of 100 kV [30].

**Cell cycle analysis**

The cells were washed, fixed and stained with PI (as mentioned earlier). The cell cycle analysis was performed [29] by FACS Calibur cytofluorometer (Becton Dickinson) for INT-407 cells using ‘Cell Quest’ software (Becton Dickinson) and FACS Canto II (Becton Dickinson) for HCT-15 cells using ‘FACS Diva’ software.

**Statistical analysis**

The data were analysed using the Student’s t-tests and one-way ANOVA; P<0.05 was taken as statistically significant.

**RESULTS**

The present study was carried out to assess cell cycle arrest and apoptosis induced by EAEC in the cultured human intestinal epithelial cell lines of two different origins, INT-407 (an epithelial cell line derived from human embryo small intestine) and HCT-15 (human colon carcinoma cell line). Initially, PI staining was done to evaluate the extent of cell death in EAEC-T8 (a clinical isolate harbouring AAF II) and EAEC-pT8 (a plasmid cured variant of EAEC-T8) infected (3 h) INT-407 cells at different periods (12–36 h), as shown in Fig. 1 (a). PI is a fluorescent dye that intercalates into the DNA strands, and the extent of PI incorporation in the cells is monitored by flow cytometry [26]. A significantly higher percentage of dead cells were found at each period in the case of EAEC-infected cells as compared to the uninfected cells. Since EAEC-infected INT-407 cells cultured for 24 h revealed sufficient extent of cell death, this period was used for the subsequent experiments for both the cell lines. The flow cytometric analysis of the PI-stained, EAEC-infected and uninfected HCT-15 cells (cultured for 24 h) are shown in Fig. 1 (b). An increase (P<0.001) in the percentage of dead cells was noted in the case of EAEC-infected cells as compared to the uninfected cells. Since EAEC-infected INT-407 cells cultured for 24 h revealed sufficient extent of cell death, this period was used for the subsequent experiments for both the cell lines. The flow cytometric analysis of the PI-stained, EAEC-infected and uninfected HCT-15 cells (cultured for 24 h) are shown in Fig. 1 (b). An increase (P<0.001) in the percentage of dead cells was noted in the case of EAEC-infected cells as compared to the uninfected cells, and maximum percentage of dead cells (44.9±2.46 %) was found with EAEC-T8 infection.

![Graph showing apoptosis index in INT-407 and HCT-15 cells](image)

**Fig. 3.** Evaluation of EAEC-induced apoptosis (in view of apoptotic index) in INT-407 cells and HCT-15 cells by CDD-ELISA after 24 h incubation with different strains of EAEC. Each bar is the mean±SD of values obtained from the three independent experiments of each set done in duplicates.
Phospholipids are known to be translocated from inner to the outer leaflet of plasma membrane during early stage of apoptosis [31], and the accumulation of phosphatidylserine (PS) on the outer leaflet of the apoptotic cell membrane is monitored using fluorochrome-conjugated annexin-V, a calcium-dependent PS-binding protein. PI cannot stain live or early apoptotic cells owing to an intact plasma membrane. However, in late apoptotic cells because of the loss of membrane integrity, PI can pass through the membranes and intercalate into nucleic acid [32, 33]. Thus, in this study, the cells were stained with annexin-V-FLUOS and PI to assess the percentage of early and late apoptotic cells. Fig. 2 reveals the representative dot plots (Fig. 2a) and bar diagrams (Fig. 2b) of FACS analysis of the cells stained with annexin-V-FLUOS and PI. INT-407 and HCT-15 cells infected with EAEC-T8 revealed 16±1.1 % and 12.8±1.4 % early apoptotic cells (annexin-V positive) and 29.5±1 % and 19.4±0.97 % late apoptotic cells (annexin-V and PI positive), respectively. The uninfected INT-407 and HCT-15 cells showed only 4.1±0.7 % and 0.7±0.09 % early apoptotic cells and 6.4±1 % and 7.2±1.18 % late apoptotic cells, respectively. Furthermore, the number of early apoptotic cells in the case of both the cell lines infected with EAEC-pT8 was found to be comparable to that of respective uninfected cells. However, INT-407 cells infected with EAEC-pT8 revealed ~8 % increase in the number of late apoptotic cells, while no such increase was noted in the case of EAEC-pT8-infected HCT-15 cells.
Since DNA fragmentation is the hallmark of apoptosis [34], the fate of the genomic DNA of the EAEC-infected and uninfected cells was assessed by CDD ELISA and TUNEL assay. CDD-ELISA is a photometric quantitative, sandwich-enzyme immunoassay, which measures the apoptotic index in view of mono- and oligo-nucleosomes present in the cytoplasmic fraction of the apoptotic cells, using mouse monoclonal antibodies against DNA and histones. The apoptotic index of both the cell lines infected with EAEC-T8 was found to be more as compared to the EAEC-pT8-infected respective cells (Fig. 3).

