Muc17 protects intestinal epithelial cells from enteroinvasive \textit{E. coli} infection by promoting epithelial barrier integrity

Silvia Resta-Lenert, Srvidhara Das, Surinder K. Batra, and Samuel B. Ho

1Department of Medicine, University of California San Diego, and VA San Diego Healthcare System, San Diego, California; 2Department of Biochemistry and Molecular Biology, University of Nebraska, Medical Center, Omaha, Nebraska

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\textbf{Resta-Lenert S, Das S, Batra SK, Ho SB.} Muc17 protects intestinal epithelial cells from enteroinvasive \textit{E. coli} infection by promoting epithelial barrier integrity. \textit{Am J Physiol Gastrointest Liver Physiol} 300: G1144–G1155, 2011. First published March 10, 2011; doi:10.1152/ajpgi.00138.2010.—The membrane-bound mucin MUC17 (mouse homolog Muc3) is highly expressed on the apical surface of intestinal epithelia and is thought to play a role in epithelial restitution and protection. Therefore, we hypothesized that MUC17 has a role in protection of the intestinal mucosa against luminal pathogens. Human intestinal cell lines were transfected by electroporation (Caco-2 and HT 29/19A) and by retroviral expression vector (LS174T, a cell line with high levels of MUC17 expression) using MUC17 siRNA. Transepithelial electrical resistance, permeability, tight-junction protein expression, adhesion, and invasion in response to enteroinvasive \textit{Escherichia coli} (EIEC) were measured in all cell lines. In some experiments, the effect of the addition of exogenous purified crude mucin or recombinant Muc3 cysteine-rich domain protein (Muc3 CRD1-L-CRD2) as preventative or protective treatment was tested. Reduction of endogenous MUC17 is associated with increased permeability, inducible nitric oxide synthase and cyclooxygenase 2 induction, and enhanced bacterial invasion in response to EIEC exposure. Bacterial adhesion is not affected. Exogenous mucin (Muc3) and recombinant Muc3CRD treatment had a small but significant effect in attenuating the effects of EIEC infection. In conclusion, these data suggest that both native and exogenous MUC17 play a role in attachment and invasion of EIEC in colonic cell lines and in maintaining epithelial barrier function.

enteropathogens; inflammatory bowel diseases; mucins; mucosal barrier

MUCOSAL SURFACES ARE PROTECTED against xenobiotics and pathogenic microorganisms by a variety of innate and adaptive mechanisms, which contribute in various measure to promote the integrity of the epithelial barrier function (58). Among the innate mechanisms, mucins prevent noxious interactions of epithelial cells with microbial pathogens and toxic chemicals by providing a physicochemical barrier, through specific mucin-microorganism interactions and cell-signaling regulation (3–5, 7, 27, 29, 37, 42). By limiting adherence or invasion of microorganisms to the epithelial cell surface, mucin can effectively hamper the ability of bacteria and viruses to colonize and invade the cells, block their spread along the mucosal surfaces, and limit the amount of microbial-produced toxins reaching mucosal cells (22, 27). To date, little is known concerning the specific roles and structure/function relationships of membrane-bound mucins in intestinal cytoprotection and barrier-function integrity. At least 21 mucin genes have been identified so far. MUC3 and MUC17 and Muc3, the mouse ortholog of human MUC17, are membrane-bound mucins and are moderately expressed in the colon (16) but are found abundantly in both goblet cells and enterocytes of the small intestine (19, 20, 36, 40). Altered mucin expression has been associated with chronic inflammation at mucosal interface and as a possible pathogenetic mechanism involved in inflammatory bowel disease and cancer (2, 8, 12, 13, 17, 22, 24–26, 32, 39, 43).

We, and others, have previously shown that certain bacteria belonging to the gut microbiota provide the epithelial cells with additional stimuli to produce mucins (3, 46). Moreover, we have shown that both recombinant Muc3 mucin (related to human MUC17) and recombinant human MUC17 stimulate cell migration in human colon cell lines without inducing proliferation and protect cells from apoptosis in response to TNF-α or Fas receptor stimulation (18, 21).

We hypothesized that MUC17 may have similar biological activity in vitro and that endogenous MUC17 mucin may contribute to barrier-function integrity and protect intestinal cells from pathogen colonization and invasion. To prove our hypothesis, we have investigated the ability of MUC17 to limit adherence, invasion, and injury, expressed as increased expression of proinflammatory molecules, derived from infection with an enteric pathogen, enteroinvasive \textit{Escherichia coli} (\textit{E. coli}) (EIEC), in human intestinal epithelial cell lines. Furthermore, our approach was aimed at demonstrating, not only the role of MUC17 in preserving the mucosal physical barrier, but also to show that exogenous addition of mucin and/or MUC17 constructs provided some degree of protection against bacterial injury.

