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Epidermal Growth Factor Inhibits *Campylobacter jejuni*-Induced Claudin-4 Disruption, Loss of Epithelial Barrier Function, and *Escherichia coli* Translocation▽

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***Campylobacter jejuni* is a leading cause of acute bacterial enteritis in humans. Poultry serves as a major reservoir of *C. jejuni* and is thought to act as a principal vehicle of transmission to humans. Epidermal growth factor (EGF) is a small amino acid peptide that exerts a broad range of activities on the intestinal epithelium. The aims of this study were to determine the effect of EGF on *C. jejuni* intestinal colonization in newly hatched chicks and to characterize its effects on *C. jejuni*-induced intestinal epithelial barrier disruption. White Leghorn chicks were treated with EGF daily, starting 1 day prior to *C. jejuni* infection, and were compared to control and *C. jejuni*-infected, EGF-treated chicks. Infected chicks shed *C. jejuni* in their feces throughout the study period. *C. jejuni* colonized the small intestine and cecum, disseminated to extraintestinal organs, and caused jejunal villus atrophy. EGF reduced jejunal colonization and dissemination of *C. jejuni* to the liver and spleen. In EGF-treated *C. jejuni*-infected chicks, villus height was not significantly different from that in untreated *C. jejuni*-infected chicks or controls. In vitro, *C. jejuni* attached to and invaded intestinal epithelial cells, disrupted tight junctional claudin-4, and increased transepithelial permeability. *C. jejuni* also promoted the translocation of noninvasive *Escherichia coli* C25. These *C. jejuni*-induced epithelial abnormalities were abolished by pretreatment with EGF, and the effect was dependent upon activation of the EGF receptor. These findings highlight EGF's ability to alter colonization of *C. jejuni* in the intestinal tract and to protect against pathogen-induced barrier defects.**

Campylobacter was first recognized as a human pathogen in the early 1970s and has since emerged as a leading cause of food-borne bacterial enteritis in the developed world (12, 22). *Campylobacter*-associated human enteritis ("campylobacteriosis") is characterized by fever, abdominal pain, intestinal inflammation, and diarrhea. While most cases of campylobacteriosis are self-limiting, infection is implicated as a risk factor for postinfectious complications, such as Guillain-Barré syndrome and bacteremia (38, 50). Moreover, *Campylobacter* is thought to be one of the most common causative microorganisms of postinfectious irritable bowel syndrome (44).

Campylobacter jejuni infection is strongly associated with the handling and consumption of poultry meat. Several studies have shown that poultry serotypes of *C. jejuni* are associated with human infection (1, 26). Furthermore, the incidence of human campylobacteriosis has been shown to coincide with *C. jejuni* contamination of poultry carcasses (18, 57). Poultry is generally colonized by *C. jejuni* at approximately 2 weeks of age (13, 24). Despite extensive intestinal colonization, chickens usually do not show overt signs of illness. Nonetheless, weight reduction (30, 52), hepatitis (2, 37), diarrhea, and mortality (8, 52, 54) have been reported for infected poultry. Moreover, *Campylobacter* can disseminate to various internal organs, such as the thymus, spleen, liver, gallbladder, and bursa of Fabricius, via mechanisms that remain obscure (10).

Intercellular tight junctions serve to restrict paracellular passage of luminal bacteria into the underlying mucosal tissues. Pathogenic microorganisms have evolved sophisticated strategies to target tight junctions and thereby circumvent the intestinal epithelial barrier. Recently, *C. jejuni* 81-176, a human-pathogenic strain, was shown to disrupt tight junctional occludin and to compromise epithelial barrier integrity (9, 33). Whether this may contribute to dysfunction of the intestinal epithelial barrier of poultry remains unclear.

Reducing *Campylobacter* colonization in poultry may reduce the risk of campylobacteriosis and its associated postinfectious sequelae in humans. Attempts to reduce *Campylobacter* colonization in poultry have focused mainly on the administration of probiotic and competitive exclusion bacteria (40, 64) and diet formulations (16). These strategies have had variable effectiveness.

Epidermal growth factor (EGF) is a 53-amino-acid peptide with a broad range of bioactivities on the intestinal epithelium, including the stimulation of cellular proliferation, differentiation, and intestinal maturation (49). EGF is produced and secreted from numerous sites along the intestinal tract as well as in saliva, bile secretions, and breast milk (49). Many cell types, including those of epithelial lineage, express the EGF receptor (EGFR) (62, 63). EGFR is found along the length of the intestine (60) and on both the luminal and basolateral surfaces of epithelial cells (62, 63). EGFR exists in many species, including humans, cattle, pigs, dogs, mice, rats, and poultry, and the receptor in chickens recognizes human EGF (32,

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49, 60, 62, 63). Previous studies have shown that EGF administration plays a protective role in a variety of intestinal insults by either reducing injury (4) or accelerating repair (7, 21, 51). Of particular interest to the context of this study is the finding that EGF can reduce colonization of the intestinal epithelium by enteropathogens (4–6). Administered prophylactically to weanling rabbits challenged with attaching-effacing *Escherichia coli*, oral EGF prevents enteropathogenic *E. coli* (EPEC)-induced diarrhea, reduces intestinal colonization, and prevents a reduction in weight gain (4). The effects of EGF on *C. jejuni* infection have yet to be investigated. Whether or not EGF may alter infection by an invasive pathogen such as *C. jejuni* remains obscure.

The aims of this study were (i) to investigate the effects of *C. jejuni* infection in poultry, (ii) to characterize the effects of *C. jejuni* on intestinal epithelial tight junction structure and function, and (iii) to assess the effects of EGF on these microbe-host interactions.

MATERIALS AND METHODS

Bacteria and enumeration. *C. jejuni* strain RM1221, isolated from a chicken carcass, was kindly provided by William G. Miller (United States Department of Agriculture) and maintained in 40% glycerol at −70°C. For use in all experiments, *C. jejuni* was grown on Karmali agar (Oxoid, Nepean, Ontario, Canada) for 48 h at 37°C in gas jars (Oxoid). Microaerobic conditions (≈5% O_2, 10% CO_2, 85% N_2) were generated with CampyGen sachets (Oxoid). To prepare an inoculum for in vitro and in vivo studies, a single colony was suspended in Casamino yeast extract broth (25 ml containing 3% Casamino Acids, 0.4% yeast extract, 0.05% KH_2PO_4, and 0.1% $FeSO_4$ [all from Sigma, St. Louis, MO]). The broth culture was incubated microaerobically at 37°C (100 rpm) for 17 h (late log phase). Bacterial concentrations were estimated using optical density readings and confirmed by CFU enumeration on brucella agar (Becton Dickinson, NJ).