In the TUNEL assay, TdT-mediated incorporation of FITC-labelled dUTP at the 3' hydroxyl ends of the degraded dsDNA and ssDNA in the apoptotic cells is assessed by flow cytometry. Fig. 4(a) shows the representative histograms of FACS analysis of both the cell lines cultured under different conditions. A significantly higher percentage of FITC-dUTP incorporated cells was detected in the hypodiploid region (M2 region) of the histograms in the case of EAEC-infected cells as compared to the respective uninfected cells, and maximum number of apoptotic cells was detected in the case of EAEC-T8 that infected both the cell lines, as was also reflected in the bar diagrams (Fig. 4b).

Fig. 5(a) reveals the representative photomicrographs of the uninfected and EAEC-infected INT-407 cells, stained with FITC-labelled dUTP in presence of TdT enzyme as well as PI. Cells were examined with a rhodamine-FITC set filter under fluorescent microscope. Normal cellular morphology was noted in the case of uninfected cells. Both FITC-dUTP and PI were found to be incorporated in the fragmented DNA in EAEC-infected cells, as characterized by the condensed chromatin. Moreover, such double staining of the cells clearly indicates that the extent of apoptosis is more in EAEC-T8-infected cells in comparison to the EAEC-pT8-infected cells. Fig. 5(b) depicts transmission electron microscopic analysis of HCT-15 cells cultured under different conditions. No morphological alterations were observed in the case of the uninfected cells, as characterized by the well-preserved plasma membrane and nucleus containing nucleolus and euchromatin. EAEC-infected cells showed the characteristic features of apoptotic cell death including chromatin condensation, cytoplasmic vacuolation and apoptotic body formation as indicated by different arrows.

Since cell cycle alteration and apoptosis are directly linked [35, 36], cell cycle arrest was also assessed in EAEC that infected both the cell lines. The representative histograms of cell cycle analysis of EAEC that infected both the cell lines are shown in Fig. 6(a). Redistribution of the cells was noted in different phases of the cell cycle, as also reflected in the bar diagrams (Fig. 6b). The percentage of cells was significantly increased in the G2-M phase in the case of both the cell lines infected with EAEC as compared to respective uninfected cells, and maximum increase was found with EAEC-T8 infection. The S phase population in the case of both the cell lines infected with EAEC-pT8 was comparable to that of respective control cells, while it was found to be increased significantly in the case of EAEC-T8-infected cells.

**DISCUSSION**

Bacterial adherence to target tissue is an essential event for any infection to occur. After attachment, the pathogens use their full genetic potential to secure a specific niche within the host that permits its replication and survival [10, 11, 37]. Host cell death may be one of the strategies developed by micro-organisms to ensure their survival and invasion of target tissue [9, 38]. Enteropathogens including diarrheagenic *E. coli* have been identified as mediators of programmed cell death in professional phagocytes [13–15].

Apoptosis of intestinal epithelial cells has been defined as a delayed cellular response to bacterial infection as compared to macrophage apoptosis [39]. It can either be beneficial or detrimental to the pathogen. Apoptosis induced by *Pseudomonas aeruginosa* was shown to favour host defence [40]. However, *Salmonella* was found to utilize apoptosis as a virulence strategy [41]. It was reported that YpkA, the *Yersinia* effector protein, could induce apoptosis in a number of cell types [42]. EPEC-mediated apoptosis of human intestinal epithelial cell lines was found to be contact dependent. Further, it was reported that the bundle-forming pilus (BFP) and autotransporter proteins EspC, EspF and Map of EPEC might be the major contributors to this process [12, 16, 17, 43–45]. It was demonstrated that enterohaemorrhagic *E. coli*-induced apoptosis in HEp-2 cells could augment the level of outer leaflet phosphatidylethanolamine receptor and thus offer an advantage for bacterial attachment [46]. Studies have shown that EAEC-042 could alter the morphology of T84 cells (human colonic adenocarcinoma cell line), ultimately leading to cell death, which was further substantiated by *in vitro* organ culture model [47, 48]. Moreover, different toxins of this
organism were shown to induce cytotoxic effects in intestinal mucosa [19, 20]. However, the specific features of programmed cell death were not indicated in these reports.

In the present study, we have provided evidence that EAEC infection of cultured human intestinal epithelial cells resulted in cell cycle alteration and apoptosis. EAEC-T8, an AAF-II harbouring strain, was used to infect INT-407 (human embryo small intestinal epithelial cell line) and HCT-15 (human colon carcinoma cell line) cells. These cell lines were chosen based on their significant relevance to natural niche of EAEC infection [45]. The plasmid of EAEC is known to carry most of the virulence genes [49]; thus, the plasmid-cured strain was also used to assess the contribution of the plasmid to EAEC-induced cell cycle modulation and programmed cell death. Earlier, we have reported that EAEC-T8 revealed stacked brick pattern of adherence to INT-407 cells at 3 h, while the plasmid-cured strains could not reveal such type of adherence pattern [25, 50]. In the present study, similar pattern of adherence of EAEC-T8 was also noted in the case of HCT-15 cells. Thus, both the cell lines were infected for 3 h with EAEC.