\textbf{MATERIALS AND METHODS}

\textbf{Bacteria and growth conditions.} A stock culture of EIEC was maintained at 4°C on trypticase soy agar slants (Becton-Dickinson Microbiology Systems, Cockeysville, MD). Bacterial strains were kindly provided by Dr. J. Fierer (EIEC strain O29:NM, UCSD). \textit{E. coli} was grown in microaerophilic conditions overnight at 37°C in static, trypticase soy broth (Difco, Detroit, MI), harvested by centrifugation and quantified by determination of colony-forming units (CFU), as previously described (46, 47).

\textbf{Cell growth conditions and treatments.} HT29, HT29/19A (clone derived from HT-29) and Caco-2 cells (American Type Culture Collection, Manassas, VA) were grown in McCoy’s 5a culture medium (Life Technologies, Gaithersburg, MD) plus 5% fetal calf serum (Life Technology, Carlsbad, CA). Cell cultures were grown at 37°C in a humidified atmosphere with 5% CO₂, 95% O₂ and were subcultured after being washed with Earle’s balanced salt solution (Life Technologies) using trypsin-EDTA (Life Technologies) (46, 47). These cell lines do not express the full array of mucins, and/or some of the mucin molecules may be mutated or defective compared with normal colonic tissue, which may constitute a limitation to our experimental design.
with 5.0 ml 1 M HEPES, nonessential amino acids, 2 mML-glutam-
These cells were grown in 10% MEM LS174T medium (10% MEM,
Caco-2) and high levels of MUC3, by mRNA and protein analysis.
(M17TR) was used as described previously (40). Antibody against
RT-PCR and immunoblotting were performed to confirm a decrease
sphere. Antibiotics were removed at least 24 h before experiments.
However, short-lived unpolarized monolayers can be produced by
culture conditions and are unable to form stable junctional complexes.
Tama
tamine, 1 mM sodium pyruvate, and 1,500 mg/l sodium bicarbonate).
These cells were grown in 10% MEM LS174T medium (10% MEM,
and high levels of MUC3, by mRNA and protein analysis.
In some experiments, transient knockdowns were used by subjecting
Caco-2 cells to MUC1 gene silencing by electroporation with an Ammaxa
nucleofector system (Lonza, Walkersville, MD) according to the manu-
facturer’s instructions. siRNA reagents contained three pooled siRNA
duplexes [mRNA accession no.: NM_001040105.1, 1) GAAGC-UAGUUCACUCCUA, 2) CACUCUGCUAGAAGUAAU, 3) CAA-
CCUCGUUGACACUAA; Santa Cruz Biotechnology, Santa Cruz,
and 100 pmol of siRNA was transfected into cell suspensions. Cells
were then seeded onto permeable supports and incubated for 3 days.
In some experiments, a nonspecific control siRNA (scrambled, 100 pmol)
was used as negative control (Santa Cruz Biotechnology).
Moreover, in some experiments, we used the LS174T cell line, which
produces very high levels of MUC1 (95% > HT29, 5% >
Caco-2) and high levels of MUC3, by mRNA and protein analysis.
These cells were grown in 10% MEM LS174T medium (10% MEM,
with 5.0 ml 1 M HEPES, nonessential amino acids, 2 mML-glutami-
tamine, 1 mM sodium pyruvate, and 1,500 mg/l sodium bicarbonate).
Cell medium was changed every other day, and cells were passaged
before confluence. LS174T cells will remain unpolarized under any
culture conditions and are unable to form stable junctional complexes.
However, short-lived unpolarized monolayers can be produced by
heavy cell seeding (105-107 cells/ml per insert) and careful monitoring
of cell attachment. Usually cells were used within 18–24 h from
initial seeding (100% confluent by contrast-phase microscopy).
The LS174T MUC17 RNAi and scramble RNAi clones were stably
transduced with a small hairpin RNA sequence, cloned, and expres-
sed in a retroviral expression vector pSUPER.Retro.Puro (OligoEngine,
Seattle, WA) secreted from Phi-NX cells (293T cell-derived), target-
ing MUC17 expression. The MUC17-specific sequence was deter-
determined using the search algorithm of Drimacoan’s siDESIGN
Center (http://www.drimacoan.com/DesignCenter/DesignCenterPage.aspx;
Drimacoan, Lafayette, CO). A 19-nucleotide target sequence 5’-
atacaacagtggcttca-3’ occurring 5445 downstream of the initiation
codon was selected as the best candidate target. This resulted in a cell
line with a suppressed expression of MUC17, labeled LSsi. A second
small hairpin RNA with an unspecific scrambled sequence was used
to create LSsc as an experimental control. Targeted clones were
identified by antibiotic selection, 3 μg/ml puromycin (Invitrogen, San
Diego, CA) for LSsi or G418 at 400 μg/ml (EMD chemicals;
Calbiochem, Gibbstown, NJ) for LSsc. Permanent cell lines were
created by continuous antibiotic selection with either puromycin or
G418. LS174T cells were grown in MEM (Mediatech, Herndon, VA)
supplemented with 10% FBS, HEPES, nonessential amino acids,
L-glutamine, sodium pyruvate, sodium bicarbonate, and its corre-
sponding antibiotic [Puromycin used at 3 μg/ml for LSsi, G418
