RESEARCH ARTICLE

Human intestinal epithelial cell-derived molecule(s) increase enterohemorrhagic *Escherichia coli* virulence

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Abstract

To better understand the role of host cell-derived molecules on enterohemorrhagic *Escherichia coli* (EHEC) infection, we studied EHEC virulence gene expression when exposed to cell-free spent (conditioned) medium (CM) from HCT-8 intestinal epithelial cells. Exposure to HCT-8 CM for 1 h and 3 h increased the expression of 32 of 41 EHEC locus of enterocyte effacement (LEE) virulence genes compared with fresh medium (FM). Expression of the Shiga toxin 1 (*stx1B*) gene was up-regulated at 1 h of exposure. Seventeen genes encoded by prophage 933W, including those for Stx2, were also up-regulated at both time-points. The increase in 933W prophage expression was mirrored by a 2.7-fold increase in phage titers. Consistent with the increase in virulence gene expression, we observed a fivefold increase in EHEC attachment to epithelial cells when exposed to CM. The increase in EHEC attachment was abolished when CM was heated to 95 °C or treated with proteinase K to degrade the proteins. The host cell-derived molecule(s) were larger than 3 kDa, which suggests that the molecule(s) that increase EHEC virulence and attachment are protein-based.

Introduction

The human gastrointestinal (GI) tract is colonized by approximately 10^{14} commensal bacteria consisting of hundreds of bacterial species (Berg, 1996). The introduction of pathogenic bacteria such as enterohemorrhagic *Escherichia coli* (EHEC) into the human GI tract results in colonization of host cells and leads to the onset of bloody diarrhea and hemolytic uremic syndrome. EHEC infections pose a serious clinical problem as they are often associated with complications and permanent disabilities, including neurological defects, hypertension, and renal insufficiency (Frankel *et al.*, 1998). Understanding the mechanisms underlying EHEC pathogenicity could lead to better approaches for attenuating the deleterious consequences associated with GI tract infections.

Enterohemorrhagic *Escherichia coli* virulence is significantly affected by host molecules present in the GI tract microenvironment. Sperandio *et al.* (2003) demonstrated that the eukaryotic hormone epinephrine (epi) restores the type 3 secretion system (T3SS) of EHEC in a EHEC *luxS* mutant, and suggested that signaling occurs between epithelial cells and EHEC. Clarke *et al.* (2006) also reported that the QseC sensor kinase is a bacterial adrenergic receptor for epi/norepinephrine (NE), and the EHEC *qseC* mutant has attenuated virulence in a rabbit model. Previous work from our lab has shown that phenotypes that affect EHEC infection – motility, chemotaxis, biofilm formation, gene expression, and attachment to epithelial cells – are increased in EHEC exposed to epi and NE (Bansal *et al.*, 2007). Similar observations have been made by Vlisidou *et al.* (2004), who showed that NE increases adhesion of EHEC to cecal mucosa, colonic mucosa, and the ileum. These studies suggest that EHEC virulence and infectivity are enhanced by host-derived molecules present in the GI tract.

Apart from hormones, other factors produced by eukaryotic cells that promote or mitigate pathogen infection have also been identified. Mrsny *et al.* (2004) identified that hepoxilin A_3 (HXA_3) recruits polymorphonuclear leukocytes to the site of infection. They also discovered (McCormick, 2007) that the *Salmonella typhimurium* T3SS protein, SipA, promotes a lipid signal transduction cascade that leads to production of HXA_3, and that lung epithelial

cells produce HXA_3 in response to *Pseudomonas aeruginosa* infection. Similarly, Ju *et al.* (2006) discovered a novel 40-kDa secreted protein, named lipopolysaccharide recognition protein (LRP), purified from the plasma of larvae of the large beetle *Holotrichia diomphalia*, helps in the clearance of *E. coli in vivo* but not *Staphylococcus aureus* or *Candida albicans*. Jandu *et al.* (2009) reported that, of the EHEC LEE virulence genes, only *escRSTU* is up-regulated in the presence of HEp-2 human epithelial cells. However, that study did not distinguish whether the recognition of secreted host factors (i.e. in the culture medium) or colonization and contact with host cells, or both, led to the observed changes in EHEC gene expression.

In this study, our goal was to determine whether host cell-derived molecules can act as inter-kingdom signals and activate the virulence machinery of EHEC in a manner similar to NE, in the absence of host cells. We hypothesized that EHEC infection is influenced by molecules derived from intestinal epithelial cells and investigated alterations in EHEC gene expression upon exposure to conditioned medium from intestinal epithelial cells. Our results suggest an increase in EHEC virulence and colonization that is triggered by intestinal epithelial cell-derived protein-based molecule(s) that are > 3 kDa.

Materials and methods

Growth and maintenance of eukaryotic cells and bacteria

The human colon-cancer cell line HCT-8 (ATCC, Manassas, VA), derived from enterocytes at the junction of the large and small bowel, was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% horse serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, at 37 °C in 5% CO_2, according to standard ATCC protocols (henceforth referred to as RPMI medium).

The T84 cell line, of colonic adenocarcinoma origin, and HeLa cells, a cervical cancer line of epithelial origin, were obtained from ATCC and were maintained in ATCC-recommended media during normal growth and culture.

Escherichia coli O157:H7 EDL933 (EHEC, ATCC 43895) was purchased from ATCC and, unless stated otherwise, was grown and maintained in Luria–Bertani (LB) medium at 37 °C.

Collection of fresh and conditioned medium

Roswell Park Memorial Institute medium without antibiotics and with heat-inactivated (65 °C, 1 h) horse serum (henceforth referred to as antibiotic-free RPMI) was used to prepare fresh (FM) and spent conditioned medium (CM). Low-passage-number HCT-8 cells were cultured in 10-cm diameter Petri-dishes until ~80% confluence in normal RPMI medium. The cells were then washed twice with sterile phosphate-buffered saline (PBS) (9 g L^{-1} sodium chloride, 0.795 g L^{-1} sodium phosphate dibasic, and 0.144 g L^{-1} potassium phosphate monobasic) to remove traces of antibiotic containing medium and antibiotic-free RPMI was added to the Petri-dish. The Petri-dishes were then incubated at 37 °C in 5% CO_2 for 24 h to generate CM. An empty Petri-dish was similarly washed twice with PBS and antibiotic-free RPMI was added to it to generate FM. CM from the intestinal epithelial cell line T84 and the cervical epithelial cell line HeLa were similarly generated by growing cells for 24 h in antibiotic-free media. The pH of FM and CM were adjusted to 7.4 ± 0.1 pH units and were stored at 4 °C until further use. No differences in the specific growth rate of EHEC (FM: 0.70 ± 0.02 h^{-1}; CM: 0.70 ± 0.01 h^{-1}) were observed.