E. coli C25, a nonpathogenic, streptomycin-resistant strain, was generously provided by E. Deitch (New Jersey Medical School). Stock cultures of *E. coli* C25 were maintained at −70°C in MacConkey broth (Becton Dickinson) coating Microbank porous beads (Pro-Labs Diagnostics, Richmond Hill, Ontario, Canada). At the time of study, one bead was placed into 25 ml of MacConkey broth and incubated at 37°C (100 rpm) for 12 h (late log phase). Bacterial concentrations were estimated using optical density readings and confirmed by CFU enumeration on MacConkey agar.

Chicken model. White Leghorn chicks (Rochester Hatcheries, Westlock, Alberta, Canada) were separated by treatment group and housed in a level 2 biohazard compliant facility at the Life and Environmental Sciences Animal Resource Centre (University of Calgary). All experimental procedures were carried out according to the guidelines established by the Canadian Council of Animal Care. All procedures were approved by the University of Calgary Committee on Animal Care.

Day-of-hatch chicks were arbitrarily divided into the following four treatment groups: (i) controls (n = 11); (ii) *C. jejuni*-infected chicks (n = 15); (iii) *C. jejuni*-infected, EGF-treated chicks (n = 15); and (iv) EGF-treated chicks (n = 8). EGF was administered by oral gavage with a blunt feeding needle. Starting on day 0 (day of arrival), chicks were treated daily with phosphate-buffered saline (PBS) or human recombinant EGF (100 μg/kg of body weight in PBS; AB Biopharma, Calgary, Alberta, Canada). This concentration is consistent with the dosage used in other in vivo studies and is within the physiological range that intestinal microbes may encounter (4, 20). On day 1, chicks were fed sterile broth (500 μl) or *C. jejuni* (500 μl containing 10^9 CFU/ml) by gavage.

Weight gain, signs of diarrhea (pericloacal soiling), and fecal shedding of *C. jejuni* were examined at the same time each day (±1 h). To assess fecal shedding, *C. jejuni* was isolated from cloacal swabs by streaking of samples onto *Campylobacter* blood-free selective agar containing antibiotic selective supplement SR0155E (CCDA+ agar; Oxoid) and incubating the agar dishes at 37°C under microaerobic conditions. Agar dishes were examined for the presence of *C. jejuni* at 48 h and were scored as positive or negative.

Chicks were sacrificed by cervical dislocation on day 14, and the intestinal tract, from the gizzard to the large intestine, was removed aseptically. Tissue from the jejunum was taken 5 cm distal to the duodenal loop. A 3-cm-long section of jejunum was removed and divided into three equal segments for sample analysis (i.e., bacterial enumeration, disaccharidase activity, and histopathologic examination). Segments of liver, spleen, and cecum were also removed and prepared for bacterial enumeration.

Isolation and quantification of *C. jejuni* from chick tissues. Luminal digesta were removed from the jejunum by washing with PBS. Jejunum samples were then weighed, homogenized, and sonicated in brucella broth (1:10 [wt/vol]). Spleen and liver samples were removed aseptically, weighed, homogenized, and sonicated in brucella broth (1:5 [wt/vol]). Tissue homogenates were serially diluted and spot plated on CCDA+ agar, and dishes were incubated microaerobically at 37°C for 48 h. Colonies were enumerated at a dilution yielding 30 to 300 CFU per dish. Bacterial colonization was expressed as $\log_{10}$ CFU/g of tissue.

Sucrase activity. Jejunal sucrase activity was measured as an additional marker of mucosal integrity. Jejunal segments (1 cm) were weighed and homogenized in 2.5 mM EDTA (1:10 [wt/vol]). Samples were snap-frozen and stored at −70°C. Sucrase activity was measured according to the method of Dahlqvist (11). In brief, each sample (100 μl) was coincubated with substrate (100 μl of 19.1-mg/ml sucrose in maleate buffer) in two separate borosilicate test tubes (VWR, West Chester, PA). One set of tubes, used to measure background endogenous glucose in the sample, was incubated in boiling water for 2 min to denature the enzymes and stop the reaction. The second set of tubes was placed in a 37°C water bath for 30 min, followed by 2 min in boiling water. Glucose oxidase reagent (3.0 ml) was added to each tube, and the tubes were incubated at 37°C for 1 h. A colored product of *O*-dianisidine was produced based on the amount of glucose liberated in the sample. The samples were transferred from the tubes to a 96-well plate, and the colorimetric reaction was quantified by reading the absorbance (A_{450}) of the samples on a microplate reader (ThermoMax microplate reader; Molecular Devices, Sunnyvale, CA). The total protein concentrations of the samples were determined by the Bradford protein assay (Bio-Rad, Mississauga, Ontario, Canada), and sucrase activity was expressed as units/g protein. Both protein and sucrase concentrations were determined relative to a standard curve.

Crypt-villus measurements. For histopathologic examinations, tissue from the jejunum was fixed in fresh 2% paraformaldehyde for 24 h at 4°C. Tissue was embedded in paraffin wax, sectioned (5 μm), stained with hematoxylin and eosin, and examined under a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). Assessments were blinded by covering slide labels prior to measurements. Crypts and villi were measured using a calibrated micrometer. Measurements were obtained from 10 uninterrupted crypt-villus units per slide. The mean of these 10 measurements was considered an individual observation for each group. Photomicrographs were obtained using a Photometrics CoolSnap camera (Roper Scientific, Tucson, AZ) and were processed with Openlab (version 3.03; Improvision, Guelph, Ontario, Canada).