(neomycin) used for LSsc] maintained at 37°C in 5% CO2
atmosphere. Antibiotics were removed at least 24 h before experiments.
RT-PCR and immunoblotting were performed to confirm a decrease
in MUC17 expression. The antibody against a MUC17 core protein
(M17TR) was used as described previously (40). Antibody against
β-actin was purchased from Invitrogen (Carlsbad, CA). Purified
porcine stomach mucin, type III, containing a mixture of crude
MUC1 and MUC3 mucins; 1% wt:vol, was obtained from Sigma
(St. Louis, MO).
One cysteine-rich domain (CRD) Muc3 recombinant protein
Muc3CRD1-L-CRD2-His6 (abbreviated in the text as Muc3CRD) (18,
34) was used and consisted of the following sequence, followed by a
COOH-terminal 8-His tag: cmmgfwstgdklcpnffgddrcenvnv-
vncenggtdwlglckctslfetygprceelvseviepvtvaa-
vesvsvtvstqsekyqlrkdrseefsnftktfkqalyagipe-
yegvikiarnkskisv dvdyvilkakygtpentldtvknlet-
kiknatevqvdvnncsallcfnstakvqnsatsvnpnpe-
tckkeagedfakftvlqkgdkwcfipcagystksncgkyc-
cqlqrsgpqcclldttthwysgenccdwiqkkslygy-y-
HHH. This is a portion of the Muc3 extracellular region and contains
two epidermal growth factor (EGF)-like CRDs (CRD1 and CRD2) connected
by an intervening linker segment with sea urchin sperm protein, enteroki-
nase, and agrin (SEA) module (L-SEA), as described (18, 21, 34). This
recombinant protein is similar to that described previously, known as
Muc3EGF1,2 (18), and was shown to be biochemically active in
several human colon cancer cell lines (18, 21, 34).
Adherence assay. Bacteria from log-phase growth cultures were
treated twice in PBS, pelleted by centrifugation, and suspended at a
concentration of 1 × 106 CFU/ml in cell growth medium. Bacterial
suspension (1 ml) was diluted in 1 ml of cell growth medium and the
suspension (overlaid on cells grown on glass slides pretreated with
mucin or recombinant Muc3CRD (see above) or medium, as negative
control. The treated cells were then incubated at 37°C for 1 h in an
atmosphere of 5% CO2. After incubation, cells were washed with
PBS, fixed in absolute methanol, Gram stained, and observed by light
microscopy. Adherence of EIEC was measured by counting Gram-
negative bacteria per 100 cells in 20 random microscopic fields.
Alternatively, bacterial adhesion was measured by CFU count, i.e.,
the number of adherent bacteria (CFU/ml) = total bacteria (CFU/ml) −
intracellular bacteria (CFU/ml), where the total number of bacteria
includes extracellular and intracellular, and where the intracellular
bacteria were determined after killing of extracellular E. coli
by gentamicin treatment (see Invasion assay below for procedure).
Invasion assay. Confluent epithelial cell monolayers were treated
with mucin (Sigma, type III, from pig stomach, containing a mixture
of crude MUC1 and MUC3 mucins; 1% wt:vol) or recombinant Muc3
(Muc3CRD, 1 μg/ml) for 1 h in serum-free medium. Then serum-free
medium containing exponentially grown bacteria, at a multiplicity
of infection of 5:1–20:1 (or medium alone (uninfected controls) was
added to the apical surface. After 1 h at 37°C, cells were washed and
incubated in serum-free medium with gentamicin (50 μg/ml) for 1 h
at 37°C. Treatment with gentamicin effectively kills all extracellular
bacteria as previously shown (46, 47) and is a widely used method for
invasion assay with gentamicin-sensitive Gram-negative bacteria. In
control experiments, gentamicin had no effect on any of the param-
eters measured. Furthermore, no significant bacterial overgrowth was
observed during the duration of the experiment under all conditions
tested. Cells were then maintained at 37°C, 5% CO2 in serum-
and antibiotic-free medium. All treated monolayers had 50% of the culture
medium changed every 12 h after infection to avoid detrimental
effects from variations in pH. Cell invasion and bacterial survival
were checked between 3 and 24 h after infection to test the reproduc-
ibility of the infection protocol. Cell lysates and supernatants from
treated monolayers and controls were checked by CFU counts on
trypticase soy agar. EIEC invasion was expressed as a percentage
of intracellular bacteria compared with total cell-associated bacteria.
In situ hybridization and immunofluorescence assay. Probe EC1531
(5’sccctagtgtgccgccgtatca-3’ specific for E. coli 23S rRNA, labeled with
CY3, was used for visualization of E. coli cells as previously described (47).
Hybridization was performed by adding hybridization solution (10% formalde-
hyde, 0.1 M Tris pH 7.2, 0.9 M NaCl) containing 2 ng probe EC1531/μl to slide chambers.
After incubation in humidified chambers overnight at 37°C, the slides
were washed in buffer (0.1 M Tris pH 7.2, 0.9 M NaCl) three times for 5 min,
and the slides were briefly rinsed in distilled water and left to dry
at room temperature. Cover slips were mounted with VectraShield
and the slides examined with an Olympus fluorescence microscope (550 nm excitation, 570 nm emission).

