Transforming Growth Factor- β Regulation of Epithelial Tight Junction Proteins Enhances Barrier Function and Blocks Enterohemorrhagic *Escherichia coli* O157:H7-Induced Increased Permeability

Kathryn L. Howe, Colin Reardon, Arthur Wang, Aisha Nazli, and Derek M. McKay

From the Department of Pathology and Molecular Medicine, Intestinal Disease Research Programme, McMaster University, Hamilton, Ontario, Canada

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is an enteric pathogen that causes potentially fatal symptoms after intimate adhesion, modulation of intestinal epithelial signal transduction, and alteration of epithelial function (eg, barrier disruption). Although the epithelial barrier is critical to gut homeostasis, only a few agents, such as transforming growth factor (TGF)- β , can enhance or protect epithelial barrier function. Our aims were to delineate the mechanism(s) behind TGF- β -induced barrier enhancement and to determine whether TGF- β could prevent EHEC-induced barrier disruption. Using monolayers of the human T84 colonic epithelial cell line, we found that TGF- β induced a significant increase in transepithelial electrical resistance (a measure of paracellular permeability) through activation of ERK MAPK and SMAD signaling pathways and up-regulation of the tight junction protein claudin-1. Additionally, TGF- β pretreatment of epithelia blocked the decrease in transepithelial electrical resistance and the increase in transepithelial passage of [3 H]-mannitol caused by EHEC infection. EHEC infection was associated with reduced expression of zonula occludens-1, occludin, and claudin-2 (but not claudin-1 or claudin-4); TGF- β pretreatment prevented these changes. These studies provide insight into EHEC pathogenesis by illustrating the mechanisms underlying TGF- β -induced epithelial barrier enhancement and identifying TGF- β as an agent capable of blocking EHEC-induced increases in epithelial permeability via maintenance of claudin-2, occludin, and zonula occludens-1 levels. (*Am J Pathol* 2005, 167:1587-1597)

Infection with the intestinal pathogen enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) causes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in some individuals, the latter being a result of EHEC-derived Shiga toxins.¹ Enteropathogenic *E. coli* (EPEC) is a related pathogen that causes much morbidity and mortality by eliciting watery diarrhea.² Antibiotics, although useful in certain cases of EPEC infection, are primarily ineffective and may even be harmful during EHEC infection, leaving no effective medical therapy for infected individuals.³ Thus, understanding the interaction(s) between EHEC and the enteric epithelium may be important in defining novel therapeutic targets.

Under normal circumstances the epithelial lining of the gut forms a semipermeable barrier, the selectivity of which is in large part determined by the intercellular tight junctions. The tight junctions consist of interdigitating occludin molecules and one or more claudins that are linked to the actin cytoskeleton via a complex of proteins including the zonula occludens (ZO) 1, 2, and 3.^{4,5} Recent evidence suggests that members of the claudin family play a critical role in tight junction formation and determine the permeability characteristics in a variety of tissues including the gut.⁶ Claudin-1 in particular is believed to regulate epithelial permeability. Evidence includes the lethality of claudin-1-deficient mice,⁷ reduced claudin-1 expression after interferon (IFN)- γ -induced barrier defects in thyrocytes,⁸ increased claudin-1 expression leading to barrier enhancement of airways and kid-

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Address reprint requests to Derek M. McKay, Ph.D., Intestinal Disease Research Programme, McMaster University, HSC-3N5C, 1200 Main St. West, Hamilton, ON, Canada L8N 3Z5. E-mail: mckayd@mcmaster.ca.

ney epithelia,^{9,10} and the significant redistribution of claudin-1 at the onset of rotavirus-induced paracellular permeability in intestinal epithelial monolayers.¹¹

Investigations with model epithelial cell lines have shown that EHEC forms intimate attaching and effacing lesions on host cells by eliciting and modulating a variety of intracellular signaling pathways to reorganize the cytoskeleton while evoking a significant increase in epithelial permeability. This diminished epithelial barrier is accompanied by loss of ZO-1 from the tight junction region¹² and may be of pathological significance by allowing pathogens and antigens unimpeded access to the mucosa. Indeed, the paracellular pathway may be one way for Shiga toxin to enter the circulation because the receptor for the toxin has not been identified on human gut epithelium.¹³ Thus, agents capable of enhancing or maintaining the epithelial barrier, through preservation of the tight junction or via modulating intracellular signaling pathways that compete with those activated by bacteria, may be of value in reversing the effects of EHEC infection.

Transforming growth factor (TGF)- β is a multifunctional cytokine with immunosuppressive properties produced by many cell types including intestinal epithelia and can enhance epithelial barrier function. For instance, monolayers of human colon-derived epithelia treated with TGF- β display an increase in transepithelial electrical resistance (TER).¹⁴ Moreover, TGF- β also prevents or reduces epithelial barrier disruption caused by infection with *Cryptosporidium parvum*, exposure to IFN- γ , or exposure to conditioned medium from activated immune cells.^{14–17} However, neither the ability of TGF- β to ameliorate EHEC-induced barrier disruption nor the mechanisms underlying preservation of epithelial barrier function have been reported. Thus, the aims of the present study were 1) to define the mechanism behind TGF- β enhancement of epithelial barrier function in terms of intracellular signaling pathways and tight junction protein expression, 2) to characterize the effects of EHEC on tight junction protein expression and distribution, and 3) to determine whether TGF- β could block the EHEC-induced increase in epithelial permeability.