Proteinase and heat-inactivation of proteins

To degrade proteins present in fresh and conditioned media, 200 μg mL^{-1} proteinase K (Sigma-Aldrich, St. Louis, MO), a broad-spectrum serine protease, was added and the media incubated for 1 h at 37 °C. Proteinase K activity was then neutralized by adding 100 μg mL^{-1} of the serine protease inhibitor Pefabloc (Sigma-Aldrich), and incubating further at 37 °C for 16 h. The treated media were stored at 4 °C until use. Protein denaturation was verified by separating proteins on 4–20% SDS-PAGE gel and confirming the presence of a low molecular weight protein smear. Proteins from fresh and conditioned media were also heat-inactivated by incubating at 95 °C for 15 min. The heat-denatured media were then cooled to room temperature and stored at 4 °C until use.

Fractionation of media through centrifugal filters

Fractionation of FM and CM was achieved using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA) with a molecular weight cut-off of 3 kDa. FM and CM, 4 mL each, were loaded onto the top chamber of the filters and centrifuged at 4000 ***g*** for 30 min at 25 °C. Fractions containing small molecules (< 3 kDa) were collected at the bottom (filtrate). Serum-free RPMI was added to the resulting top fraction to make a final volume of 3 mL. The media were stored at 4 °C until use.

RNA isolation and DNA microarrays

Overnight cultures of EHEC were diluted in LB medium to a turbidity of 0.1 at 600 nm. The cells were allowed to

grow to a turbidity of 1.0 at 600 nm at 37 °C and the EHEC cells were then resuspended in either fresh or conditioned medium. The cultures were then allowed to grow for 1 or 3 h before cell pellets were collected by centrifugation and stored at −80 °C. Transcriptome profiling was carried out after 1 and 3 h of exposure to CM based on our prior work showing dynamic changes in LEE gene expression in the first 4 h after signal addition (Bansal *et al.*, 2008).

Total RNA was isolated from the cell pellets (Ren *et al.*, 2004a, b) and RNA quality was assessed using gel electrophoresis. *Escherichia coli* GENOME 2.0 arrays (Affymetrix, Santa Clara, CA) containing 10 208 probe sets for all 20 366 genes present in four strains of *E. coli*, including EHEC, were used to profile changes in gene expression using RNA samples for each treatment. Hybridization was performed for 16 h and the total cell intensity was scaled automatically in the software to an average value of 500. The data were inspected for quality and analyzed according to the procedures described by the manufacturer (Affymetrix Data Analysis Fundamentals), which include using premixed polyadenylated transcripts of the *Bacillus subtilis* genes (*lys*, *phe*, *thr*, and *dap*) at different concentrations. As done in our previous work (Bansal *et al.*, 2008), genes were identified as differentially expressed if the expression ratio (between conditioned and fresh medium cells at different time-points) was > 1.5 and if the change in the *P*-value was < 0.05 (based on the standard deviation between values measuring relative changes in expression; Ren *et al.*, 2004a, b). The differentially expressed genes were annotated using gene ontology definitions available in the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx). The expression data are deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible as GEO ID: GSE22285.

A total of four microarrays were used in this study at the two time points for samples in fresh and conditioned media. The genes were sorted into various functional categories, and the role of conditioned medium in regulation of these genes was analyzed.

In vitro colonization assays

Adhesion of EHEC to HCT-8 cells was performed as previously described (Bansal *et al.*, 2007). Low-passage-number HCT-8 cells were cultured in standard 24-well tissue culture plates and grown at 37 °C in 5% CO_2 until ~80% confluence. Next, HCT-8 monolayers were washed two times with PBS to remove traces of antibiotics, and different media to be tested were added to individual wells. Approximately 10^7 cells of a freshly grown EHEC culture (OD of ~0.8 at 600 nm) were added to each well and incubated for 3 h at 37 °C and 5% CO_2. Loosely attached cells were removed by washing the wells two times with PBS, and the HCT-8 cells were lysed in the wells using 0.2% Triton X-100 in PBS. The cell suspension in each well was vigorously vortexed, and serial dilutions of the bacteria were spread on LB plates. Colonies were counted after 24 h incubation at 37 °C.

Intracellular phage titer assay

Intracellular phage levels were determined using a protocol adapted from a previously published method (Köhler *et al.*, 2000). Since EHEC cells were exposed for only 3 h in nutrient-rich FM or CM, and no significant cell lysis was observed during this period, phage titers were determined in cell extracts instead of in culture supernatants. Briefly, EHEC and *E. coli* DH5α were cultured overnight in LB medium. The next day, EHEC was re-inoculated in LB medium at a starting turbidity of 0.1 at 600 nm and grown at 37 °C till a turbidity of 1.0 was reached. The cells were then collected through centrifugation and resuspended in fresh and conditioned media. The cultures were further incubated at 37 °C for additional 3 h. EHEC cells were again collected and were lysed using chloroform to collect the intracellular phage. At the same time, *E. coli* DH5α was freshly inoculated and grown till a turbidity of 1.0. The EHEC phages were diluted 10-fold in four to six serial dilutions in 1 mL *E. coli* DH5α. A 1-mL aliquot of the above dilutions was added to 5 mL of molten LB-top agar (8 g L^{-1} agar, 0.1% glucose, 5 mM $CaCl_2$) and poured over LB-bottom plates (10 g L^{-1} agar, 0.1% glucose, 5 mM $CaCl_2$). The plates were incubated at 37 °C for 24 h. The next day, the resulting Stx2 plaques were counted.

qRT-PCR

DNA microarray data was corroborated using qRT-PCR (Bansal *et al.*, 2007). The primers were designed using PRIMERQUEST online software (Table 1). qRT-PCR was performed using iScript one-step RT-PCR kit with SYBR green (Bio-Rad Laboratories, CA) on a MyiQ single-color real-time PCR detection system (Bio-Rad Laboratories). The threshold cycles, as calculated by the MYIQ optical system software (Bio-Rad Laboratories), were used to determine the relative changes between samples. The experiments were run in triplicate in 20-μL reactions and 50 ng of total RNA was used for each reaction, with the final forward and reverse primer concentrations at 0.15 μM each. After amplification, template specificity was ensured through melting curve analysis. *rrsG* was used as the housekeeping gene for normalizing the data.