Cell culture. All in vitro experiments were performed using SCBN cells. SCBN cells are nontumorigenic canine intestinal epithelial cells that form polarized confluent monolayers. This cell line has been used extensively to study epithelial pathophysiology and epithelial barrier function. The monolayers express tight junctional proteins, including claudin-4, and have a high transepithelial electrical resistance (TER) (15). SCBN cells (passages 21 to 29) were grown in tissue culture flasks at 37°C with 5% CO_2 and 96% humidity in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, 0.8 mg/ml tylosin, and 200 mM L-glutamine (all from Sigma). Cell culture medium was replenished every 2 to 3 days, and 12 h prior to all experiments, cells were given medium without antibiotics and FBS. Cells were passaged using 5× trypsin-EDTA (Sigma). Trypsinized cells (400 μl; 2.0×10^4 cells/ml) were seeded into Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) for immunohistochemistry experiments. For adherence and invasion experiments, cells (2.0 ml; 2.0×10^4 cells/ml) were added to six-well plates (Costar, Cambridge, MA). For Western blotting experiments, cells (4.0 ml; 2.0×10^4 cells/ml) were added to sterile 60-mm by 15-mm tissue culture dishes (Becton Dickinson). To investigate in vitro permeability and bacterial translocation, cells (500 μl; 2.0×10^5 cells/ml) were added to the apical compartments of Transwell filter units (1.13-cm^2 filter; 0.4-μm or 3-μm pore size [Costar]).

In vitro studies. All in vitro experiments contained the following groups: (i) controls, (ii) *C. jejuni*-treated chicks, (iii) *C. jejuni*- and EGF-treated chicks, and (iv) EGF-treated chicks. *C. jejuni* was added to the apical compartment of SCBN monolayers at a multiplicity of infection of 100. Control monolayers received an equivalent volume of sterile broth. EGF (100 ng/ml) was applied to the apical compartment, 20 min prior to challenge with *C. jejuni*, as previously described (5, 6). The volume of medium in each well was 500 μl. The volume of "challenge" solution added to each well was 60 μl. Monolayers were incubated microaerobically at 37°C. To assess whether effects were due to EGFR activation, monolayers were pretreated for 30 min with the selective EGFR tyrosine kinase inhibitor PD153035 (1 μM; Calbiochem, La Jolla, CA), or vehicle, in some

experiments. To assess whether EGF had direct microbicidal activity, EGF (100 ng/ml) was added to a bacterial broth culture, and bacteria were enumerated hourly (for 5 h) as described above.

Adherence and invasion. Confluent SCBN monolayers were treated for 3 h with or without *C. jejuni* and/or EGF (as described above). To measure adherence, monolayers were washed three times with sterile PBS and permeabilized with 1% Triton X-100 (30 min at 37°C; Sigma). To measure invasion, monolayer cells were treated with 100 μg/ml of gentamicin (Sigma) for 1 h (37°C) to kill extracellular bacteria. Cells were then washed three times with sterile PBS and permeabilized with 1% Triton X-100 (30 min at 37°C). The number of *C. jejuni* cells in permeabilized extracts was enumerated as described above. Bacterial numbers were $\log_{10}$ transformed before statistical analysis.

Paracellular permeability. (i) TER. SCBN cells were grown to confluence (TER, ≥1,000 Ω/cm^2) for 7 days on Transwell filters (0.4-μm pore size). To confirm monolayer confluence, TER was measured using an electrovoltohmmeter (World Precision Instruments, Sarasota, FL) as previously described (15). Changes in TER were assessed 2 h and 6 h after inoculation with *C. jejuni*.

(ii) Dextran flux. The flux of a 3-kDa nonabsorbable tracer solute across confluent polarized monolayers was assessed as previously described (15). Briefly, SCBN cells were grown to confluence on Transwell filters (0.4-μm pore size) and treated with or without *C. jejuni* and/or EGF (as described above). Following 6 h of incubation, monolayers were washed twice with sterile Ringer's solution, a fluorescein isothiocyanate (FITC)-conjugated dextran probe (500 μl [100 μM in Ringer's solution]; Molecular Probes, Eugene, OR) was added to the apical chamber, and 1 ml of Ringer's solution was added to the basolateral chamber. After 3 h, two samples (300 μl) were taken from the basolateral chamber, and relative fluorescence was calculated using a microplate fluorometer (Spectra Max Gemini; Molecular Devices). Values were expressed as the percent apical FITC-dextran/cm^2/h that crossed the Transwell membrane.

(iii) Bacterial translocation. SCBN cells were grown to confluence on Transwell filters (3.0-μm pore size) and treated with or without *C. jejuni* and/or EGF (as described above). After 2, 4, 6, 8, 12, and 24 h, the basolateral medium was removed and serially diluted, and *C. jejuni* was enumerated as described above. To assess whether *C. jejuni* facilitates translocation of nonpathogenic, noninvasive bacteria, monolayers were treated with or without *C. jejuni* and/or EGF (as described above). After 6 h, monolayers were inoculated apically with *E. coli* C25 (multiplicity of infection, 100) and incubated at 37°C. After 3 h, the basolateral medium was removed and serially diluted, and *E. coli* cells were enumerated as described above.

Immunofluorescence microscopy. SCBN cells were grown to confluence on chamber slides and treated with or without *C. jejuni* and/or EGF (as described above). After 6 h, cells were washed once with PBS, fixed with −20°C methanol for 20 min, washed three times with PBS (for 10 min each time), and permeabilized with 0.5% Triton X-100 (Sigma) for 15 min. Nonspecific antibody binding was blocked by incubation with FBS (Sigma) for 15 min. Monolayers were washed three times with PBS (for 10 min each time) and incubated for 1 h (37°C) with a mouse anti-claudin-4 monoclonal antibody (1:200 dilution; Zymed Laboratories). Cells were washed with PBS and then incubated for 1 h (37°C) with Cy3-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:100 dilution; Sigma). Monolayers were mounted with Aqua Poly-Mount (Polysciences, Warrington, PA) and visualized using a Leica DMR fluorescence microscope. Photomicrographs (12 to 15 areas for each of eight monolayers per group from two separate experiments) were obtained using a Photometrics CoolSnap camera (Roper Scientific, Tucson, AZ) and processed with Openlab (version 3.03; Improvision, Guelph, Ontario, Canada).