Materials and Methods

Cell and Bacterial Culture

T84 cells were grown and maintained in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with 10% fetal bovine serum, 1.5% HEPES, and 2% penicillin-streptomycin (all from Life Technologies, Grand Island, NY) at 37°C, 5% CO₂. Enterohemorrhagic *E. coli* (EHEC) O157:H7 (strain CL-56)¹² and enteropathogenic *E. coli* (EPEC) O127:H6 (strain E2348/69)¹⁸ were maintained on 5% sheep blood agar plates (PML Microbiologicals, Mississauga, Canada) at 4°C, cultured overnight in Luria-Bertani broth at 37°C with continuous agitation, diluted in Luria-Bertani broth, and then grown for 4 to 6 hours (to mid-logarithmic growth phase) before T84 monolayer in-

fection. The bacterial suspension was pelleted (2500 rpm, 20 minutes), resuspended in antibiotic-free T84 cell culture medium, and 10⁸ CFU were added to the apical surface of epithelial monolayers at an MOI of 100 (multiplicity of infection; 100 infectious units/epithelial cell). A laboratory strain of nonpathogenic *E. coli*, HB101, was used as a control and was cultured as described above.¹⁹

Measurement of Epithelial Barrier

T84 cells were seeded onto filter supports (10⁶ cells/well, surface area 1 cm²; Costar Inc., New York, NY) and grown to confluence (≥ 6 days), at which point TER was monitored by a voltmeter and chopstick electrodes (Millicell-ERS; Millipore, Bedford, MA). To assess the effects of TGF- β , the cytokine was added to the basolateral surface of T84 monolayers (human recombinant TGF- β ₁, 10 ng/ml; R&D Systems, Minneapolis, MN), and TER was recorded at 4, 8, 16, 24, 48, and 72 hours after treatment. Based on previous studies showing that TGF- β has barrier-enhancing effects at doses of 1 to 100 ng/ml,^{14,20} we selected a dose of TGF- β of 10 ng/ml for this study. The acute effects of TGF- β were assessed by wash out experiments, in which monolayers were exposed to TGF- β for 1 hour, rinsed twice with culture media, and maintained daily in fresh culture media with TER recorded at 24-hour intervals. For pharmacological inhibition of ERK MAPK, T84 monolayers were pretreated with the MEK1 inhibitor PD98059 (apical and basolateral, 1 hour, 25 μ M; Calbiochem, San Diego, CA²⁰), exposed to TGF- β (10 ng/ml, 1 hour), rinsed, and maintained with fresh culture media for 72 hours, and TER was then recorded.

In bacterial co-culture experiments, T84 monolayers were treated with TGF- β (10 ng/ml) 45 minutes before infection, with TGF- β remaining for the duration of infection, and TER recorded 16 hours later (a time point at which EHEC infection reduces epithelial barrier function¹²). In additional studies, 2.5 μ Ci of [³H]-mannitol (~ 180 d; Sigma Chemical Co., St. Louis, MO) was added to the apical side of filter-grown monolayers at the end of the experiment. As an inert small molecule, mannitol will cross the epithelium predominantly via the paracellular pathway. Four hours later 10- μ l and 500- μ l aliquots of culture medium were obtained from the apical and basal culture well, respectively, and radioactivity determined in a scintillation counter (Becton Dickinson, Mississauga, Canada). The transepithelial passage of the labeled mannitol was calculated.

Adenoviral Infection

T84 cells were seeded as described above and, at 4 hours after seeding, infected for 16 hours with replication-deficient adenovirus constructs encoding SMAD7 (Ad-SMAD7, MOI = 50; a generous gift from Dr. ten Dijke, The Netherlands Cancer Institute, Leiden, The Netherlands).^{21,22} Cells were rinsed, grown for 3 days, and then

treated with TGF- β (10 ng/ml, 24 hours) and TER recorded 72 hours after cytokine exposure.

Immunoprecipitation for SMAD2/3

T84 monolayers were seeded onto filter supports (3×10^6 cells/well, surface area 4.7 cm^2 ; Costar Inc.), grown to confluence, serum-starved for 24 hours, and treated with TGF- β (10 ng/ml, 5 to 60 minutes). Whole cell protein extracts were prepared, protein concentrations determined,²³ and samples (1 mg/ml in phosphate-buffered saline) incubated with gentle agitation at room temperature for 2 hours with mouse anti-human SMAD2/3 antibody (2 $\mu\text{g/ml}$; Santa Cruz Biotechnology, Santa Cruz, CA), followed by centrifugation and incubation with protein A-conjugated beads (Easy-View; Sigma-Aldrich, St. Louis, MO). Immunoprecipitated SMAD2/3 was recovered following the manufacturer's protocol and loaded immediately onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel.

Immunoblotting

Samples were prepared from T84 monolayers as described above $\pm$ TGF- β (10 ng/ml) $\pm$ bacteria (MOI = 100). Proteins (40 $\mu\text{g/sample}$) were electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and blocked in 5% bovine serum albumin in Tris-buffered saline (containing 0.1% Tween-20, TBST) or 5% milk diluted in TBST, according to the manufacturer's directions.