Table 1. Sequences of primers used for qRT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
escU	CAAGCTCTTGTCGTTGTTGCCT	TCCTTGGCTTCTCGTTTCACCT
eae	GCTGGCCCTTGGTTTGATCA	GCGGAGATGACTTCAGCACTT
fepE	CGCAATTGTCGCATTGAGCGAA	ACTCTTTCACCACCAACGCAGA
stx1B	CGCCTGATTGTGTAACTGGA	TGAATCCCCCTCCATTATGA
C1433	GGGTATGGACCAGATGGAAA	CCTTACGACGGTCCTCTCTG
fliF	ATCAAAATAACCGCCAGCAG	GTTGCTGAGAGGCAATGGTT
flhD	CTCCGAGTTGCTGAAACACA	GTGGCTGTCAAAACGGAAGT
wcaG	CGGCGGATTTCATCTATCAG	GATTTTGGCAATGGCGTAAG
wcaF	GGCAACAATATTTGCCTGGT	ATGTGCGCCAATGGTTATTT
wcaH	GAAAGACGAAACGCTGGAAG	GCTCATCCGGCAGTAACAGT
rrsG	TATTGCACAATGGGCGCAAG	ACTTAACAAACCGCCTGCGT

Results

Gene expression profiling in EHEC exposed to CM

Significant changes in the EHEC transcriptome were observed upon exposure to CM for 1 and 3 h. As the first step in determining genes that were differentially regulated in CM relative to FM, a fold-change cut-off of 1.5 was used (Fig. 1a and b). Statistically significant changes were then sorted based on a *P*-value cut-off of 0.05. Using this strategy, we determined that approximately 1400 genes (~ 28% of total genome) were up-regulated by CM at both 1 and 3 h, whereas about 200 genes (~4%) were down-regulated at both time-points. In addition, about 100 genes (~2%) were up-regulated at 1 h and down-regulated at 3 h, or vice-versa (Fig. 1c). We mainly focused on the 1400 genes that were up-regulated at both time-points in our discussion, since most of the virulence-related genes are in this category. Examples are genes belonging to the LEE operon, flagella genes, prophage 933W genes, iron-related, and biofilm-related genes (Fig. 2). Table 2 gives a partial list of the differentially regulated genes identified in this study.

The LEE pathogenicity island consists of 41 genes distributed in five operons (LEE1 through LEE5), whose products are involved in EHEC virulence and infection (Hacker & Kaper, 2000). In our study, 32 of these 41 genes (78%) were up-regulated by CM at either 1 or 3 h. The positive regulator of entire LEE island, *ler*, was up-regulated 1.5-fold at 1 h, whereas its expression was not significantly altered at 3 h. The *eae* gene, which encodes the adhesin intimin (Frankel *et al.*, 1998), was up-regulated 1.7-fold at 3 h. The *tir* gene encodes a receptor for intimin that is secreted into host cells (Frankel *et al.*, 1998) and was induced by 1.5-fold at 3 h. *espADF* genes, up-regulated significantly at both time-points, encode proteins that are involved in the type III secretion system of EHEC, of which EspF is encoded at the far end of LEE. The highest induced genes in LEE belonged to the *esc* operon (*escCDFRSTU*), which also encodes type III secretion system in EHEC (Table 2).

Shiga toxin 1 (Stx1) of EHEC is responsible for cleavage of host ribosomal RNA, thus disrupting protein synthesis and killing infected cells (Kaper *et al.*, 2004). In our study, CM up-regulated expression of the *stx1A* and *stx1B* genes that are involved in Stx1 production at 1 h, suggesting an increase in EHEC virulence. EHEC also contains Stx2, which is encoded by the 933W prophage. Although we did not see any change in *stx2A* and *stx2B* genes at any of the time-points, 17 of 27 (63%) 933W prophage genes were up-regulated at both time-points (Table 2). Thus, CM also caused a direct or indirect increase in toxin production by EHEC.

Several genes associated with flagella were also up-regulated by CM (Table 2), and induction of these genes is associated with increase in virulence (Lane *et al.*, 2005). For example, the flagellar genes (*flhA, fliF*, and *fliN*) were all up-regulated. However, *flhD*, the master regulator of flagellar genes, was down-regulated at 1 h but unchanged at 3 h. Colanic acid is a capsular exopolysaccharide that is involved in biofilm formation (Prigent-Combaret *et al.*, 1999) and we observed an increase in nine of 12 genes (75%) of the *wca* operon (Table 2), which is involved in colanic acid biosynthesis, at both time-points. Increase in motility and biofilm formation of EHEC is correlated with increased virulence (Sperandio *et al.*, 2003; Bansal *et al.*, 2007), and demonstrates the consistency in our data.

Poirier *et al.* (2008) have shown that genes involved in iron availability, specifically the iron transport operons *fit* and *chu*, are up-regulated along with the Shiga toxin genes in *E. coli* O157:H7 during infection of human macrophages, and they proposed that iron uptake systems may help *E. coli* O157:H7 survival in limiting nutrient environments. In our study we observed that iron uptake, iron transport, and hemoglobin transport genes were up-regulated in the presence of CM (Table 2), which suggests that CM up-regulates EHEC virulence.