Western blotting for claudin-4. Confluent SCBN monolayers were treated with or without *C. jejuni* and/or EGF (as described above). After 6 h, cells were lysed for 30 min (4°C) in cell lysis buffer (200 μl; 1% Igepal CA-630, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate in PBS [all from Sigma]) containing a protease inhibitor tablet (Complete mini; Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged (10,000 × *g*, 10 min, 4°C). Protein concentrations in the supernatants were measured using the Bradford protein assay (Bio-Rad). Proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham, Buckinghamshire, England). Membranes were incubated for 1 h in 5% nonfat milk powder (in Tris-buffered saline containing 0.1% Tween 20 [TTBS]) and then for 1 h with mouse anti-claudin-4 monoclonal antibody (1:1,000 dilution in TTBS plus 2% bovine serum albumin [Zymed]), washed with TTBS, and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:10,000 dilution in TTBS plus 2% bovine serum albumin [Santa Cruz Biotechnologies, Santa Cruz, CA]). Blots were washed with TTBS, visualized using an ECL detection kit (Amersham), and scanned using a Fluor-S-Max multi-imager (Bio-Rad). Bands were quantified by densitometry using Quantity One software (Bio-Rad).

Statistical analysis. All data were expressed as means ± standard errors of the means (SEM) and compared by one-way analysis of variance. Post hoc tests were performed if a P value of <0.05 was obtained, using Tukey's test for multiple-comparison analysis of parametric data. For nonparametric data, comparisons were made using Kruskal-Wallis multifactorial analysis of variances, followed by Dunn's test for multiple-comparison analysis. P values of ≤ 0.05 were considered statistically significant.

RESULTS

EGF reduces *C. jejuni* colonization and extraintestinal dissemination in day-of-hatch chicks. Parameters of *C. jejuni* infection and intestinal pathology were assessed in day-of-hatch chicks assigned to one of the following four treatment groups: (i) controls ($n = 11$), (ii) *C. jejuni*-infected chicks ($n = 15$), (iii) *C. jejuni*-infected chicks treated with daily oral EGF ($n = 15$), and (iv) EGF-treated chicks ($n = 8$). At no time in the study did any of the chicks exhibit pericloacal soiling or show signs of lethargy. *C. jejuni* was isolated from cloacal swabs of all *C. jejuni*-infected chicks, but from none of the controls, throughout the 14-day study period (data not shown). The numbers of chicks for which *C. jejuni* was isolated from cloacal swabs were not significantly different between the *C. jejuni*-infected and *C. jejuni*-infected, EGF-treated groups (data not shown). All chicks weighed significantly more on day 14 than on day 0. On the day of arrival, control, *C. jejuni*-infected, *C. jejuni*-infected plus EGF-treated, and EGF-treated chicks weighted 39.3 g ± 0.8 g, 38.9 g ± 0.9 g, 39.1 g ± 0.8 g, and 40.5 g ± 1.2 g, respectively. At the end of the study period (day 14), their respective weights were 129.6 g ± 3.0 g, 126.9 g ± 2.2 g, 129.4 g ± 2.0 g, and 127.0 g ± 3.1 g; these values were not significantly different.

On day 14, *C. jejuni* was isolated from the ceca, jejuna, livers, and spleens of *C. jejuni*-infected and *C. jejuni*-infected, EGF-treated chicks (Fig. 1). *C. jejuni* was not recovered from controls. *C. jejuni* was recovered from the ceca of all experimentally infected chicks; the cecum had the highest level of *C. jejuni* colonization, and there were no differences in the level of colonization between the *C. jejuni*-infected and *C. jejuni*-infected, EGF-treated groups. Significantly more *C. jejuni*-infected chicks shed *C. jejuni* (12 of 15 chicks [80%]), and greater numbers (3.78 $\log_{10}$ CFU/g) were recovered from their jejuna than from those of *C. jejuni*-infected, EGF-treated chicks (2 of 15 chicks [13%]; 0 $\log_{10}$ CFU/g). *C. jejuni* was isolated from the liver (5.65 $\log_{10}$ CFU/g) for 80% of the chicks and from the spleen (5.18 $\log_{10}$ CFU/g) for 93% of the chicks from the *C. jejuni*-infected group. *C. jejuni* was isolated from the liver (0 $\log_{10}$ CFU/g) and spleen (2.40 $\log_{10}$ CFU/g) for 40% and 53%, respectively, of *C. jejuni*-infected, EGF-treated chicks. While fewer EGF-treated than untreated animals had *C. jejuni* in the liver or spleen, the differences in median *C. jejuni* counts did not reach statistical difference between both groups. EGF did not affect *C. jejuni* viability in vitro, suggesting that EGF does not have a direct microbicidal effect (data not shown).

Effects of EGF on *C. jejuni*-induced jejunal villus atrophy. A small but statistically significant decrease in jejunal villus height was measured at 14 days postinfection in chicks infected with *C. jejuni* in comparison to control chicks (Fig. 2). In contrast, villus lengths for *C. jejuni*-infected, EGF-treated