Phospho-SMAD2/3, SMAD2/3, and SMAD7

Blots were probed with mouse anti-human phosphoserine antibody (phospho-SMAD2/3), rabbit anti-human SMAD2/3, or goat anti-human SMAD7 diluted in bovine serum albumin-TBST (1:1000, 1:1000, and 1:750, respectively; 1 hour at room temperature; Abcam, Cambridge, UK), washed in TBST, and exposed to the appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology).

FLAG

Blots were probed with mouse anti-FLAG diluted in milk-TBST for 1 hour at room temperature (1:1000, Sigma-Aldrich), washed in TBST, and exposed to horse-radish peroxidase-conjugated rabbit anti-mouse IgG (1:3000, 1 hour room temperature; Santa Cruz Biotechnology).

Phospho-ERK1/2 and Tight Junction Proteins

Blots were either probed for 1 hour at room temperature (mouse anti-human phospho-ERK1/2, 1:800; rabbit anti-human ERK1/2, 1:3000; Santa Cruz Biotechnology) or overnight at 4°C (rabbit anti-human claudin-1, 0.5 $\mu\text{g/ml}$; rabbit anti-human claudin-2, 1.0 $\mu\text{g/ml}$; mouse anti-

human claudin-4, 1.5 $\mu\text{g/ml}$; rabbit anti-human occludin, 0.25 $\mu\text{g/ml}$; rabbit anti-human ZO-1, 1.5 $\mu\text{g/ml}$; Zymed Laboratories, San Francisco, CA), washed in TBST ($5\times$) and then exposed to the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000 to 1:15,000, 1 hour room temperature; Santa Cruz Biotechnology). Immunoreactive proteins were visualized using chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ) and exposure to Kodak XBL film (Eastman Kodak Co., Rochester, NY). Initial tight junction protein immunoblots were stripped and reprobed for β -actin (goat anti-human actin, 1:200; bovine anti-goat 1:400; Santa Cruz Biotechnology) to assess total protein loading. Subsequently, we used claudin-4 expression as an internal control because this did not change with infection or TGF- β treatment and is a tight junction protein. The densitometry ratio between the tight junction protein of interest and the internal control from each extraction was compared between control (ie, naïve) and treated epithelia.

Confocal Microscopy

T84 cells were seeded onto filter supports (10^5 cells/well, surface area 0.33 cm^2) and either treated with TGF- β for 72 hours (10 ng/ml) or infected with EHEC O157:H7 for 16 hours (MOI = 100) $\pm$ TGF- β pretreatment for 45 minutes (10 ng/ml) (as described above). Monolayers were rinsed in phosphate-buffered saline, fixed in 4% formalin, permeabilized with 0.1% Triton X-100 (Mallinckrodt Inc., Paris, KY), and blocked for 30 minutes in blocking solution [5% bovine serum albumin/5% goat serum (Sigma-Aldrich) in 0.1% Triton X-100]. Primary and secondary antibodies were diluted in blocking solution (rabbit anti-human claudin-1, 15 $\mu\text{g/ml}$; mouse anti-human claudin-2, 2 $\mu\text{g/ml}$, or rabbit anti-human ZO-1, 2 $\mu\text{g/ml}$, Zymed Laboratories; Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse IgG, 1.5 $\mu\text{g/ml}$, Molecular Probes, Eugene, OR) and exposed to monolayers for 1 hour at room temperature. After extensive washing, filters were excised from the polystyrene filter supports, and mounted on glass slides in Gel/Mount anti-fade reagent (Biomedex Corp., Foster City, CA). Images were acquired using an inverted laser-scanning microscope (LSM 510, Axiovert 100 mol/L; Zeiss, Oberkochen, Germany) equipped with argon (450 to 514 nm). Immunoreactive claudin and ZO-1 were excited using the 488-nm laser and collected using a standard fluorescein isothiocyanate filter set. For each experiment, image acquisition (ie, confocal microscope settings) and processing was identical between controls and TGF- β -, EHEC-, or TGF- β + EHEC-treated cells. Images are presented as either *en face* to illustrate the distribution of the tight junction protein immunoreactivity or as a composite Z-stack reconstruction, which shows the monolayer in transverse profile with the basally located nuclei identified by propidium iodide staining (red) and the tight junction proteins by fluorescein isothiocyanate-labeled secondary antibodies (green).

[³H]-Thymidine Incorporation

T84 cells were seeded onto 96-well plates (4×10^4 cells/well) and grown for 4 days. [³H]-Thymidine (1 μ Ci/well; DuPont-New England Nuclear, Wilmington, DE) was added for the duration of TGF- β treatment (10 ng/ml, 72 hours). Cells were harvested onto glass-fiber filter disks and assayed in 5 ml of aqueous counting scintillant for β -ray emission, and counts per minute were determined in a scintillation counter (Becton Dickinson). Results are expressed as mean cpm $\pm$ SEM.

Bacterial Growth

EHEC was cultured to mid-logarithmic growth phase (described in Cell and Bacterial Culture), resuspended in antibiotic-free T84 cell culture media $\pm$ TGF- β (10 ng/ml), and incubated at 37°C with continuous agitation for 16 hours. Bacterial growth was determined by spectrophotometric analysis of each sample (OD_{600} : $0.1 = 10^9$ CFU). In similar experiments, supernatants from naïve T84 monolayers or TGF- β -treated T84 cells were added to EHEC in mid-logarithmic growth phase, cultured as above, and OD_{600} measured.