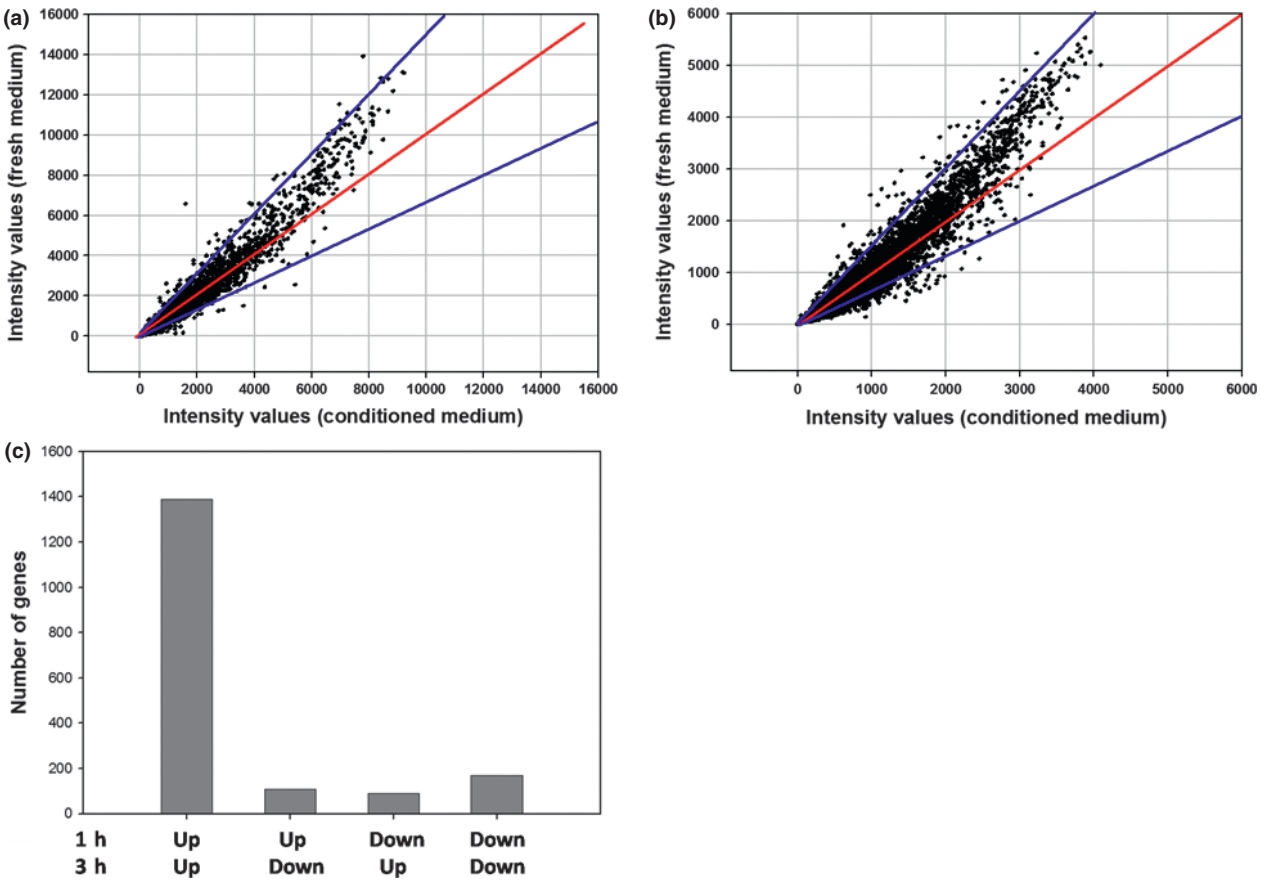

Fig. 1. Determination of EHEC genes differentially regulated by CM. EHEC cells were exposed to FM or CM for either 1 or 3 h, and the RNA was isolated. As a first step, differentially regulated genes were determined using a fold change cut-off of 1.5 (blue line). Red line represents genes that were unchanged at (a) 1 h and (b) 3 h exposure to CM. (c) After using a *P*-value cut-off of 0.05, the number of genes differentially regulated in presence of CM at different time points is shown by the bars.

Validation of transcriptome data

Selected genes from the LEE pathogenicity island, iron uptake, colonic acid, and flagellar genes were profiled using qRT-PCR and the fold-increase in expression in CM relative to FM determined. The LEE genes whose expression was validated include *escU* (2.8-fold up-regulation in the arrays at 3 h, 3.2 ± 0.1 in qRT-PCR), *eae* (1.7-fold up-regulation in the arrays at 3 h, 3.7 ± 0.1-fold in qRT-PCR), and *ler* (1.5-fold up-regulation in the arrays at 1 h, 1.7 ± 0.2-fold in qRT-PCR). Changes in the expression levels of lysis protein S homolog C1433 (1.9-fold up-regulation in the arrays at 3 h; 2.1 ± 0.3-fold in qRT-PCR), *fepE* (2.0-fold up-regulation in the arrays at 3 h, 2.5 ± 0.1-fold in qRT-PCR), *flhD* (1.5-fold down-regulation in the arrays at 1 h, 1.8 ± 0.1-fold in qRT-PCR), *fliF* (1.9-fold up-regulation in the arrays at 3 h, 1.5 ± 0.2-fold in qRT-PCR), *wcaH* (3.0-fold up-regulation in the arrays at 3 h, 2.5 ± 0.1-fold in qRT-PCR), *wcaF* (3.0-fold up-regulation in the arrays at 3 h, 3.2 ± 0.4-fold in qRT-PCR), *wcaG* (2.8-fold up-regulation in the arrays at 3 h, 2.2 ± 0.3-fold in qRT-PCR), and *stx1B* (2.1-fold up-regulation in the arrays at 1 h, 1.7 ± 0.2-fold in qRT-PCR) were also validated.

EHEC colonization and phage production in CM

We performed an *in vitro* attachment assay where we found CM increased EHEC attachment by 4.9-fold (Fig. 3a), suggesting that the recognition of epithelial cell-derived product(s) by EHEC through inter-kingdom signaling could play a role in EHEC infections. Based on the increase in expression of prophage 933W genes through microarrays, intracellular phage titers were determined in EHEC exposed to FM and CM. In CM, the intracellular phage titer increased by 2.7-fold (Fig. 3b), suggesting an increase in EHEC virulence. Thus, transcriptome data were corroborated with phenotypic assays.

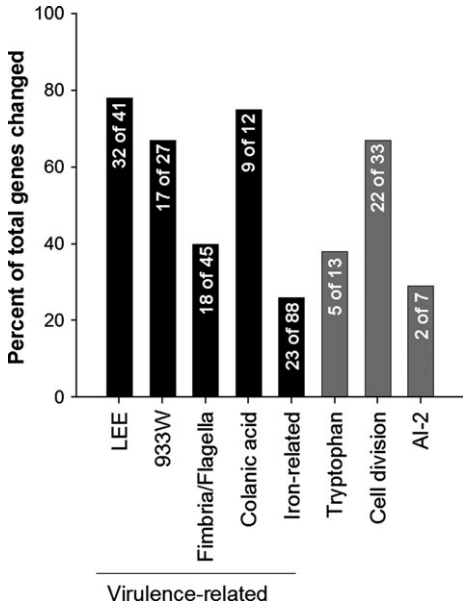

Fig. 2. EHEC virulence-related genes regulated by CM. The bar graph represents important classes of virulence-related genes that were regulated by CM. Genes were classified based on molecular, cellular, or biological function using descriptions provided by Affymetrix. The numbers in each column represent the number of genes in each category showing differential expression.

Identification of HCT-8-derived molecule

The molecule(s) responsible for increased EHEC virulence are heat-sensitive, as heating the CM to 95 °C abolished the increase in virulence (Fig. 4a). The molecule(s) also appear to be protein-based, as proteinase K-treated CM failed to increase EHEC attachment to HCT-8 cells (Fig. 4b). To determine whether a component of the serum used in the media contributes to the increase in colonization, we repeated the *in vitro* attachment experiment using CM from serum-free and reduced (1%) serum-containing media. Exposure to these CM did not increase EHEC attachment (Fig. 4c and d), suggesting the importance of serum components in generating the active molecule.