Fig. 6. (a) Representative dot plots indicating the gated cells and histograms of FACS analysis of EAEC-induced altered distribution of cells in various phases of the cell cycle in INT-407 cells and HCT-15 cells, as assessed by PI staining after 24 h incubation with different strains of EAEC. (b) Bar diagrams indicating the cell population (%) in different phases of the cell cycle. Each bar is the mean±SD of values obtained from three independent experiments of each set, performed triplicates. One-way ANOVA: **P<0.01 (INT-407 cells) and ***P<0.001 (HCT-15 cells) (cells+EAEC-T8 vs cells only in S phase); **P<0.01 (INT-407 cells) and ***P<0.001 (HCT-15 cells) (cells+EAEC-T8 vs cells +EAEC-pT8 in S phase); **P<0.01 (cells+EAEC-T8 vs cells only in G2-M phase); *P<0.05 (cells+EAEC-pT8 vs cells only in G2-M phase).
Initially, the cell death in EAEC that infected both the cell lines (cultured for 24 h) was assessed by PI staining, and the maximum extent of cell death was found in the case of EAEC-T8-infected cells. Since PI staining cannot distinguish apoptotic cells from necrotic cells, different methods were used to assess apoptosis. Annexin-V-FLOUS along with PI was used to determine early and late apoptotic cells in view of the difference in plasma membrane integrity and permeability [51]. EAEC-T8 that infected both the cell lines revealed the maximum number of early and late apoptotic cells, while EAEC-pT8-induced increase in the number of late apoptotic cells was noted only in the case of INT-407 cells. The difference in the virulence potential of wild-type and plasmid-cured EAEC-T8 strains might be responsible for the variation in the extent of apoptosis induced by these strains. Moreover, the cell lines (INT-407 and HCT-15) used in the experiments are from two different origins of the intestine, which may be another cause for their differential response. Furthermore, the reduction in apoptosis in the case of EAEC-pT8-infected cells as compared to EAEC-T8-infected cells clearly revealed the importance of plasmid in this process. Similar results were obtained in the case of EPEC, wherein the epithelial cells (HEp-2, HeLa and Caco-2) infected with the plasmid-encoded BFP* EPEC strains resulted in higher number of cells having PS exposed on the outer leaflet of the plasma membrane as compared to the BFP* EPEC-infected cells [43].

EAEC-induced apoptosis in intestinal epithelial cells was further confirmed by DNA fragmentation in EAEC-infected cells, as was assessed by CDD-ELISA and TUNEL assay. In both these assays, maximum extent of apoptosis was noted in the case of EAEC-T8-infected cells, which was well correlated to our previous observations. Our findings were further substantiated by the characteristic apoptotic morphology in EAEC-infected INT-407 cells, as was assessed by fluorescence microscopy. Jones et al. [52] also demonstrated such alteration in Helicobacter pylori-infected human gastric epithelial cell line [52]. We have observed distinct ultrastructural apoptotic features in EAEC-infected HCT-15 cells by transmission electron microscopy, which are in accordance with the reports of Abul-Milh et al. [43].

Bacterial infection was shown to induce cell cycle modulation, which might have an important contribution to the pathogenesis caused by these organisms [53]. Studies have supported a direct link between cell cycle alteration and apoptosis [35, 36]. Samba-Louaka et al. [12] observed that cycle inhibiting factor, a plasmid-encoded protein of EPEC could alter the distribution of cells in G1-S and G2-M phases in IEC-6 cells [12]. Furthermore, cycle inhibiting factor was found to induce apoptosis in these cells [54]. In another study, cytolethal distending toxin Campylobacter jejuni was also shown to contribute to the pathogenesis of HeLa cells by arresting cells at G2-M phase through accumulation of inactive, tyrosine-phosphorylated form of cyclin-dependent kinase and apoptosis [8]. Our observations regarding arrest of maximum number of cells at the S and G2-M phases of the cell cycle in the case of EAEC-T8 that infected both the intestinal epithelial cell lines are in good agreement with the previous reports.

To our knowledge, this is the first report regarding EAEC-induced cell cycle modulation and apoptosis in cultured human intestinal epithelial cells, to which plasmid-borne factors of EAEC might have a major contribution. We hypothesize that the aggregative adherence of this enteric pathogen to the intestinal epithelium might initiate a signalling mechanism that may be responsible for cell cycle arrest and induction of apoptosis in epithelial cells. It is possible that the plasmid-encoded factors of wild-type EAEC-T8 might induce PS exposure to the outer leaflet of the plasma membrane of intestinal epithelial cells leading to its early apoptosis, while both plasmid- and chromosome-encoded factors might be involved for late apoptosis following DNA fragmentation in these cells. These might result in inhibition of cell turnover in the intestinal epithelium and thus invasion of EAEC to deeper mucosa leading to the pathogenesis.