Interleukin (IL)-8 Enzyme-Linked Immunosorbent Assay

T84 cells were seeded onto filter supports (10^6 cells/well, surface area 1 cm²), grown to confluence, and infected with EHEC $\pm$ TGF- β pretreatment (as described above) for 16 hours. Subsequently, culture medium samples were collected from the basolateral wells and frozen at -20°C until the concentration of IL-8 was determined by commercial enzyme-linked immunosorbent assay (R&D Systems). Detection limit of the assay was 16 pg/ml.

Statistical Analysis

Data are presented as means $\pm$ SEM. Data were analyzed using one-way analysis of variance followed by posthoc comparisons using Tukey's test, or Student's *t*-test, with *P* < 0.05 accepted as the level of statistical significance.

Results

TGF- β -Induced Increase in Epithelial Barrier Function Is Mediated by ERK MAPK and SMAD Signaling Pathways

Figure 1 shows that TGF- β (10 ng/ml) evoked a time-dependent increase in TER that was significant at 16 hours after treatment and more pronounced at 72 hours when there was a twofold increase greater than pretreatment values. Epithelial TER was similarly increased 72 hours after a single 1-hour pulse with TGF- β , indicating that acute exposure to this cytokine has long-term effects (Figure 1, bar labeled 72h^A). Indeed, our earlier publica-

Figure 1. Bar graph showing increased TER across T84 cell monolayers after continuous TGF- β treatment (10 ng/ml, 4 to 72 hours) or after a 1-hour pulse with cytokine (denoted as 72h^A). TER values were recorded before cytokine treatment for comparison against values at the end of each time point (indicated as percent pretreatment value), and these were compared against naïve, time-matched controls (mean $\pm$ SEM, **P* < 0.05, *n* = 3 to 10 monolayers). Pretreatment TER values were $609 \pm 189 \Omega\text{cm}^2$ and $607 \pm 158 \Omega\text{cm}^2$ for naïve controls and pre-TGF- β treatment, respectively.

tion showing that 1-hour TGF- β treatment causes alterations in epithelial ion transport 16 hours later supports this observation.²⁰ Reflecting the high TER baseline of control monolayers, there was no significant difference in [³H]-mannitol flux across control and TGF- β -treated epithelial monolayers.

TGF- β is known to activate the ERK MAPK pathway and mobilize SMAD proteins in multiple cell types.²⁴ Figure 2A is an immunoblot showing ERK 1/2 activation (ie, increased phosphorylation) in whole cell protein extracts of T84 cells after 15 and 30 minutes of TGF- β exposure. Figure 2B shows increased levels of phosphorylated SMAD2/3 30 to 60 minutes after cytokine treatment, indicating SMAD2/3 activation. SMAD7 is a naturally occur-

Figure 2. TGF- β activates ERK MAPK and SMAD signaling pathways in colonic epithelia. Immunoblot of protein extracts from serum-starved TGF- β -treated (10 ng/ml) T84 epithelial cells probed for phosphorylated ERK1/2 (p-ERK) followed by stripping and reprobing for total ERK1/2 (A), serine-phosphorylated SMAD2/3 (p-SMAD2/3) followed by stripping and reprobing for total SMAD2/3 (B), or SMAD7 protein expression (C). All panels depict representative images from three separate experiments (arrows highlight lanes where protein expression is visibly different from untreated controls).

Figure 3. Inhibition of ERK MAPK and SMAD signaling pathways prevents the TGF- β -induced increases in TER. T84 cell monolayers were pretreated with the inhibitor of ERK MAPK signaling PD98059 (PD, 25 μ mol/L) (**A**) or infected with an adenovirus encoding inhibitory SMAD7 (Ad-SMAD7, MOI = 50) $\pm$ TGF- β (10 ng/ml) (**B**) and TER values (percent change from pretreatment) were compared against naive controls and TGF- β -only treated cells (means $\pm$ SEM, * P < 0.05, n = 9 to 12 monolayers; average control TER values were 784 ± 251 Ω /cm²). **B, inset:** FLAG-tagged adenoviral SMAD7 protein expression confirming adenoviral infection.

ring inhibitor of the SMAD signaling pathway that is typically induced after TGF- β activation and provides a negative feedback mechanism for TGF- β signaling.²⁴ In TGF- β -treated T84 epithelia, SMAD7 expression was increased 4 hours after cytokine exposure and remained elevated at 24 hours (end of experiment, Figure 2C). Inhibition of ERK MAPK and SMAD signaling via the pharmacological inhibitor PD98059 and via Ad-SMAD7, respectively, completely prevented the TGF- β -induced increase in epithelial TER (Figure 3).