Conditioned medium was also fractionated using a centrifugal filter with a 3-kDa molecular weight (MW) cut-off. The filtrate showed no increase in EHEC attachment (data not shown), whereas the fraction containing large molecules (> 3 kDa) showed a 1.9-fold increase (Fig. 4e). Together, these results suggest that the active molecule is protein-based and not an organic molecule or a small peptide. However, further fractionation using filters with different MW cut-offs was not successful, as high abundance proteins in horse serum (e.g. albumin which accounts for ~ 55% of serum; Anderson & Anderson, 2002) clogged the membrane and prevented fractionation by centrifugal filtration.

Production of active molecule by other epithelial cell lines

Since CM from the HCT-8 intestinal epithelial cell line increased attachment of EHEC to host cells, we investigated whether a similar increase would be obtained with T84 and HeLa cell-CM as well. The T84 cell line is of colonic adenocarcinoma origin (McCool *et al.*, 1990), similar to HCT-8, and has been extensively used to study gastrointestinal infections *in vitro*, whereas HeLa is a cervical cancer line of epithelial origin and has been used for EHEC attachment studies (Sperandio *et al.*, 2003; Walters & Sperandio, 2006). CM from T84 cells caused a similar increase in EHEC attachment (Fig. 5), which was expected, as these two cell lines are intestinal in origin. CM from HeLa cells also resulted in an increase in EHEC attachment (Fig. 5), suggesting that the molecule(s) could be a general epithelial cell-derived factor and not specific to intestinal epithelial cells.

Discussion

It is becoming increasingly evident that cell–cell signaling inside the GI tract plays a key role in commensal survival, pathogen colonization, and host defense. An important component of this is inter-kingdom signaling, i.e. the recognition of eukaryotic signaling molecules by commensal bacteria and pathogens, and vice versa. The importance of inter-kingdom signaling interactions in pathogen infection is evident from our prior work (Bansal *et al.*, 2007) showing that host hormones up-regulate EHEC virulence. In the current study, we demonstrate that EHEC virulence gene expression and attachment are increased in the presence of molecule(s) synthesized by human intestinal epithelial cells.

As determined through microarrays, the coordinated increase in expression of 32 of 41 LEE genes strongly suggests a global increase in EHEC virulence upon exposure to CM. In addition, Stx phage genes are also induced, suggesting an increase in Stx production and EHEC virulence. Induction of flagellar genes has been associated with virulence (Lane *et al.*, 2005) and was observed in the current study (Table 2) along with colonic acid genes that are involved in surface colonization and biofilm formation.

Since the time points at which EHEC gene expression was profiled (1 and 3 h) are relatively early in the infection process, we speculate that genes responsible for both migration and attachment are simultaneously up-regulated in CM, and divergent regulation of flagellar and

Table 2. Important EHEC genes up-regulated in the presence of conditioned medium

Probe set ID	Gene symbol	1-h fold change	3-h fold change	Description
Virulence/LEE genes				
1761795_s_at	*ler*	1.5*	1.2	Activator of LEE
1759158_s_at	*stx1B*	2.1*	−1.1	Hemolysis
1768192_s_at	*stx1A*	1.7*	−1.1	Negative regulation of protein biosynthesis
1760207_s_at	*tir*	1.2	1.5*	Cell adhesion
1759472_s_at	*sepQ*	1.2	1.7*	
1764730_s_at	*sepL*	1.7*	1.7*	
1768968_s_at	*mviN*	1.3	1.5*	Pathogenesis
1761587_s_at	*espF*	1.5*	1.5*	
1761936_s_at	*espD*	1.5*	1.7*	Pathogenesis
1769270_s_at	*espA*	1.5*	1.7*	
1767609_s_at	*escU*	1.9*	2.8*	Transport
1764491_s_at	*escT*	2.3*	2.5*	Protein targeting
1763133_s_at	*escS*	2.1*	2.5*	Transport
1762528_s_at	*escR*	2.3*	2.3*	Type III protein (virulence-related) secretor activity
1761477_s_at	*escF*	1.7*	1.7*	Pathogenesis
1767897_s_at	*escD*	1.3	1.5*	
1767809_s_at	*escC*	1.4	1.5*	Transport
1760011_s_at	*eaeH*	1.6*	2.3*	Attaching and effacing protein
1764248_s_at	*eaeH*	1.3	1.7*	Cell adhesion
1766942_s_at	*eae*	1.3	1.7*	Cell adhesion
1763952_s_at	*ECs4480*	2.0*	1.4	Pathogenesis
1759147_at	*ECs3718*	1.3	1.7*	Pathogenesis
1765580_s_at	*Z4196*	1.6*	1.9*	Type III protein (virulence-related) secretor activity
1766891_s_at	*Z4190*	1.5*	2.1*	Type III protein (virulence-related) secretor activity
1763006_s_at	*Z4189*	1.5*	2.5*	Type III protein (virulence-related) secretor activity
1769199_s_at	*Z4180*	1.0	1.5*	Type III protein (virulence-related) secretor activity
933W Prophage genes				
1765575_s_at	*Z1459*	1.4	1.9*	Carbohydrate metabolism
1766027_s_at	*c1433*	1.3	1.9*	Lysis protein S homolog from lambdoid prophage DLP12
1763115_s_at	*c1555*	1.5*	1.1	Putative DNA N-6-adenine-methyltransferase of bacteriophage
1768861_s_at	*Z1458*	1.5*	1.9*	Regulation of transcription
1763468_s_at	*Z1495*	1.9*	1.1	
1759811_s_at	*Z1494*	1.7*	1.1	
1762616_s_at	*Z1493*	1.7*	1.3	
1760240_s_at	*Z1490*	1.6*	1.2	
1764484_s_at	*Z1487*	1.6*	1.4	
1760789_s_at	*Z1486*	1.6*	1.4	
1767326_s_at	*Z1482*	1.6*	1.3	
1767349_s_at	*Z1480*	1.6*	1.4	
1765250_s_at	*Z1478*	1.5*	1.1	
1762000_s_at	*Z1476*	1.5*	1.7*	
1760248_s_at	*Z1467*	1.5*	1.7*	
1761222_s_at	*Z1469*	1.4	1.9*	
1767360_s_at	*Z1468*	1.3	1.5*	
Fimbrial and flagellar genes				
1766324_s_at	*sfmH*	1.6*	1.6*	Fimbrial assembly protein
1763064_s_at	*sfmD*	1.7*	1.1	Putative outer membrane protein
1766811_s_at	*sfmC*	1.9*	1.2	Putative chaperone
1767430_s_at	*fliP*	1.5*	1.7*	Flagellar biosynthetic protein FliP precursor
1765241_s_at	*fliN*	1.6*	1.2	Flagellar motor switch protein FliN
1763750_s_at	*fliF*	1.3	1.9*	Flagellar M-ring protein
1762824_s_at	*flhD*	−1.5*	−1.3	Regulator of flagellar biosynthesis
1767873_s_at	*flhA*	1.5*	1.3	Flagellar biosynthesis protein FlhA
1767422_s_at	*ppdD*	1.6*	1.5*	Prepilin peptidase-dependent protein D precursor