Electrophysiological studies. Cell monolayers were pretreated with medium alone, mucin, and recombinant MUC17 as described above. Infected and noninfected cell monolayers were tested for transepithelial electrical resistance (TEER) using a "chopstick" voltohmeter and in Ussing chambers (WPI, Sarasota, FL) and for chloride secretion in modified Ussing chambers at various times after infection, as described previously (43). Mucosal and serosal baths contained Ringer’s solution (composition in mM: 115 NaCl, 25 NaHCO3, 0.4 KH2PO4, 2.4 K2HPO4, 2.4 K3HPO4, 1.2 MgCl2, 1.2 CaCl2, 10 glucose, pH 7.4), which was gassed with 5% CO2-95% O2 and maintained at 37°C. The monolayers were continuously short circuited by application of short-circuit current (Isc); open-circuit potential difference was measured every 1–5 min, and TEER was calculated using Ohm’s law.

In case of electrical resistance of LS174T cells, TEER across infected and control monolayers was measured at various times using only a chopstick voltohmeter with the inserts maintained at a constant temperature (37°C) and 5% CO2 gassing. Measurements were expressed in Ω × cm² after subtracting mean values of resistance obtained from cell-free inserts.

Permeability assay. Polarized cell monolayers were pretreated with mucins or medium for 1 h, infected with EIEC as described above, and harvested after incubation at 37°C in 5% CO2 atmosphere for 12 h. Fluorescein sulphonic acid (FS; Molecular Probes, Eugene, OR; molecular weight 478 Da, 200 μg/ml) or FITC-D10 (molecular weight 10,000 Da, 20 mg/ml; Sigma) was added to the basolateral side (46–48). Dishes were incubated at 37°C in a 5% CO2 incubator for 1 h with gentle mixing at 15-min intervals. After the initial incubation, at 15-min intervals up to 2 h thereafter, the FS and FITC-D10 content of apical (Ap) and basolateral (Bl) samples was measured as fluorescence intensity of 1:250 dilutions in distilled water (492-nm excitation and 515-nm emission wavelengths). Monolayer permeability was determined as FS or FITC-D10 clearance: [FS]Bl/[FS]Ap/[FS]Bl, corrected for insert area (nl/cm² per h). A standard curve using incremental concentrations of FS or FITC-D10 was constructed for each assay, and permeability was expressed as nanograms per milliliter in the cleared fluid ([FS]Bl) (48).

Immunoblot analysis. Mucin pretreated infected and uninfected cells grown on 24-mm-diameter Millicell HA filters were harvested at various times after infection and processed as previously described.
(46, 47). Proteins of interest were immunoprecipitated by overnight incubation at 4°C on a mixer with an appropriate dilution of specific antibody [anti-zonula occludens (ZO)-1 and anti-occludin, and anti-phosphorylated occludin from Santa Cruz Biotechnology] in cold lysis buffer supplemented with additional phenylmethylsulphonyl fluoride (20 μM). Immunoblotting for MUC17 core protein was performed using anti-MUC17TR as described previously (34). Proteins were electrophoresed on 7.5% or 4.5–15% polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred onto blotting membranes (Polyscreen PVDF; NEN, Boston, MA). After being blocked (PBS/Tween supplemented with 1% nonfat dry milk), blots were incubated with primary and secondary antibodies (horseradish peroxidase-conjugated antimouse IgG, antirabbit IgG, as appropriate) for 60 min at room temperature. Proteins were visualized by chemiluminescence reagents (ECL Plus; Amersham, Piscataway, NJ) and exposed to X-OMAT film (Eastman Kodak, Rochester, NY).

RNA isolation. Total RNA was isolated from cell monolayers using the guanidine isothiocyanate-cesium chloride cushion ultracentrifugation technique with modifications to minimize mRNA shearing, as previously described (17). RNA was stored in 0.3 M sodium acetate with 2.5 volumes of ethanol at −70°C.