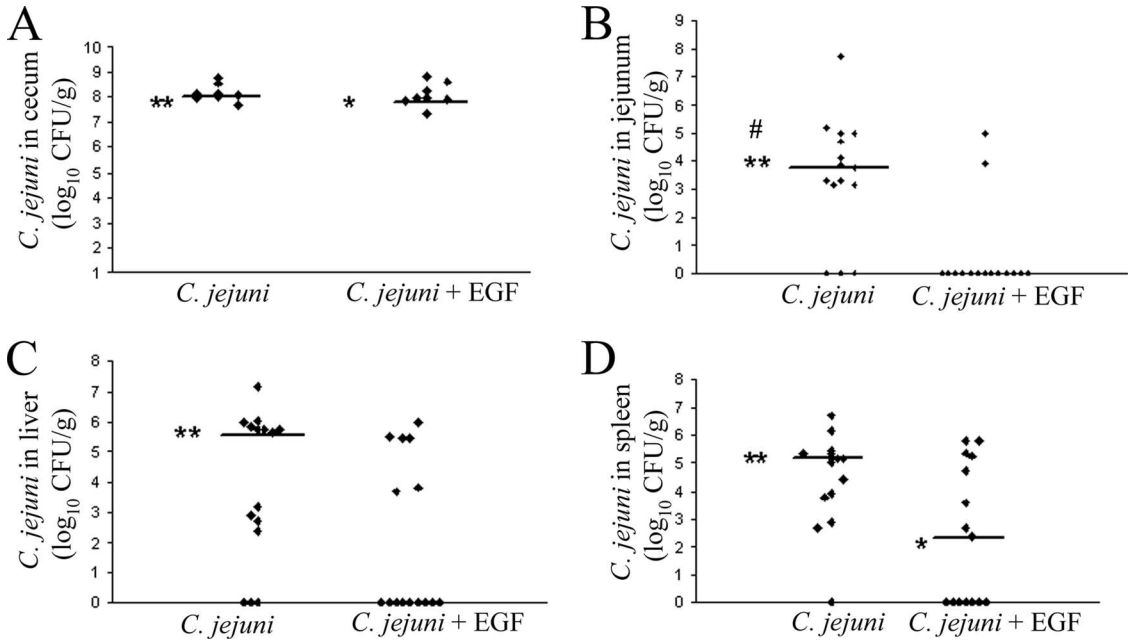

FIG. 1. EGF reduces *C. jejuni* colonization and extraintestinal dissemination in chicks. Day-of-hatch chicks were inoculated with or without *C. jejuni* RM1221 and treated daily with or without EGF, as described in Materials and Methods. The graphs show recovery ($\log_{10}$ CFU/g tissue) of live *C. jejuni* from the ceca (A), small intestines (B), livers (C), and spleens (D) of White Leghorn chicks 14 days after inoculation with 500 μl of a broth containing 1.0×10^9 CFU/ml live *C. jejuni. C. jejuni* was recovered from the ceca of all experimentally infected chicks (A), and there was no difference between the median values for both groups. (B) Daily treatment with EGF (100 μg/kg) significantly reduced *C. jejuni* colonization in the small intestine. No *C. jejuni* was recovered from the livers or spleens of control uninfected chicks. While fewer EGF-treated than untreated chicks had *C. jejuni* in the liver (40% versus 80%) and spleen (53% versus 93%), differences between median CFU values for untreated (5.65 $\log_{10}$ and 5.18 $\log_{10}$ CFU/g from the liver and the spleen, respectively) and EGF-treated (0 $\log_{10}$ and 2.40 $\log_{10}$ CFU/g from the liver and the spleen, respectively) chicks failed to reach statistical significance. The lines indicate medians. **, $P < 0.001$ compared to control; *, $P < 0.01$ compared to control; #, $P < 0.01$ compared *C. jejuni* plus EGF treatment.

chicks were not significantly different from values obtained for control chicks or from those measured in untreated *C. jejuni*-infected animals. Neutrophil infiltration or overt inflammation was not seen in any of the samples. Although there was a trend of reduced jejunal sucrase activity for *C. jejuni*-infected chicks compared to control values, the change failed to reach statistical significance ($P = 0.15$) (Fig. 3).

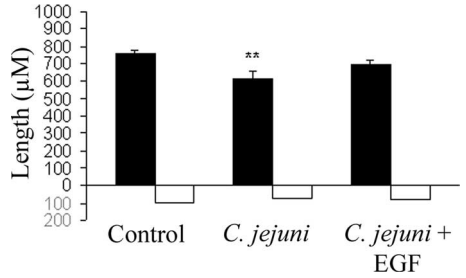

FIG. 2. Effects of EGF on *C. jejuni*-induced jejunal villus atrophy. Day-of-hatch chicks were inoculated with or without *C. jejuni* RM1221 and treated daily with or without EGF, as described in Materials and Methods. Jejunal villus height (black bars) and crypt depth (white bars) were measured at 14 days postinfection ($n = 7$ or 8). The villus-crypt junction is represented by 0. Values represent means ± SEM. **, $P \leq 0.01$ compared to control. Differences between values for untreated *C. jejuni*-infected chicks and EGF-treated *C. jejuni*-infected chicks failed to reach statistical significance ($P = 0.15$).

EGF reduces *C. jejuni* invasion into epithelial cells. *C. jejuni* adhered to and invaded SCBN epithelial cells (Fig. 4). Apical exposure of EGF prior to *C. jejuni* challenge did not affect *C. jejuni* adherence. However, it caused a significant reduction of *C. jejuni* invasion into SCBN cells.

EGF prevents *C. jejuni*-induced increase in paracellular permeability. TER was significantly reduced in *C. jejuni*-infected SCBN monolayers 2 h after infection (Fig. 5). The TER of *C. jejuni*-infected, EGF-treated monolayers was significantly

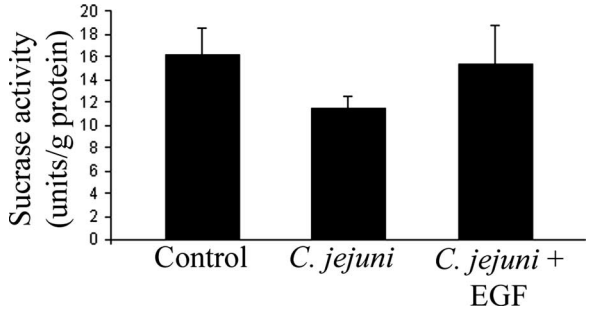

FIG. 3. Jejunal sucrase activity. Day-of-hatch chicks were inoculated with or without *C. jejuni* RM1221 and treated daily with or without EGF, as described in Materials and Methods. Sucrase activity in the jejunum was measured at 14 days postinfection ($n = 7$ to 11). Values represent means ± SEM.