Table 2. Continued

Probe set ID	Gene symbol	1-h fold change	3-h fold change	Description
1763745_s_at	*ycbV*	1.5*	1.5*	Putative fimbrial-like protein
Multidrug resistance				
1759593_s_at	*emrK*	1.7*	1.6*	Multidrug resistance protein K
1761644_s_at	*emrD*	1.3	1.6*	Multidrug resistance protein D
1762168_s_at	*emrB*	1.1	1.9*	Multidrug resistance; probably membrane translocase
Iron-related genes				
1765511_s_at	*fixX*	1.4	1.9*	Ferredoxin-like protein
1762110_s_at	*fixC*	1.5*	1.7*	FixC protein
1760251_s_at	*fixB*	1.2	1.7*	Probable flavoprotein subunit, carnitine metabolism
1762922_s_at	*fhuD*	1.2	1.9*	Ferrichrome-binding periplasmic protein precursor
1761740_s_at	*fhuC*	1.1	1.6*	Ferrichrome transport ATP-binding protein fhuc
1766821_s_at	*fhuB*	1.2	1.9*	Ferrichrome transport system permease protein FhuB
1760499_s_at	*fepG*	1.1	1.6*	Ferric enterobactin transport protein
1768501_s_at	*fepE*	1.9*	2.0*	Ferric enterobactin (enterochelin) transport
1768432_s_at	*fepB*	1.6*	1.1	Ferric enterobactin-binding periplasmic protein precursor
1769044_s_at	*chuY*	2.1*	1.2	
1765359_s_at	*chuW*	3.5*	1.7*	
1760730_s_at	*chuU*	2.6*	1.5*	Putative permease of iron compound ABC transport system
1768770_s_at	*chuT*	2.6*	1.2	
1763313_s_at	*chuS*	2.8*	1.9*	Putative heme/hemoglobin transport protein
1762260_s_at	*chuA*	2.1*	1.7*	
Colanic acid genes				
1761929_s_at	*wcaJ*	1.6*	2.6*	Putative colanic biosynthesis UDP-glucose lipid carrier transferase
1759709_s_at	*wcaI*	1.1	2.8*	Putative colanic acid biosynthesis glycosyl transferase wcaI
1759993_s_at	*wcaH*	1.2	3.0*	GDP-mannose mannosyl hydrolase
1765792_s_at	*wcaG*	1.7*	2.8*	GDP-4-keto-6-L-galactose reductase
1766922_s_at	*wcaF*	1.5*	3.0*	Putative colanic acid biosynthesis acetyltransferase wcaF
1759224_s_at	*wcaE*	1.1	2.3*	Putative colanic acid biosynthesis glycosyl transferase wcaE
1763157_s_at	*wcaD*	1.4	1.5*	Putative colanic acid polymerase
1763999_s_at	*wcaC*	1.0	2.0*	Putative colanic acid biosynthesis glycosyl transferase wcaC
1768788_s_at	*wcaA*	1.1	1.5*	Putative colanic acid biosynthesis glycosyl transferase wcaA

*Statistically significant change in expression at P-value < 0.05.

attachment/colonization genes is likely to be seen only at later stages of infection.

It should be pointed out that the observed changes in gene expression upon exposure to CM cannot be unequivocally assigned only to the > 3-kDa CM fraction, as other components present in the CM could also contribute to the observed changes in gene expression. However, since these changes in gene expression upon exposure to CM are relative to those observed in FM, they are not simply due to a component present in serum.

In addition to changes in gene expression, CM increased the EHEC attachment to host cells and intracellular phage titers. Although titers of Stx2-converting phages were not specifically determined, a prior study (Köhler *et al.*, 2000) has shown that in several Shiga toxin producing strains, including EDL 933W, an increase in overall phage titers directly correlates with increase in Stx levels. Therefore, the measured phage titers indirectly suggest an increase in the production of Stx. Along with the increase in LEE gene expression, this observation suggests that EHEC exposed to CM is more virulent. It has been proposed that EHEC only expresses its virulence genes after specific recognition of the intestinal environment, as these sets of genes are not expressed in rich media such as LB (Russell *et al.*, 2007). A recent review proposes that EHEC colonization of the intestine is mediated by adhesins other than intimin (Farfan & Torres, 2012). Although this model suggests that EHEC utilizes its long polar fimbriae to interact with the intestinal mucosa, how these adhesion factors are selectively expressed under specific environmental conditions is not fully understood. Based on our data, it is intriguing to speculate that such sensing of its environment by EHEC is achieved, in part, through these epithelial cell-derived molecules.

Our data on CM from different epithelial cell lines from intestinal and cervical origin are consistent in that each CM resulted in similar increase in EHEC attachment. As expected, T84 and HCT-8 CM induced EHEC attachment, since they are of intestinal origin. While it was surprising that HeLa cell CM caused an increase in