RT-PCR. Cells were seeded onto T25 flasks, n = 3. When cells reached close to 85% confluency, RNA was extracted with TRI Reagent (Ambion, Austin, TX) and then treated with TurboDNase (Ambion). RNA extract was reverse transcribed with M-MLV (Invitrogen) and then screened for MUC17 with PCR (forward primer: GGG CCA GCA TAG CTG TAG GGA GTT CA and reverse primer: GCC ACA GGA ATT GTG GGA GTT CA). PCR product was viewed by gel electrophoresis.

Statistical analysis. Group data are expressed as means ± SE. Analyses between multiple groups were determined using one-factor ANOVA with 95% confidence intervals. Post hoc ANOVA analyses were determined by Fisher’s protected least-significant difference using the Statview software program (version 5.0.1; SAS Institute, Cary, NC) and SigmaPlot software (Systat, San Jose, CA). Analysis between two groups was determined using the two-tailed unpaired Student t-test.

Fig. 2. Effect of MUC17 RNA silencing on bacterial adhesion and invasion. Cells were transiently transfected with MUC17 siRNA (si) or control scrambled siRNA (sc). After 24 h cells were treated with bacteria and gentamicin as described in MATERIALS AND METHODS. A: adhesion of enteroinvasive Escherichia coli (EIEC) is similar in MUC17 siRNA (si) and control scrambled siRNA (sc)-treated cells. This is exemplified here for the Caco-2 cell line. Data for all 3 cell lines are summarized in Table 1. B: extent of EIEC invasion in control (sc) and MUC17 siRNA (si)-treated cells. Cells with reduced expression of MUC17 because of silencing have increased loads of intracellular bacteria. This is true for all 3 cell lines. Naïve cells had similar results as scrambled cells and are not shown for simplicity. C: Caco-2 cells, sc and si, were either pretreated with mucin or Muc3 cysteine-rich domain (CRD) or medium alone, and then tested for EIEC invasion as described in MATERIALS AND METHODS. Pretreatment with crude mucin afforded some protection against invasion compared with untreated cells. [Mean ± SE; N = 6; *P < 0.05, **P < 0.01, infected silenced (si) vs. infected scrambled (sc); #P < 0.05, infected (sc) treated vs. infected (sc) untreated; ^P < 0.05, ^^P < 0.01, infected (si) treated vs. infected (si) untreated]. CFU. colony-forming unit.
RESULTS

MUC17 limits adhesion and invasion of bacteria into epithelial cells. To test the hypothesis that MUC17 has a role in protecting intestinal epithelial cells from enteroinvasive bacterial infections, we investigated the influence of MUC17 on the ability of EIEC to adhere to and invade intestinal epithelial cells expressing MUC17 or cells in which the ability to synthesize MUC17 protein had been silenced. In Fig. 1, we show the mRNA and protein expression levels of MUC17 in naïve and MUC17-silenced cells. Both mRNA and protein expression were significantly suppressed by the siRNA treatment.

Bacterial adhesion was measured by CFU count (number of adherent bacteria/total bacteria) summarized in Table 1, and by in situ hybridization against E. coli 23S rRNA. In Table 1 and Fig. 2A, we show that the number of adherent bacteria was not significantly different in naïve, scrambled, or siRNA-treated cells by both test methods. For simplicity, only one cell line is shown (Caco-2), the one with the moderate expression of MUC17. Similar results were obtained with the other two cell lines used in these studies (LS174T and HT29/19A).

Then we tested the hypothesis that E. coli could more easily gain entry of cells deficient in MUC17 compared with naïve or scrambled cells able to express normal levels of MUC17. We measured this by CFU count (number of bacteria/total bacteria extracellular bacteria) and by in situ hybridization against E. coli 23S rRNA after treatment with gentamicin to Fig. 3. Effect of MUC17 RNA silencing on transepithelial electrical resistance (TEER). Cell monolayers were treated according to MATERIALS AND METHODS, and TEER was tested using a voltmeter or in Ussing chambers applying Ohm’s law. A: reduction of MUC17 does not alter significantly TEER compared with naïve cells. However, the addition of mucin or Muc3 CRD-induced increased electrical resistance in MUC17 siRNA-treated cells. For simplicity, some of the HT29/19Asi data, similar to Caco-2, are not shown. B: effect of reduction of MUC17 on TEER after infection with EIEC (multiplicity of infection, MOI = 10). Cells in which MUC17 had been silenced succumbed faster to the deleterious effect of EIEC on electrical resistance. However, treatment with mucin or Muc3CRD rescued silenced cells as observed previously. (Means ± SE; N = 6; ***P < 0.001, naïve mucin infected vs. naïve mucin; ****P < 0.001, naïve Muc3CRD infected vs. naïve Muc3CRD) B: moreover, neither mucin nor Muc3 CRD treatment had any significant effect on chlorid secretion after EIEC infection. (Means ± SE; N = 6; ###P < 0.001, si mucin infected vs. si mucin; ***P < 0.001, si Muc3CRD infected vs. si Muc3CRD).

