Interleukin-6 Induces Keratin Expression in Intestinal Epithelial Cells

POTENTIAL ROLE OF KERATIN-8 IN INTERLEUKIN-6-INDUCED BARRIER FUNCTION ALTERATIONS

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Keratin 8 (K8) and keratin-18 (K18) are the major intermediate filament proteins in the intestinal epithelia. The regulation and function of keratin in the intestinal epithelia is largely unknown. In this study we addressed the role and regulation of K8 and K18 expression by interleukin 6 (IL-6). Caco2-BBE cell line and IL-6 null mice were used to study the effect of IL-6 on keratin expression. Keratin expression was studied by Northern blot, Western blot, and confocal microscopy. Paracellular permeability was assessed by apical-to-basal transport of a fluorescein isothiocyanate dextran probe (FD-4). K8 was silenced using the small interfering RNA approach. IL-6 significantly up-regulated mRNA and protein levels of K8 and K18. Confocal microscopy showed a reticular pattern of intracellular keratin localized to the subapical region after IL-6 treatment. IL-6 also induced serine phosphorylation of K8. IL-6 decreased paracellular flux of FD-4 compared with vehicle-treated monolayers. K8 silencing abolished the decrease in paracellular permeability induced by IL-6. Administration of dextran sodium sulfate (DSS) significantly increased intestinal permeability in IL-6−/− mice compared with wild type mice given DSS. Collectively, our data demonstrate that IL-6 regulates the colonic expression of K8 and K18, and K8/K18 mediates barrier protection by IL-6 under conditions where intestinal barrier is compromised. Thus, our data uncover a novel function of these abundant cytoskeletal proteins, which may have implications in intestinal disorders such as inflammatory bowel disease wherein barrier dysfunction underlies the inflammatory response.

Keratins are a family of structural proteins that form the intermediate filaments of the cytoskeleton in epithelial cells (1, 2). They are among the most abundant cytoskeletal proteins and constitute up to 5% of total cellular proteins in the intestinal epithelia. At least 49 keratins subtypes have been identified so far (3). These proteins are encoded by a large multigene family whose individual members can be divided into type I (acidic) and type II (neutral and basic) classes on the basis of their sequence. The prototype structure of all intermediate filament proteins, including keratins, consists of a structurally conserved central coiled-coil helix termed the “rod” that is flanked by non-helical N-terminal “head” and C-terminal “tail” domains (4). Most of the structural heterogeneity of the different keratins resides in their head and tail domains, which also contain all of the known phosphorylation sites (1, 5). The type I and type II keratins are regulated in a pair-wise and tissue-specific pattern in epithelial tissues of various types including the intestine (6). Epithelial cells express keratin pairs that are assembled as obligate heteropolymers of type I (keratins K9-K20) and type II (K1-K8) keratin (1, 2, 4, 7). Thus, all epithelial cells express at least one type I and one type II keratin in an overall 1:1 stoichiometry. Each keratin pair has a specific and characteristic tissue distribution pattern. For example, the thick external barrier such as the skin express primarily K1 and K5 (type II) and K10 and K14 (type I), whereas the internal epithelia such as the intestine express principally K8 (type II) and K18 (type I) and with variable levels of K7, K19, and K20 depending on the cell type (1, 8, 9).

One clearly delineated function of keratins in many tissues is to protect cells from mechanical and non-mechanical forms of injury (1, 10, 11). Impressive progress has been made over the past decade or so in linking at least 14 of the keratins to a number of skin, oral, esophageal, and liver diseases (6, 12). However, a full appreciation of keratin function in the intestine has been lagging. Recent studies have reported K8 missense mutation in a subset of patients with inflammatory bowel disease. These mutations have been shown to result in incompetent keratin polymerization that may lead to increased epithelial fragility (13, 14). Based on these observations, K8 might play a pathogenic role in inflammatory bowel disease. This is substantiated by animal data obtained from K8 or K18 null mice. For example, the phenotype resulting from K8 deletion has demonstrated an important role for keratins in epithelial barrier integrity and inflammation (15, 16). K8 null mice in C57BL/6 background die in utero predominantly due to placental barrier dysfunction mediated by tumor necrosis factor-α (17). On the other hand, K8 null mice in FVB/N background survive to adulthood but develop colonic hyperplasia, rectal prolapse, and Th-2-mediated colitis that is amenable to antibiotic treatment (15). Although the mechanism by which K8 null mice develop barrier dysfunction or colitis is not known, it is hypothesized that...
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the absence of K8 leads to mistargeting of cellular proteins (such as anion-exchanger 1,2), altered susceptibility to injury, and antigen processing culminating in a mucosal inflammatory response (15, 18). This model underscores the importance of intestinal epithelial keratin in injury and inflammation.