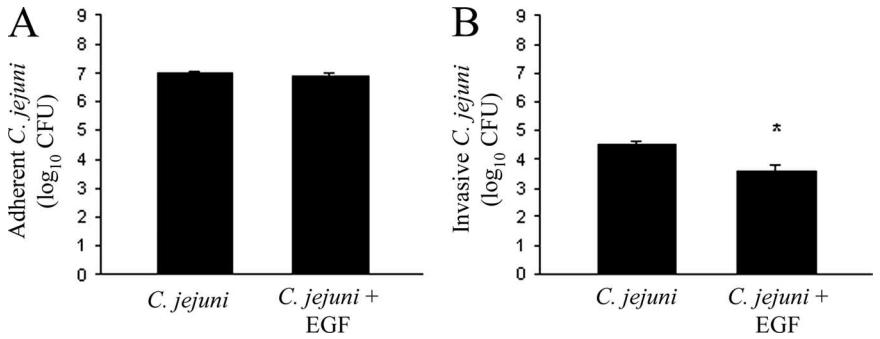

FIG. 4. EGF reduces *C. jejuni* invasion of intestinal epithelial cells. SCBN monolayers were pretreated with or without EGF and inoculated with or without *C. jejuni* RM1221, as described in Materials and Methods. *C. jejuni* adherence (A) and invasion (B) were measured at 3 h postinoculation ($n = 6$). Values represent means ± SEM. *, $P \leq 0.05$ compared to *C. jejuni*-infected group.

higher than that of *C. jejuni*-infected monolayers and was no different from that of controls. Similar results were observed 6 h after *C. jejuni* infection. *C. jejuni* also significantly increased the transepithelial flux of FITC-dextran across SCBN monolayers (Fig. 6). Pretreatment with EGF significantly reduced the *C. jejuni*-induced increase in paracellular permeability. Pretreatment with PD153035, a specific inhibitor of EGFR tyrosine kinase activity, prevented the protective effect of EGF. Both EGF and PD153035 alone had no effect on dextran flux. Experimental challenge induced a noticeable reduction in TER across control monolayers, possibly reflecting the addition of bacterial broth (to optimize bacterial survival) to the cell culture medium.

EGF prevents *C. jejuni*-induced disruption of tight junctional claudin-4. Tight junctional claudin-4 appeared as a continuous and uniform pericellular staining pattern in control monolayers (Fig. 7). After 6 h of incubation with *C. jejuni*, epithelial monolayers exhibited disrupted pericellular claudin-4 staining and punctate granular cytoplasmic accumulation of claudin-4. Pretreatment with EGF prevented these abnormalities. *z* axis reconstructions of the monolayers were consistent with the *x-y* axis observations. Furthermore, Western blotting and densitometry analyses indicated that epithelial cells exposed to *C. jejuni* for 6 h expressed significantly less total claudin-4 than control cells did (Fig. 8). In contrast, claudin-4 expression in *C. jejuni*-infected, EGF-treated cells was not different from control values.

***C. jejuni* facilitates the translocation of a noninvasive *E. coli* strain, and this effect is inhibited by EGF.** *C. jejuni* was not recovered from the basolateral chamber of confluent SCBN monolayers at any time for up to 24 h postinoculation (data not shown). To assess whether *C. jejuni* could facilitate the translocation of nonpathogenic, noninvasive *E. coli* (C25), confluent SCBN monolayers were challenged with *C. jejuni* for 6 h, followed by apical *E. coli* inoculation. *E. coli* was recovered from the basolateral chambers of 4 of 12 (33%) Transwells treated with *C. jejuni* and was not recovered from the basolateral chambers of control or *C. jejuni*-infected, EGF-treated monolayers ($n = 12$).

DISCUSSION

This study characterizes pathogenic mechanisms of *C. jejuni* infection in newly hatched chicks and assesses potential benefits of exogenous EGF. *Campylobacter* infection in poultry may be asymptomatic (45, 64), although diarrhea, reduced weight

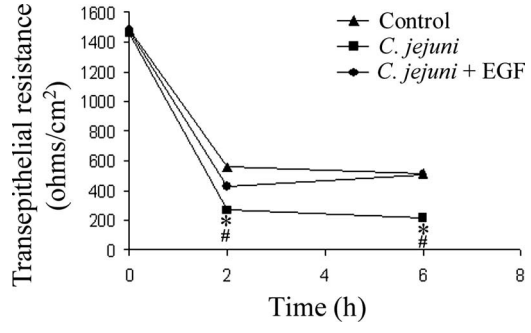

FIG. 5. EGF prevents *C. jejuni*-induced decrease in TER of intestinal epithelial monolayers. SCBN monolayers were pretreated with or without EGF and inoculated with or without *C. jejuni* RM1221, as described in Materials and Methods. TER was measured at 2 h and 6 h postinoculation ($n = 6$). Values represent means ± SEM (SEM are too small to be visible). *, $P \leq 0.001$ compared to control; #, $P \leq 0.05$ compared to *C. jejuni* plus EGF treatment.

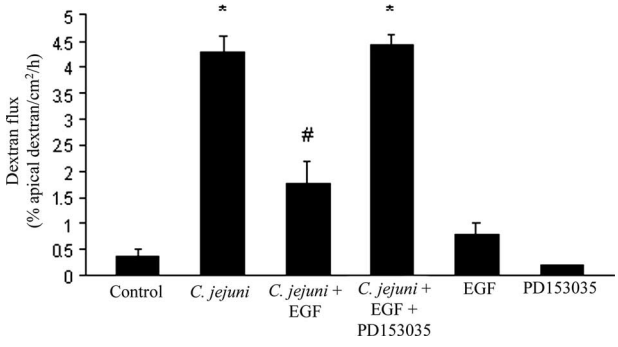

FIG. 6. EGF prevents *C. jejuni*-induced increase in paracellular permeability of intestinal epithelial monolayers. Pretreatment with an inhibitor of EGFR tyrosine kinase activity (PD153035) prevents EGF's protective effect. SCBN monolayers were pretreated with or without EGF and/or PD153035 and then inoculated with or without *C. jejuni* RM1221, as described in Materials and Methods. Flux of a 3-kDa FITC-dextran probe was measured at 6 h postinoculation ($n = 9$). Values represent means ± SEM. *, $P < 0.001$ compared to control and *C. jejuni* plus EGF treatment; #, $P < 0.01$ compared to control.