Fig. 4. Effect of MUC17 on ion transport. A: scrambled and silenced cells grown on inserts were infected with EIEC and mounted in Ussing chambers to test for ion transport as described in MATERIALS AND METHODS. Chloride secretion, measured as current and expressed as $I_{sc}$, is not affected by MUC17, i.e., MUC17 has little role in protecting intestinal epithelial cells from the deleterious effects of EIEC on ion transport. (Means ± SE; N = 6; ###P < 0.001, naïve mucin infected vs. naïve mucin; ****P < 0.001, naïve Muc3CRD infected vs. naïve Muc3CRD) B: moreover, neither mucin nor Muc3 CRD treatment had any significant effect on chloride secretion after EIEC infection. (Means ± SE; N = 6; ***P < 0.001, naïve mucin infected vs. naïve mucin; ***P < 0.001, naïve Muc3CRD infected vs. naïve Muc3CRD).
eliminate extracellular bacteria (not shown). This experiment demonstrated that greater numbers of EIEC were able to invade MUC17-silenced Caco-2 and LS174T cells compared with naïve or scrambled cells (Fig. 2B). The number of intracellular bacteria was substantially the same for naïve (not shown), scrambled, and MUC17 siRNA HT-29/19A cells because this cell line has a very low basal level of MUC17. These data strongly suggest that native MUC17 plays a role in invasion of EIEC in epithelial cells.

We then investigated whether a crude mucin mixture or the recombinant Muc3CRD protein offered protection against adhesion and invasion by EIEC. There was no difference in adhesion among untreated and pretreated Caco-2sc (Table 1). Neither was adhesion significantly altered by mucin or Muc3CRD pretreatment in any of the silenced cell lines used in these studies (Table 1). Similarly, invasion levels were not different among untreated and pretreated scrambled cells (Fig. 2C; only Caco-2 shown for simplicity). However, when we compared pretreatment with crude mucin or Muc3CRD in MUC17-silenced cells, we observed a significant reduction in the number of invading bacteria compared with untreated cells (Fig. 2C). These data support the hypothesis that reduced levels of MUC17 in intestinal epithelial cells may play a role in progression of infection by allowing greater invasion by pathogenic enteroinvasive bacteria in acute and chronic infections of the gut, and possibly in conditions of chronic intestinal inflammation as exemplified by inflammatory bowel disease, where altered mucins contribute to the pathogenesis of this illness.

Effect of MUC17 mucin on TEER and ion transport. To determine the role of MUC17 in maintaining epithelial barrier integrity, we measured TEER after exposure to EIEC or medium alone in naïve, scrambled, and MUC17 siRNA-polarized human colon cancer cells. For the purpose of simplifying the figures, only data obtained from naïve cells are shown because the results for both naïve and scrambled cells were similar in all tests. We used HT29/19A and Caco-2 cells, which we have shown to be susceptible to EIEC infection (46). LS174T cells were used in similar experiments. However, this cell line required postconfluent conditions because it is unable to form stable monolayers and its nonpolarized status may somehow influence measurement of TEER. In some experiments, cells were pretreated with mucin or Muc3CRD to test the ability of this compound to correct the defect artificially induced by MUC17 gene silencing.

Baseline TEER, i.e., TEER measured before infection, was not affected either by silencing of MUC17 or by the addition of exogenous mucin or Muc3CRD to naïve or scrambled cells (Fig. 3A). After EIEC infection, all MUC17 gene-silenced cells, including the LS174T cell line (not shown), demonstrated a significantly more rapid and profound loss in TEER compared with naïve cells (Fig. 3B). Naïve HT29/19A and Caco-2 results are in agreement with previous published results (46, 47). Pretreatment of silenced cells with mucin and Muc3CRD inhibited the decrease in TEER induced by infection with EIEC (Fig. 3B) and improved TEER in a significant manner compared with controls. This may depend on some of the signaling effects shown in vitro against proinflammatory molecules (17).

We tested then the ability of MUC17 to protect cells from altered ion transport, another effect of EIEC infection (46). Chloride secretion, measured as $I_{sc}$ in Ussing chambers, was not altered by lack of MUC17 or treatment with mucin or Muc3CRD in naïve or silenced Caco-2 and HT29/1c19A cells (Fig. 4, A and B). The LS174T cells could not be tested for ion transport in any of the experimental conditions because of rapid loss of monolayer integrity in Ussing chambers.

These data support the hypothesis that MUC17 contributes to epithelial physiological function integrity and protects, in
part, epithelial cells from barrier disruption induced by enteric pathogen infection. However, these effects do not extend to amelioration of EIEC-induced ion-transport abnormalities.