Loss of barrier function provided by epithelial cells is thought to be the initial inciting event that underlies injury and inflammation in many intestinal disorders including shock, trauma, sepsis, and inflammatory bowel disease. Such barrier defects result in the migration of antigenic material, previously confined to the intestinal lumen, into the submucosa exposing lamina propria immune cells to naive antigens eliciting inflammatory response and epithelial injury that characterize these diseases (19, 20). Cytokines play a central role in regulating epithelial barrier function during inflammation. We have previously shown that interleukin-6 (IL-6)2 plays a protective role in epithelial barrier function (21), in contrast to interferon-γ or tumor necrosis factor-α, which decreases barrier function (22).

IL-6 is a potent immunoregulatory cytokine with pro- and anti-inflammatory properties. Its expression under physiological conditions is important for the host response to a number of infections, and under pathological states excessive secretion of IL-6 may play a major role in the pathogenesis of many diseases including inflammatory bowel disease (23–25). In addition to its effect on immune cells, we and others have shown that intestinal epithelial cells are an important source of IL-6 (26), and intestinal epithelial cells express IL-6 receptors at the same density or higher than monocytes (27–30). IL-6 acts on epithelial cells in an autocrine or paracrine fashion to activate the classical ST1AT (signal transducers and activators of transcription) signaling pathway as well as the NF-kB signaling pathway (29). In this study we addressed the role and regulation of intestinal epithelial keratins, K8 and K18, by IL-6 and IL-6-mediated barrier function alterations.

MATERIALS AND METHODS

Reagents and Antibodies—All tissue culture supplies were obtained from Invitrogen. Reagents for SDS-PAGE and nitrocellulose membranes were from Bio-Rad. Anti-K8 monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies (used for confocal microscopy at 1:400 dilution) were a kind gift from Dr. Bishr Omary (31). Anti-K8/18 mouse monoclonal antibody (used for Western blot at 1:1000 dilution) and anti-phospho-K8 Ser-431 and Ser-73 were obtained from Labvision (1:1000 dilution), and anti-tubulin mouse monoclonal antibody was from Sigma. Phosphatase inhibitor mixture and fluorescein isothiocyanate-dextran-FD 4 were from Sigma. The One-step RT-PCR kit was obtained from Qiagen (Valencia, CA). Oligofectamine and Lipofectamine 2000 were from Invitrogen. Human K8 RNAi was from Invitrogen. Recombinant IL-6 was obtained from R&D Systems (Minneapolis, MN) and used at 100 ng/ml, added to the basolateral medium (29, 32). This dose of IL-6 was chosen based on our previous data with respect to signaling as well as functional responses of IL-6 in Caco2-BBE cells (26, 29, 32).

Cell Culture—Caco2-BBE cells were grown as confluent monolayers in Dulbecco’s Vogt modified Eagle’s medium supplemented with 40 mg/liter penicillin, 90 mg/liter streptomycin, and 10% newborn calf serum. Confluent stock monolayers were subcultured by trypsinization. Experiments were performed on cells plated for 8–10 days on permeable supports of 0.33 cm2 or 4.5 cm2 inserts (Costar, Cambridge, MA). Inserts (0.4-μm pore size, Costar, Cambridge, MA) rested in wells containing media until steady-state resistance was achieved (29, 32).