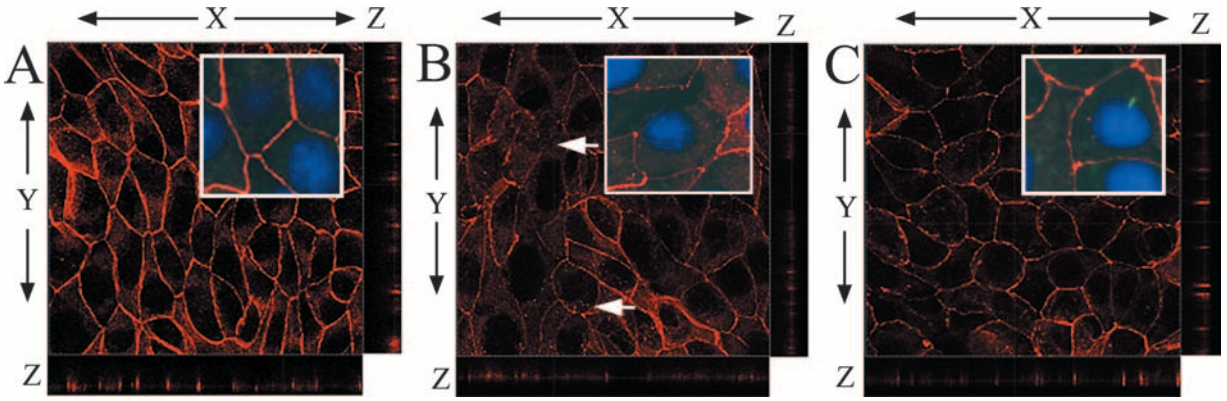

FIG. 7. EGF prevents *C. jejuni*-induced disruption of tight junctional claudin-4. SCBN monolayers were pretreated with or without EGF and inoculated with or without *C. jejuni* RM1221, as described in Materials and Methods. Monolayers were immunofluorescently stained for claudin-4 (red) at 6 h postinoculation and viewed by confocal laser microscopy. Representative confocal photomicrographs and *z*-axis reconstructions are shown. (A) In control monolayers, claudin-4 is localized at the paracellular regions. (B) *C. jejuni* causes disruption in the pericellular localization of claudin-4, with diminished, punctate, irregular staining (arrows). (C) Pretreatment with EGF inhibits claudin-4 disruption. High magnification insets show claudin-4 staining (red), Hoechst nuclear staining (blue), and *C. jejuni* staining (green).

gain, and mortality have been reported for infected birds (30, 52, 53). In our study, *C. jejuni* was recovered from the jejuna and ceca of infected chicks for up to 14 days postinfection, indicating that the intestinal tract was colonized. While infected chicks did not exhibit significant weight loss or diarrhea, *C. jejuni* was isolated from extraintestinal organs, and jejunal villus atrophy was evident. Consistent with the findings described here, jejunal villus atrophy has been reported for chicks experimentally infected with *C. jejuni* (8). Daily oral treatment with EGF reduced *C. jejuni* colonization in the jejunum. While EGF treatment reduced the number of chicks in which translocation occurred, median *C. jejuni* counts for both groups did not reach a statistical difference. *C. jejuni* produces cytolethal distending toxin, which induces cell cycle arrest (31) and may target the highly proliferating crypt epithelial cells; this may explain the observed villus atrophy in infected birds. Further research is needed to assess how this may be affected by treatment with EGF, a protein known for its mitogenic and antiapoptotic effects. While previous research has established the anti-infective properties of EGF, this study is the first to characterize the effects of the peptide on infection by an enteroinvasive pathogen such as *C. jejuni*.

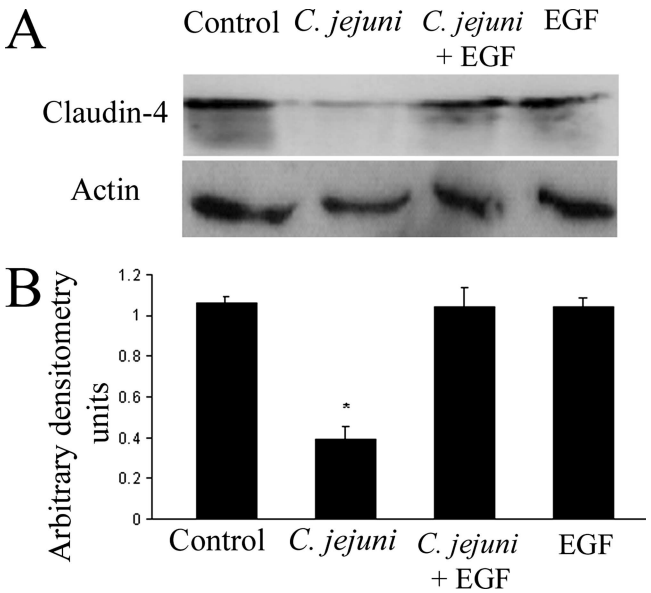

FIG. 8. EGF prevents *C. jejuni*-induced reduction in expression of claudin-4 in intestinal epithelial monolayers. SCBN monolayers were pretreated with or without EGF and inoculated with or without *C. jejuni* RM1221 for 6 h, as described in Materials and Methods. Expression of claudin-4 was examined by Western blotting (A) and quantified by densitometry (B). Values represent means ± SEM ($n = 3$). *, $P < 0.05$ compared to control.

In an attempt to identify mechanisms of EGF action that may explain our in vivo observations, we used a model intestinal epithelium to measure the effects of EGF on *C. jejuni* adherence, invasion, and translocation and *C. jejuni*-induced barrier dysfunction. Because chicken intestinal epithelial cell lines are not available, a polarized intestinal cell line with high TER was used. Our results indicated that *C. jejuni* adheres to and invades these intestinal epithelial cells in culture, in agreement with previous reports using other model systems (14, 19).

C. jejuni binds to the intestinal epithelium by adhesins specific for glycoconjugate receptors in mucus or on the brush border membrane (36, 42). Studies have shown that EGF modulates glycan expression on host epithelial cells (23), suggesting that EGF could prevent *C. jejuni* intestinal colonization in chicks by reducing the number of adhesin receptor sites. However, our in vitro studies do not support this hypothesis, as we observed that EGF does not reduce *C. jejuni*-epithelium adherence. This is not unexpected, since *C. jejuni* utilizes several different adhesins to bind to epithelial cells (25, 28, 48), and thus, interfering with the binding of one particular adhesin (such as those that bind glycoconjugates) does not necessarily preclude the binding of other adhesins to epithelial cell surfaces. However, importantly, in our study, EGF treatment inhibited invasion of intestinal epithelial cells by *C. jejuni*. It is well recognized that *C. jejuni* invasion is necessary for intestinal colonization. For example, invasion-defective *C. jejuni* flagellar mutants are unable to colonize the cecum of chicks (43). Thus, EGF may prevent *C. jejuni* colonization of the

chicken intestinal tract by preventing *C. jejuni* from invading the intestinal epithelium.