TGF- β Stimulates Increased Expression of the Tight Junction Protein Claudin-1

To determine the structural mechanism behind the epithelial barrier enhancement, we compared tight junction protein expression between TGF- β -treated T84 monolayers and untreated controls. TGF- β exposure (10, 50, or 100 ng/ml, 72 hours) resulted in twofold to threefold up-regulation of claudin-1, whereas neither claudin-2 nor claudin-4 were significantly affected

(Figure 4). Figure 4B (i) shows densitometry analysis of the representative blot shown in Figure 4A, and Figure 4B (ii) shows quantification of the increased claudin expression in six epithelial preparations exposed to TGF- β (10 ng/ml, 72 hours) compared to whole cell protein extracts from time-matched controls. Supporting the TER data (Figure 3), inhibition of ERK MAPK and SMAD signaling reduced the TGF- β -induced increases in claudin-1 expression (Figure 4C). The increased claudin-1 could have been retained solely in a central cytosolic position. However, confocal microscopy revealed a chicken-wire pattern of claudin-1 immunoreactivity that is typical of tight junction protein expression at the margins of polarized epithelia (Figure 4D). Moreover, assessment of z axis reconstructions showed that the claudin-1 immunoreactivity was similar in control and TGF- β -treated monolayers and was mostly confined to the apical lateral membranes. However, there was a tendency for claudin-1 to extend further down the lateral membrane in epithelia exposed to TGF- β (immunostaining was noted in the cytosolic compartment in both treated and control epithelial preparations, consistent with normal protein production and turnover). Neither occludin nor ZO-1 expression were consistently or significantly affected by TGF- β treatment (data not shown). The increased claudin-1 expression was not simply a reflection of increased proliferation because [³H]-thymidine incorporation was actually reduced after 72 hours of TGF- β exposure ($31,477 \pm 4861$ versus 5294 ± 612 cpm, control versus TGF- β respectively, n = 12; P < 0.001).

TGF- β Prevents Epithelial Barrier Dysfunction Caused by EHEC

TGF- β protects T84 monolayers against loss of barrier function caused by infection with *C. parvum*.¹⁵ Thus, we tested whether TGF- β (10 ng/ml) could prevent the barrier dysfunction caused by other pathogenic bacteria by challenging TGF- β -treated T84 monolayers with EHEC O157:H7 (MOI = 100, 16 hours). Figure 5 shows that a 45-minute TGF- β pretreatment prevented the decrease in TER caused by EHEC infection. EHEC infection caused an $\sim$ 30-fold increase in transepithelial flux of [³H]-mannitol: control = $0.49 \pm 0.13\%$ versus EHEC infection = $10.63 \pm 2.36\%$ of apical mannitol (based on cpm; n = 10 T84 monolayers from four experiments; P < 0.05). TGF- β pretreatment of the epithelial monolayer reduced the EHEC barrier disruption as gauged by [³H]-mannitol flux (control = 0.79 ± 0.09 , EHEC = 21.34 ± 3.42 , TGF- β + EHEC = $11.61 \pm 2.51\%$ of apical mannitol; n = 3 monolayers from one of two experiments).

Infection with the related enteric pathogen EPEC also causes epithelial barrier disruption.¹⁸ Similar to EHEC infection, TGF- β pretreatment protected T84 monolayers against barrier dysfunction induced by EPEC [$103.1 \pm 1.7\%$ versus $57.2 \pm 3.3\%$ pretreatment TER, respectively; * P < 0.01 compared to control pretreatment values ($95.2 \pm 3.3\%$); n = 3 monolayers].

Figure 4. TGF- β increases epithelial claudin-1 expression. **A:** A representative immunoblot of protein extracts from TGF- β -treated (72 hours) T84 cell monolayers shows increased claudin-1 expression (arrows) with exposure to all three TGF- β concentrations, whereas claudin-2 and claudin-4 were not consistently increased (blots re probed for β -actin as a control; $n = 3$). The densitometry analysis (integrated pixel intensity of claudin-1 was compared to the β -actin) of this blot is shown on the left portion of **B (i)** whereas the right portion (**ii**) shows the statistically significant increase in claudin-1 induced by TGF- β (10 ng/ml, 72 hours exposure, $n = 6$ T84 cell protein extracts). **C:** Representative immunoblots ($n = 2$) showing that TGF- β -induced increases in claudin-1 are reduced by pretreatment with PD98059 (PD, 25 μ M/L) or infection with Ad.SMAD7 to block ERK MAPK and SMAD2/3 signaling, respectively. **D:** *En face* confocal scanning laser microscopy reveals a chicken wire pattern of claudin-1 immunoreactivity in control epithelia, which is slightly more prominent in TGF- β -treated T84 cell monolayers (images representative of $n = 8$ monolayers). The corresponding Z-stack series below each panel (**i**) shows that the majority of the claudin-1 immunoreactivity (green) is in the lateral cell membrane in the apical compartment of the cell above the nucleus (red) and that TGF- β treatment may result in more claudin-1 along the lateral cell membrane. Original magnifications, $\times 1260$.

EHEC Infection Causes Reduced Tight Junction Protein Expression

Infection with pathogenic bacteria, such as EHEC, EPEC, and *Shigella flexneri*, decreases expression of tight junctions and associated proteins.^{12,25–28} However, little is known about their effects on claudin protein expression. Figure 6A shows representative immunoblots from an analysis of tight junction proteins in our model system. Corroborating our earlier immunolocalization studies,¹² ZO-1 expression was reduced by $34 \pm 3\%$ 16 hours after EHEC infection ($n = 9$ T84 cell extracts from three experiments). Similarly, occludin expression was reduced by $27 \pm 5\%$ by EHEC infection compared to controls ($n = 6$ T84 cell extracts from two experiments). Of the three claudins examined, only claudin-2 was significantly reduced by EHEC: claudin-2 expression was significantly reduced by $16 \pm 2\%*$ compared to controls ($n = 15$ T84 cell extracts from five experiments, $*P = 0.03$; Figure 6B).