Role of MUC17 in intestinal epithelial permeability. We then explored the effect of MUC17 on intestinal epithelial cell permeability before and after EIEC challenge in Caco-2 and HT29/cl19A, the two intestinal epithelial cell lines able to grow stable cell monolayers. Lack of MUC17 increased baseline permeability in both our cell line models. These results agree with the TEER findings described above. After infection with EIEC, we observed a higher and more rapid increase in permeability to FS and FITC-dextran in cells silenced for MUC17 at all time points. Figure 5A shows the results for the FITC-dextran (FS not shown) permeability assay at 3 h postinfection.
This mirrors the increased transepithelial dysfunction shown in our silenced intestinal epithelial cells after enteropathogen infection. Moreover, surprisingly, silencing of MUC17 per se induced an increase in permeability in our epithelial cell models, proposing yet another new role for mucins in maintaining barrier function. Furthermore, when MUC17 siRNA-transfected cells were pretreated with crude mucin or, with a lesser effect, with Muc3CRD, the increase in permeability induced by infection was less significant compared with naïve or scrambled cell monolayers (Fig. 5B). Although we cannot comment on the possible interaction of MUC17 with other mucins in protecting epithelial barrier function and the fact that our cell model does not express some important mucins (e.g., MUC1), nevertheless, we can conclude that the presence of MUC17 significantly strengthens barrier function and that its action is not limited to decreasing bacterial invasion.

**MUC17 preserves barrier function by stabilizing tight-junction proteins.** We then investigated whether the effect of MUC17 expression in intestinal epithelial cells had any bearing on the function of tight junctions. We found that MUC17 affected both the expression and distribution of occludin and ZO-1 (Fig. 6). When normal and silenced cell lines were exposed to EIEC infection, the effect of silencing MUC17 was reflected by a more severe loss of phosphorylated occludin (Fig. 6A) and levels of ZO-1 (Fig. 6B) both by Western blot and immunofluorescence (Fig. 6C). These data mirror the findings obtained by measurement of TEER and permeability and support further the conclusion that MUC17 may have an effect on barrier function that transcends the mere physical barrier role as previously described for other mucins.

**Effect of MUC17 expression on induction of markers of inflammation.** We then investigated whether the silencing of MUC17 had any bearing on the proinflammatory response mounted by intestinal epithelial cells after EIEC infection (46). Induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) is a well-known response of epithelial cells to invasive *E. coli* (46). All three MUC17 siRNA-transfected cells demonstrated a greater induction of iNOS and COX2 protein expression following exposure with EIEC compared with naïve or scrambled cells (Fig. 7). This effect was also significantly different when heat-killed bacteria were used compared with live bacteria for the infection. These results support the evidence that MUC17, by reducing the extent of EIEC invasion, may decrease the induction of proinflammatory markers, thus contributing to cell and tissue healing after infection. Moreover, it underscores the need of live bacteria, and thus active invasion, to produce the whole spectrum of inflammatory responses after infection with EIEC.

**DISCUSSION**

Mucin-type glycoproteins are important constituents of the protective intestinal mucosal barrier (6, 19, 27). Numerous studies have described mucin abnormalities in inflammatory bowel disease and cancer, both in animal models and patient cohorts (5, 8, 15, 18, 24, 33, 39). Gene-linkage studies have demonstrated, using microsatellite marker techniques, that a predisposition to inflammatory bowel disease (IBD) is carried on chromosome 7q22, which encompasses the chromosomal locus for a family of membrane-bound mucin genes (mucins MUC3A, MUC3B, MUC12, and MUC17) (50). Rare alleles of MUC3A have been shown to be associated with ulcerative colitis, and microarray studies have shown reduced levels of MUC3 in uninvolvved mucosa of patients with ulcerative colitis (39). These data are compatible with the concept that reduced or mutated membrane-bound mucins may contribute to a susceptibility to IBD. Moreover, increased levels of some mutated mucins have been observed in active IBD and gastrointestinal cancer (9, 12, 19). Recently, involvement of other types of mucins has been demonstrated in responses against *Helicobacter pylori* (*H. pylori*) and *Campylobacter jejuni* (*C. jejuni*) infection (29, 37, 38) and during parasitic invasion (53), and the role of an intact glycome in preserving gut mucosal homeostasis has been proven crucial (1, 56). However, very little is known about the specific mechanisms involved in mucosal protection by mucin proteins.

There is increasing evidence for the role of membrane-bound mucins in maintaining intestinal mucosal integrity. Membrane-bound mucins are characterized by an extracellular region with two CRDs with EGF-like modules coupled by a membrane (IBD) is carried on chromosome 7q22, which encompasses the chromosomal locus for a family of membrane-bound mucin genes (mucins MUC3A, MUC3B, MUC12, and MUC17) (50). Rare alleles of MUC3A have been shown to be associated with ulcerative colitis, and microarray studies have shown reduced levels of MUC3 in uninvolvved mucosa of patients with ulcerative colitis (39). These data are compatible with the concept that reduced or mutated membrane-bound mucins may contribute to a susceptibility to IBD. Moreover, increased levels of some mutated mucins have been observed in active IBD and gastrointestinal cancer (9, 12, 19). Recently, involvement of other types of mucins has been demonstrated in responses against *Helicobacter pylori* (*H. pylori*) and *Campylobacter jejuni* (*C. jejuni*) infection (29, 37, 38) and during parasitic invasion (53), and the role of an intact glycome in preserving gut mucosal homeostasis has been proven crucial (1, 56). However, very little is known about the specific mechanisms involved in mucosal protection by mucin proteins.