The results presented here demonstrate that *C. jejuni* from poultry can cross the intestinal epithelial barrier and disseminate to extraintestinal organs in chicks. The ability of *Campylobacter* to cross the intestinal epithelial cell barrier has been documented previously (3, 14, 19, 29). Reports suggest that *C. jejuni* may translocate via M-cell transcytosis (61), via the paracellular pathway (39, 46, 53), and/or via the transcellular route (14). However, the mechanisms by which *Campylobacter* translocation may be regulated remain obscure. Findings from recent intestinal epithelial cell culture studies indicate that *C. jejuni* 81-176 may facilitate its own dissemination by disrupting tight junctional integrity (9, 33). Results of our in vitro studies indicate that *C. jejuni* RM1221 increases epithelial permeability in association with causing disruption of tight junctional claudin-4; however, *C. jejuni* was not detected in the basolateral chambers following apical challenge. This observation was not due to loss of bacterial viability, since live bacteria were recovered from the apical chamber. One possible explanation is that any *C. jejuni* cells that have translocated may adhere to the basolateral surfaces of epithelial cells via the adhesin CadF; this has previously been observed to delay release of *C. jejuni* from epithelial cells and to reduce the number of bacteria in the basolateral compartment of filter-grown monolayers (39). Regardless, the present observations show, for the first time, that even in the absence of detectable *C. jejuni* translocation, there are measurable changes in intestinal barrier structure and function.

Previous studies using Caco-2 (33) and T84 (9) cells have observed that *C. jejuni*-induced tight junctional disruption occurs 24 h after incubation with *C. jejuni*. Consistent with the recent demonstration of early epithelial cytotoxic effects of this bacterium (27), the present study demonstrates that claudin-4 integrity and barrier function are disrupted as early as 2 h after apical challenge with *C. jejuni*. The time taken for *C. jejuni* to disrupt monolayer integrity is consistent with previous reports investigating other pathogenic microorganisms, such as *Salmonella* or EPEC (58, 60).

Although translocation of *C. jejuni* was not detected in our in vitro studies, its effects on epithelial cells are sufficient to promote the translocation of other, noninvasive bystanding microorganisms, such as *E. coli* C25. Luminal antigens that translocate across the intestinal epithelium would activate host immune system-dependent pathological pathways. Therefore, such events may be of great clinical significance to gastrointestinal health and disease. Not surprisingly, intense research efforts are trying to identify the molecular events that regulate bacterial translocation. Using models unrelated to *C. jejuni* infection, other reports have suggested that EGF may reduce bacterial translocation (34, 47).

Ratios of claudin isoforms in the tight junctions are known to determine the barrier properties of a given tissue (17, 59), and increased expression of claudin-4 has been associated with increased epithelial barrier function (56). EGF has been found to upregulate this key tight junctional protein and to tighten epithelial junctions in canine epithelial MDCK cell lines (56). It was recently shown that abnormalities of the tight junctional protein zonula occludens 1 caused by *Giardia lamblia* or *Cryptosporidium andersoni* are prevented in epithelial cells pretreated with EGF (5, 6). In agreement with these previous studies on other tight junctional proteins and other pathogens, we observed that pretreatment of the monolayers with EGF prevented claudin-4 disruption, as demonstrated by concurring immunofluorescence observations and densitometric analyses of immunoblots. In association with this protective effect, EGF also inhibited the loss of epithelial barrier function caused by *C. jejuni*. The present findings also indicate that these effects require the activation of EGFR, as a specific inhibitor of EGFR tyrosine kinase activity prevented the epithelial rescue induced by EGF. In keeping with the effects of EGF on the tight junctional structure and function, EGF also blocked the translocation of noninvasive *E. coli* through monolayers previously exposed to *C. jejuni*.

Although our results suggest that *C. jejuni* causes *E. coli* to translocate via a paracellular mechanism (i.e., through disrupted tight junctions), we are unable to rule out the possibility that translocation may have instead occurred through a transcellular mechanism involving endocytic uptake and intracellular trafficking. While tight junctions serve to restrict paracellular passage of luminal bacteria, they also maintain cellular polarity by confining the distribution of membrane proteins between the apical and basolateral cell surfaces (55). Previous studies have shown that EPEC disrupts intestinal epithelial tight junctional structure, allowing basolateral receptors to migrate to the apical cell surface (41); this facilitates EPEC adhesion to the enterocyte surface. Also, *Yersinia pseudotuberculosis*, which indirectly disrupts enterocyte tight junctions, can bind to β_1-integrin that has relocated to the apical surface, thus facilitating epithelial invasion (35). It is possible that *C. jejuni*-induced tight junctional disruption may allow basolateral receptors to migrate to the apical cell surface and, in this manner, promote *E. coli* adherence and translocation. In this regard, we are currently investigating whether *C. jejuni* disrupts cellular polarity and examining the role that EGF may play in preventing this. Future studies will also investigate whether *C. jejuni* facilitates systemic spread of normally nonpathogenic microorganisms in vivo, whether this may facilitate the development of infection with opportunistic pathogens in poultry, and whether oral EGF may prevent these abnormalities.

In summary, our findings demonstrate that oral EGF treatment reduces *C. jejuni* colonization in the small intestine of newly hatched chicks and reduces the likelihood of *C. jejuni* translocation and systemic spread to the liver and spleen. In vitro, EGF prevented *C. jejuni*-induced claudin-4 disruptions and functional tight junctional abnormalities and inhibited the *E. coli* translocation promoted by *C. jejuni*. These protective effects of EGF depend on activation of the EGFR. Therefore, the protective effects of EGF against bacterial translocation in vivo may be explained by its ability to maintain intestinal epithelial barrier integrity. The results highlight the potential therapeutic benefits of EGF and support a role for EGF in protecting the gastrointestinal tract from colonization with enteropathogens and from microbially induced barrier defects.

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