Figure 5. Bar graph showing that T84 cell monolayers pretreated with TGF- β (45 minutes, 10 ng/ml) exhibit decreases in TER caused by EHEC (16 hours after infection). Controls consisted of untreated monolayers or infection with the nonpathogenic *E. coli* HB101 (HB101). Data are presented as the percent change in TER compared to pretreatment values (means $\pm$ SEM, $*P < 0.05$ compared to untreated control and *E. coli* HB101-treated monolayers; $n = 6$ to 12 monolayers). Note that TGF- β treatment alone causes a small, but significant increase in TER after 16 hours ($*P < 0.05$ compared to control), consistent with the time-course data presented in Figure 1. Average pretreatment TER values ranged between 632 to 931 $\Omega \cdot \text{cm}^2$.

Figure 6. EHEC reduces epithelial tight junction protein expression. **A:** Representative immunoblots for tight junction proteins in protein extracts from T84 cell monolayers 16 hours after EHEC infection (arrows indicate reduced expression; representative of six to nine monolayers from three experiments; doublets in occludin and ZO-1 immunoblots likely indicate phosphorylated and nonphosphorylated forms). **B:** Claudin-2 is significantly reduced by comparison of claudin-2:claudin-4 ratio in five experiments (each symbol indicates the average of three epithelial preparations/experiment).

Complementing TGF- β prevention of EHEC-induced increased in epithelial paracellular permeability, immunoblotting revealed that claudin-2, occludin, and ZO-1 expression was preserved in EHEC-infected T84 monolayers pretreated with TGF- β (Figure 7A). Furthermore, confocal microscopy revealed an obvious disruption of the normal distribution of claudin-2-like immunoreactivity in EHEC-treated T84 cell monolayers, an effect that was virtually completely prevented if the cells were pretreated with TGF- β (10 ng/ml, 45 minutes) (Figure 7C). In addition, the accompanying Z-stack compilation images in Figure 7C (each one below the appropriately labeled en-

face image) not only confirm the lack of claudin-2 expression in EHEC-treated epithelia, they also demonstrate that although claudin-2 can be found throughout the cell, there is clear accumulation of the immunoreactivity at the cell's apical pole and juxtaposed to a location compatible with the tight junction. The distribution of claudin-2 immunoreactivity in control cells (Ci) and in TGF- β + EHEC (TEi)-treated T84 cell monolayers was virtually indistinguishable (Figure 7C). Finally, confocal microscopic assessment revealed that epithelial monolayers treated with TGF- β displayed ZO-1 immunoreactivity patterns comparable to controls. In contrast, EHEC-infected T84 cells (MOI = 100, 16 hours) had strikingly reduced ZO-1 protein expression (matching the immunoblot analysis). TGF- β pretreatment (45 minutes, 10 ng/ml) prevented this EHEC-induced rearrangement of ZO-1 (Figure 7D).

TGF- β -Induced Barrier Protection Is Independent of an Effect on Bacterial Growth

Because cytokines can possess antimicrobial activity,²⁵ we tested whether TGF- β itself, or epithelial factors induced by TGF- β (eg, antimicrobials), could impair EHEC growth. TGF- β did not directly affect EHEC viability after 16 hours of co-incubation (OD_{600} : EHEC 1.3 ± 0.1 versus TGF- β + EHEC 1.3 ± 0.1 ; $n = 2$). Bacterial growth was similarly unaffected by supernatants from TGF- β -treated T84 monolayers (data not shown), suggesting that an epithelial-derived factor was also not involved in the preservation of epithelial barrier function via induction of bacterial cell death or inhibition of bacterial proliferation.

TGF- β Does Not Prevent EHEC-Induced IL-8 Production by Intestinal Epithelia

EHEC infection induces IL-8 production from intestinal epithelia.²⁹ Given the protective effects of TGF- β on barrier function, we tested whether pretreatment with TGF- β would prevent EHEC-induced IL-8 production from T84 monolayers. In comparison to untreated control monolayers or TGF- β -treated epithelia (which produced 108 ± 18 pg/ml and 130 ± 20 pg/ml of IL-8, respectively), EHEC-infected T84 cells produced significant amounts of IL-8 that was not prevented by TGF- β pretreatment ($193 \pm 27^*$ pg/ml and $186 \pm 27^*$ pg/ml, respectively; $*P < 0.05$ compared to control and TGF- β only; $n = 5$ to 6 monolayers).