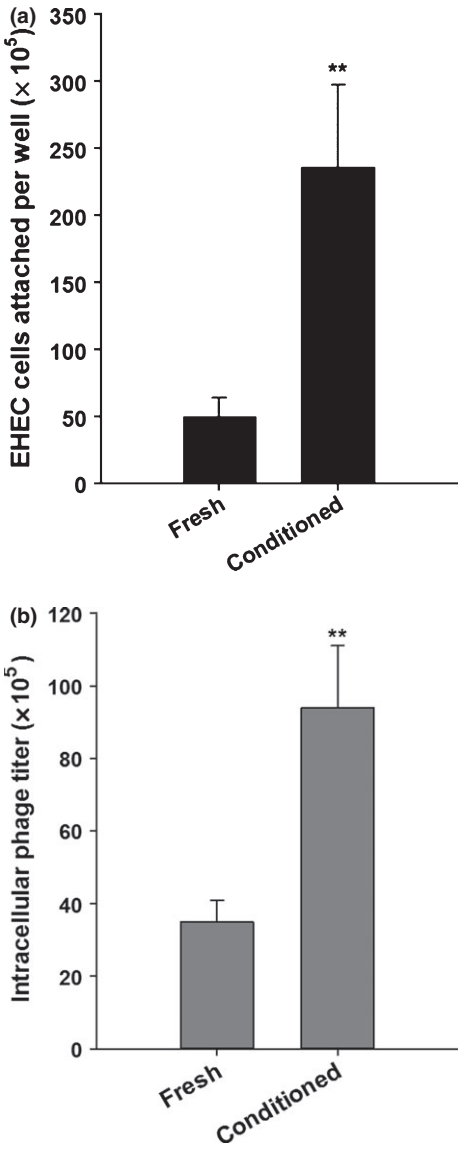

Fig. 3. EHEC virulence assays. (a) EHEC cells were attached to HCT-8 cells in FM or CM, and (b) the formation of phage particles in FM or CM. Data represent mean ± standard deviation from three independent experiments. **Statistical significance using Student's *t*-test test at $P < 0.005$.

EHEC attachment, it should be noted that several studies (Sperandio *et al.*, 2003; Walters & Sperandio, 2006) have demonstrated that HeLa is a valid *in vitro* model for EHEC attachment. Therefore, it is possible that the same molecule(s) derived from HCT-8 cells can be derived from HeLa cells as well, and are used by EHEC to increase virulence gene expression and attachment.

Eukaryotic cell factors that either mitigate or promote pathogen virulence have been identified previously (Sperandio *et al.*, 2003; Mrsny *et al.*, 2004; Dalmasso *et al.*, 2006). Dalmasso *et al.* (2006) noted that in the presence of *Saccharomyces boulardii*, a thermophilic and non-pathogenic yeast, EHEC-infected T84 cells secreted significantly lower levels of tumor necrosis factor (TNF)-α and did not activate procaspase-8 and -9, suggesting that exposure to the probiotic yeast reduces apoptosis via mitochondrial and extracellular death receptors. Sougioultzis *et al.* (2006) similarly observed that *S. boulardii* culture supernatant inhibited interleukin (IL)-8 cytokine production in IL-1β or TNF-α-stimulated human HT-29 colonocytes and THP-1 monocytes. They purified and characterized the active secreted compound as < 1 kDa in size, heat and water stable, and neither a pure protein nor a lipid. Vareille *et al.* (2007) have shown that nitric oxide (NO) produced by colonic mucosa reduces EHEC *stx* mRNA synthesis and Stx phage formation, thus reducing the harmful effects of EHEC Stx toxin. It should be noted that our study did not account for this NO-mediated reduction in Stx phage formation in CM, which suggests that the actual increase in phage titers (including that of Stx phages) upon exposure to CM could be more significant that what was observed.

A host-derived molecule that has been shown to increase pathogen virulence significantly is adenosine. Crane *et al.* (2005) have reported an enteropathogenic *E. coli* (EPEC) type III secretion system-mediated increase in ATP release in T84 cells; and that breakdown of ATP into extracellular adenosine increases EPEC infection. The authors further studied the effects of purified adenosine on EPEC virulence (Crane & Shulgina, 2009) and observed that adenosine increases EPEC growth rate in several media, inhibits formation of bundle-forming pili, and increases expression of EPEC-secreted proteins (Esps), a trend noted in our current study, where expression of several EHEC *esp* genes was up-regulated in CM (Table 2). Similarly, Patel *et al.* showed that adenosine and inosine in hypoxia-subjected intestinal epithelial cell-CM up-regulate the *P. aeruginosa* virulence factor PA-I, which is a potent barrier dysregulator (Patel *et al.*, 2007). However, it is unlikely the CM molecule that increases EHEC virulence is adenosine, as our data show that the molecule is proteinaceous and larger than 3 kDa.

Other host molecules that influence pathogen infection have also been identified. Mrsny *et al.* (2004) identified that the eicosanoid hepoxilin A_3 ($hepA_3$) is secreted at the apical surface of the epithelial cells in response to inflammatory conditions. However, $hepA_3$ is < 1 kDa in size. Sperandio *et al.* (2003) have also identified an EHEC factor, autoinducer-3 (AI-3), that is putatively structurally similar to the human hormone epinephrine, and increases EHEC virulence. Although the exact structure of AI-3 is not known, it is also < 1 kDa and non-proteinaceous.