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recombinant Muc3 mucin with two CRD domains (CRD1-L-CRD2; also termed m3EGF1,2) stimulates cell migration in human colon cell lines, without inducing EGF activation, and inhibits cytokine-induced apoptosis (18). Furthermore, administration of recombinant Muc3 mucin CRD proteins per rectum significantly reduces mucosal ulceration and apoptosis in acetic acid or dextran sodium sulfate mouse models of colitis (18). Here we have shown that significant reduction of MUC17 severely impairs the ability of intestinal epithelial cells to respond to an enteric pathogen challenge. Our experimental design included several established colon cancer cell lines commonly used as models for colonic epithelium. However, it should be understood that these cell lines may not express the normal repertoire of mucin genes present in normal colonic mucosa, and cancer cell mucins commonly have differences in extent and type of glycosylation compared with normal colon epithelial cells (28). Even with this limitation, our data unequivocally emphasize the important role of MUC17 in limiting invasion of epithelial cells by an EIEC strain, thus reducing both loss of TEER and increased permeability, which may translate in vivo into decreased fluid loss and bacterial translocation. In addition, the finding of increased permeability when MUC17 is significantly reduced, as observed in our studies, proposes a novel role for mucins in preserving epithelial barrier function and may be a contributing factor in fluid and nutrient leakage in conditions in which the mucus layer is compromised. However, MUC17, per se, was unable to improve the alteration of chloride secretion induced by EIEC. Moreover, we have shown that MUC17 contributes significantly in maintaining cell homeostasis and modulating chronic inflammatory responses because its reduced expression induced a sustained activation of signaling pathways associated with inflammation and cancer. Our data, in part, are in agreement with the recent findings of the group of McGuckin et al. (30, 38, 57) with MUC1 and infection with gastrointestinal pathogens H. pylori and C. jejuni. McGuckin and coworkers (30, 38) demonstrated that mice lacking Muc1 (Muc1−/−) were colonized by H. pylori to a greater extent than mice with Muc1 (30, 38) and that Muc1 functions as both a detachable decoy and through steric hindrance for bacterial association. In addition, McAuley et al. (37) showed that Muc1−/− mice had increased invasion of C. jejuni through the intestinal wall compared with naïve controls and that expression of Muc1 enhanced resistance to C. jejuni cytotoxic distending toxin (37). Our findings, using exogenous mucin or Muc3CRD, indicate that both these proteins have the ability, to various extents, to attenuate enteroinvasive infection and the consequent loss of barrier function. We chose to use Muc3, the mouse homolog of MUC17, because we have extensively shown that this protein has biological effects in human colon cell lines (18). We have also shown that human colon cell lines respond similarly to Muc3CRD as with a recombinant MUC17CRD protein (34). We observed that the recombinant Muc3 CRD1-L-CRD2 protein was less effective in inhibition of EIEC invasion than a crude purified mucin preparation, indicating possibly that the full-length mucin containing the large heavily glycosylated domain may be crucial to this function. We recognize that one important issue in mucin barrier protection is represented by the interaction of the various mucins and by the inherent redundancy of the mucin proteins expressed by intestinal epithelial cells. Thus, although our findings in the Caco-2 and HT29/19A cell lines have the advantage of providing relatively clear results on the protective activity of MUC17 because of a lack of expression of other key mucins in these models (28), our data have the important limitation of failing to show the role of MUC17 in concert with other mucins that have been shown to be important in protecting the intestinal epithelium against pathogens. Moreover, it still remains to be demonstrated whether MUC17 is protective for other invasive bacteria, extracellular parasites, and viruses. Furthermore, considering that synthesis of a full-length Muc3 or MUC17 recombinant protein is not feasible because of the large size of these proteins, experiments are planned with individual mucin domains or a synthetic truncated “mini”-mucin construct to determine the mechanism of the potential protective effects in primary and “normal” intestinal epithelial cell lines producing appreciable levels of normal mucin constituents. In addition, a Muc3/MUC17 knockout mouse will provide much needed data in this regard in an in vivo model. Taken together, the finding that MUC17 mucin may protect intestinal cells from EIEC infection and that some protection may be achieved by exogenous addition of mucin proteins supports our hypothesis that intestinal MUC17 plays a role in intestinal cytoprotection and restitution and represents a potential novel therapeutic agent for intestinal barrier dysfunction attributable to infection or, possibly, other injurious agents.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

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