Discussion

Intestinal epithelia serve multiple physiological roles, including the provision of a defensive barrier to protect against potentially harmful luminal contents. The importance of maintaining an intact barrier is underscored by recent reports stating increased intestinal permeability contributes to the pathogenesis of several enteric disorders, including inflammatory bowel disease.³⁰ Thus, our aims were to define the mechanism behind TGF- β -induced barrier enhancement and to determine whether

Figure 7. TGF- β pretreatment prevents EHEC-induced reductions in tight junction protein expression. **A:** Representative immunoblot showing that TGF- β (10 ng/ml) prevents EHEC (MOI = 100, 16 hours)-induced reduction in claudin-2, occludin, and ZO-1. The events are quantified in **B** using the integrated pixel intensity of the tight junction protein:claudin-4 bands ($n = 3$ to 6 separate experiments, each indicated by a different symbol). **C:** Shown is the epithelial distribution of claudin-2-like immunoreactivity both as *en face* images and in corresponding composite Z-stack images below each panel (control, Ci; TGF- β , Ti; EHEC, Ei; TGF- β +EHEC, Tei) (images taken in the x - y plane using confocal microscopy; $n = 5$ monolayers). The diffuse peripheral claudin-2 immunoreactivity in control preparations is lost in EHEC-infected cells and restored to a large extent by TGF- β pretreatment. The z axis images show that although claudin-2 immunoreactivity (green) can occur throughout the cell, the majority occurs in an apical location (nucleus shown as red based on propidium iodide staining) and is located at the tight junction (note intense punctate staining that indicates tight junction regions of adjacent cells). Similarly, and as shown in **D**, confocal microscopy confirms that TGF- β pretreatment prevents EHEC (MOI = 100, 16 hours)-induced disruption of the distribution of ZO-1. Representative images of T84 cell monolayers immunostained for ZO-1 taken in the x - y plane using confocal scanning laser microscopy ($n = 5$ to 7 monolayers; **arrows** denote loss of ZO-1 at the cell periphery and the increased punctate appearance of ZO-1 is highlighted by the **asterisk**). Original magnifications, $\times 1260$.

this cytokine could prevent epithelial barrier disruption caused by infection with pathogenic bacteria, principally EHEC. We show that TGF- β enhances the epithelial barrier via ERK MAPK and SMAD signaling pathways and up-regulates claudin-1 protein expression. Moreover, we determined that TGF- β preserves the epithelial barrier during EHEC infection and prevents EHEC-induced loss of specific tight junction and tight junction-associated proteins, namely claudin-2, occludin, and ZO-1.

This study corroborates our earlier findings²⁰ and those of others,¹⁶ showing that TGF- β enhances the epithelial barrier (at least as gauged by TER). Subsequent molecular assessment of TGF- β -treated T84 epithelia revealed an expected increase in ERK MAPK and SMAD2/3 activation as judged by phosphorylation on immunoblots. Moreover, use of inhibitors of ERK and

SMAD2/3 signaling blocked TGF- β -enhancement of barrier function. Whether these pathways regulate epithelial paracellular permeability independently or via intracellular cross-talk (Figure 8)³¹ remains to be determined. TER, although primarily reflective of tight junction permeability, can also be influenced by membrane ion channels and transporter activity. We found that TGF- β reduced expression of a Cl^- channel (ie, the CFTR).³² However, unlike the present study the ion transport effect was insensitive to ERK blockade with PD98058,²⁰ suggesting that the TGF- β -induced increased TER is due to modulation of the tight junction. Also noteworthy, is the fact that a 1-hour TGF- β pulse resulted in increased TER 72 hours later. This lasting effect of TGF- β on epithelial barrier enhancement is unlike the effects of other cytokines, for example IFN- γ , in which the cytokine-induced barrier

Figure 8. Model showing putative mechanisms of TGF- β enhancement of epithelial transepithelial resistance and prevention of EHEC-induced barrier disruption but not IL-8 production (*TGF- β ; added 45 minutes before EHEC and epithelium assessed 16 hours later).

defect is not observed with an exposure of less than 6 hours duration (D. M. McKay, unpublished observation).

Having observed physiological changes indicative of altered epithelial tight junction function, we assessed putative changes in the structural elements of the junction. TGF- β exposure led to increased protein expression of claudin-1, but not claudin-2, claudin-4, occludin, or ZO-1. The increased claudin-1 expression was reduced by use of inhibitors of ERK and SMAD2/3 signaling. Immunolocalization studies confirmed that the claudin-1 protein was being trafficked to the site of the tight junctions and that it was indeed possibly extending further down the lateral membrane compared to the pattern of immunoreactivity in control T84 cell monolayers. Our data are supported by the work of Kinugasa and colleagues,³³ who found that IL-17 enhances development of the paracellular barrier in immature T84 epithelial monolayers by activation of ERK MAPK and up-regulation of claudin-1 and claudin-2 mRNA and protein expression. However, unlike IL-17, TGF- β did not increase claudin-2 protein expression. This difference indicates cytokine-specific effects on tight junction proteins and suggests distinct roles for claudins during epithelial monolayer development versus barrier enhancement of an established monolayer. Indeed, the protein kinase C agonist bryostatin-1 enhances the barrier function of confluent T84 monolayers by recruiting claudin-1, ZO-2, and occludin to the tight junction complex.³⁴ Although the contribution of other tight junction proteins is less clear, up-regulation of claudin-1 appears to be a common mechanism by which colonic epithelial barrier function can be established and/or enhanced.