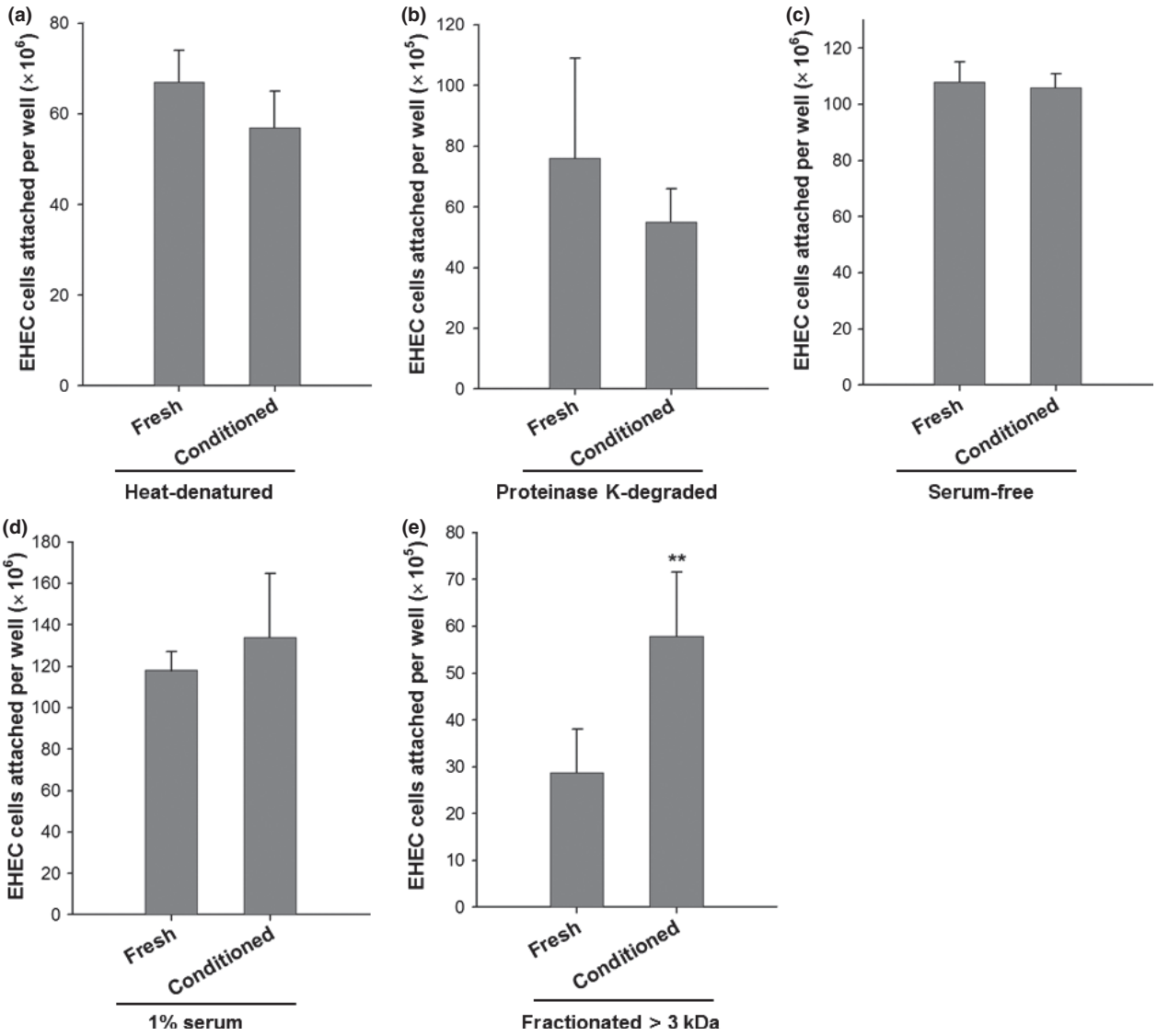

Fig. 4. EHEC colonization assay. EHEC cells were attached to HCT-8 cells in FM or CM that were (a) heat-denatured, (b) proteinase K-degraded, (c) serum-free, (d) containing 1% serum, and (e) fractionated and contained molecules > 3 kDa. Data represent mean ± standard deviation from three independent experimental wells. **Statistical significance using Student's *t*-test test at $P < 0.005$.

Two key differences between the above-mentioned studies and our results are in the size and nature of the molecule(s) that increase EHEC virulence. All the above-mentioned studies identified molecules that are small (< 1 kDa) and non-proteinaceous, whereas our results indicate EHEC virulence and attachment to HCT-8 cells is increased by a molecule that is larger than 3 kDa and proteinaceous. Although we attempted to identify the HCT-8 cell-derived molecule(s), complete characterization was difficult due to several reasons. The HCT-8 cell growth medium contained 10% heat-inactivated horse serum, and this increased the complexity of the sample significantly. Fractionation by size exclusion (gel filtration) chromatography or differential centrifugation was not successful due to an abundance of serum proteins (e.g. albumin, transferrin) that block the centrifugal filter membranes and/or prevent migration of smaller molecules through the filter. The abundance of serum proteins also made protein-based methods (e.g. two-dimensional gel electrophoresis) difficult. Precipitation of protein fractions was successful; however, none of the fractions showed any activity after resuspension. It is possible that the structure of the molecule(s) was affected during the precipitation and resuspension process; thereby, affecting activity.

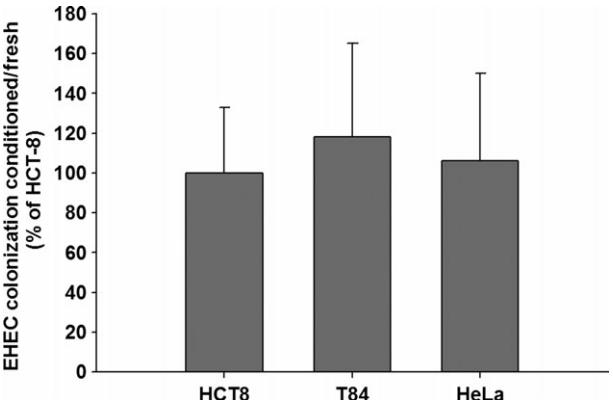

Fig. 5. Comparison of colonization in CM from T84 and HeLa cells with HCT-8. CM was collected from the intestinal epithelial cell lines HCT-8 and T84, and from the cervical epithelial cell line HeLa. The fold increase in colonized cells from T84 and HeLa CM relative to FM was determined and expressed as a percent of the fold increase in colonized cells observed with HCT-8 CM. For comparison purposes, the fold increase in HCT-8 CM relative to FM is also shown.

Although we could not identify the epithelial cell-derived molecule, our data provide information on the putative role of the molecule in EHEC infections. The fact that CM that contained either 1% serum or was serum-free failed to enhance EHEC virulence over that observed with FM, and the similar increase observed with equine (HCT-8 cells) and fetal bovine serum (T84 cells), suggest that the component(s) in serum are necessary to enhance virulence, but are not a critical requirement for virulence. This hypothesis is also supported by the observation that virulence and attachment are observed in FM as well. These suggest that the molecule(s) that result in an increase in EHEC virulence could be a breakdown product or a component of host cell surface molecules or a molecule that is processed from a serum protein component, i.e. host cells process a serum component to release molecule(s) that increase EHEC virulence.

While the difference in EHEC attachment between CM and FM is heat-inactivated, proteinase K treated, serum-free, and 1% serum CM and FM was not statistically significant, the attachment observed in these conditions with either CM or FM was 10-fold higher than that observed in 10% serum (i.e. normal) CM or FM (see Figs 3a and 4a, c, d). It is possible that heat-inactivation, proteinase K treatment, and a decrease in serum concentration reduce the levels of most, if not all, components inhibitory to bacterial growth, and this can lead to increased EHEC attachment compared with 10% serum-containing media. Moreover, it is also possible that the effect of removal of multiple inhibitory components overshadows the increase in EHEC attachment with CM, which would lead to similar attachment levels with both CM and FM under these conditions.

In summary, we show that intestinal epithelial cell-derived factors are important determinants of EHEC virulence. Phenotypic assays for host cell colonization and phage titers suggest that CM exposure up-regulates EHEC virulence and attachment to intestinal epithelial cells. Gene expression data, with the up-regulation of LEE and other virulence-related genes, further support this conclusion. Preliminary characterization indicates that the molecule(s) responsible for increasing EHEC virulence are protein-based, larger than 3 kDa, and heat-labile. Identification of host cell-derived factors can lead to approaches for modulating EHEC infection without adversely affecting the commensal microbiota.

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