In addition to barrier enhancement, TGF- β preserves intestinal epithelial barrier function after exposure to agents known to cause barrier disruption, such as IFN- γ , supernatants from superantigen-stimulated immune cells, and infection with *C. parvum*.^{14,15,17} Here we show that TGF- β protects against intestinal epithelial barrier

dysfunction caused by infection with EHEC (and EPEC), which is not due to effects on EHEC growth or viability. EHEC and EPEC infections are associated with tight junction changes. Specifically, EHEC infection disrupts the distribution of ZO-1,¹² and EPEC causes dephosphorylation of occludin and its subsequent dissociation from the tight junction.²⁶ We observed that pretreatment with TGF- β prevented the EHEC-induced reductions in occludin and ZO-1 and the disruption of ZO-1 distribution. Also, considering the important role of claudin proteins in tight junction formation³⁵ and the claudin-4 epithelial barrier disruption caused by *Clostridium perfringens*,³⁶ we examined the effects of EHEC infection on claudin expression. Our data show that in the colon-derived T84 cell line, EHEC specifically affects claudin-2 but not claudin-1 or claudin-4, as exhibited by the significant reduction in claudin-2 protein expression and dissociation from the tight junction (preliminary data suggest that EPEC similarly affects claudin-2 expression; unpublished observation). The EHEC-induced reduction in claudin-2 expression was significantly less pronounced in TGF- β -treated cells, correlating with the preservation of the other tight junction proteins (ie, occludin, ZO-1) and the maintenance of the epithelial barrier (ie, TER, [³H]-mannitol flux). At present, the exact role of claudin-2 in tight junction barrier function is unclear. Furuse and colleagues,³⁷ showed that introduction of claudin-2 into high-resistance Madin-Darby canine kidney (MDCK) epithelia caused decreased TER and led to the concept that claudin-2 forms conductive pores within tight junction strands. Indeed, claudin-2 has since been shown to induce cation-selective channels in tight junctions in the MDCK cell line.³⁸ In contrast, induction of claudin-2 expression in low-resistance MDCK cells led to a 20% increase in TER.³⁹ Furthermore, using T84 cells monolayers Nishiyama and colleagues⁴⁰ found that increased TER was associated with up-regulation of claudin-2 protein expression at the tight junction. Similarly, the loss of barrier integrity observed in IFN- γ + tumor necrosis factor- α -treated T84 cell monolayers was accompanied by reduced claudin-2 expression,⁴¹ and, conversely, the enhancement of the barrier property of Caco-2 epithelial cells (another human colon-derived cell line) by retinoic acid was associated with increased claudin-2 expression.⁴² Thus, the exact role of claudin-2 in epithelial barrier function is controversial, may be cell-type-specific, and may be influenced by the other constituents of the tight junction. Collectively our data suggest that the structural basis for barrier preservation by TGF- β lies in preventing EHEC-mediated down-regulation of claudin-2, occludin, and ZO-1 protein expression and in maintaining the normal distribution of these proteins. Because we found no evidence of direct TGF- β up-regulation of claudin-2 expression, we speculate that TGF- β antagonism of EHEC-induced disruption of epithelial barrier function could be due to interference with a bacteria-induced epithelial signaling pathway⁴³ or stabilization of claudin-2 mRNA (Figure 8).

In terms of other effects on host cell function by EHEC, Dahan and colleagues⁴⁴ determined that *S. bouardii* significantly decreased epithelial IL-8 secretion during EHEC infection by inhibiting the MAPK and nuclear factor

(NF)- κ B signaling pathways responsible for this proinflammatory response. Given the anti-inflammatory properties of TGF- β , we tested the ability of TGF- β to prevent epithelial IL-8 secretion during EHEC infection. TGF- β did not prevent EHEC-induced epithelial IL-8 secretion, indicating that the effect of TGF- β was specific for protection against EHEC-mediated disruption of the epithelial barrier. However, we cannot exclude the possibility that TGF- β may regulate other aspects of an EHEC-induced proinflammatory response. For example, TGF- β exerts anti-inflammatory properties against *Bacteroides vulgatus*- and lipopolysaccharide-mediated NF- κ B activation and subsequent IL-6 secretion in intestinal epithelia.⁴⁵ Because activation of NF- κ B regulates not only IL-8 gene expression but also the expression of several other proinflammatory mediators such as tumor necrosis factor- α and IL-6,^{46,47} TGF- β may exert anti-inflammatory properties against EHEC-induced expression of such genes. Finally, EHEC-derived Shiga toxin and its translocation across the epithelium pose a significant biological threat to renal function that may culminate in hemolytic uremic syndrome. It is therefore noteworthy that elevated levels of TGF- β are associated with reduced incidence of hemolytic uremic syndrome in children infected with EHEC O157:H7,⁴⁸ making it feasible that increased TGF- β enhances epithelial barrier function and limits toxin entry into the body.

TGF- β influences many aspects of intestinal epithelial homeostasis. We found that TGF- β enhancement of epithelial barrier function was associated with increased claudin-1 expression, an ERK MAPK- and SMAD2/3-dependent event. In addition, preservation of epithelial barrier integrity in the face of EHEC infection by TGF- β was accompanied by maintenance of the levels and distribution of claudin-2, occludin, and ZO-1 (Figure 8). Thus, awareness of TGF- β enhancement of epithelial barrier function is now complemented by data on the intracellular signaling pathways and structural modification of the tight junctions that mediate this physiological and protective effect of TGF- β in the intestine. Overall, the data presented offer compelling evidence that differential regulation of tight junction protein expression, particularly claudins, by TGF- β provides beneficial effects capable of enhancing the epithelial barrier and protecting it against severe insult by pathogenic bacteria.

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