Northern Blot—A Northern blot was performed as described previously (29). Briefly, total RNA was extracted from cells with Tri-reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer’s protocol. Total RNA (20 μg) was separated on 1% formaldehyde-agarose gel and transferred to Gene Screen Plus membranes (PerkinElmer Life Sciences). After fixation under calibrated UV light, the membranes were hybridized with 32P-labeled K8, K18, or glyceraldehyde-3-phosphate dehydrogenase cDNA and visualized by autoradiography. The probe for K8 cDNA was generated by RT-PCR using K8 specific primers 5’-atgcctcaggtgacca-3’ and 5’-tgccgtggcaggtcag-3’ (1454-bp product) and ligated into PCR2.1 vector. The sequence was verified by DNA sequencing (Emory DNA Core Facility). HindIII was used to excise the probe. Complementary cDNA clones encoding K18 and glyceraldehyde-3-phosphate dehydrogenase were purchased from Research Genetics (Huntsville, AL).

SDS-PAGE, Western Blot, and Immunoprecipitation—For Western blot analysis, colonic tissues were homogenized and extracted with lysis buffer. Samples were then centrifuged at 12,000 rpm for 10 min at 4°C, and the resulting supernatant was used for assays. Keratin-enriched cytoskeletal preparations from Caco2-BBE cells were made as described (33, 34). Briefly, Triton X-100-soluble or Triton X-100-insoluble fractions were prepared by solubilizing cells for 10 min at 4°C with buffer containing 1% Triton X-100, 5 mm EDTA, and a protease inhibitor mix (1 mm phenylmethylsulfonyl fluoride, 10 μm leupeptin, 10 μM pepstatin, and 25 μg/ml aprotinin) in phosphate-buffered saline (PBS, pH 7.4) followed by centrifugation (16,000 × g, 10 min). The supernatant was collected as the soluble fraction. The pellet was homogenized in 1 ml of 10 mm Tris-HCl, pH 7.6, 140 mm NaCl, 1.5 mm KCl, 5 mm EDTA, 0.5% Triton X-100, and the protease inhibitor mix. After 30 min (4°C), the homogenate was pelleted (16,000 × g; 10 min), and the pellet (insoluble fraction) was re-homogenized with 5 mm EDTA in PBS, pH 7.4, and dissolved in Laemmli sample buffer containing 1% β-mercaptoethanol, sonicated, and boiled for 5 min (35). The samples were separated on 7.5 or 10% polyacrylamide gels according to the method of Laemmli (35). Proteins were electrotransferred to nitrocellulose membranes and probed with primary antibody (anti K8/18, 1/1000). The membranes were incubated with corresponding peroxidase-linked secondary antibody diluted 1:2000, washed, and subsequently incubated with ECL reagents (Amersham Biosciences) before exposure to high-performance chemiluminescence films (Amersham Biosciences). For Mİ determination, polyacrylamide gels were cal-

2 The abbreviations used are: IL-6, interleukin-6; PBS, phosphate-buffered saline; RNAi, RNA interference; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; WT, wild type; DSS, dextran sodium sulfate; CREB, cAMP-response element-binding protein.
ibrated using standard proteins (Bio-Rad) with M markers within the range 10–250. Immunoprecipitation was performed as described (36, 37). Briefly, cells were solubilized in lysis buffer. After pelleting (16,000 × g; 15 min), K8 was immunoprecipitated from the supernatant using an anti-K8 antibody coupled to protein A/G-Sepharose. The beads were washed once with lysis buffer and twice with PBS (3 mM EDTA). Proteins were solubilized in 3× Laemmli sample buffer and then immunoblotted as described above with the relevant antibodies.

**Plasmid and Transient Transfection**—For RNAi studies, Caco2-BBE cells grown on 0.45 cm² filters were transfected with K8-specific stealth RNAi duplex oligoribonucleotides (siRNA) (UGGACACCUUGUAGGACUUCUGGGU) for most effective knockdown of K8. Stealth RNAi oligos are a chemically modified blunt-ended 25-bp RNA duplex that elicits the RNAi response in cells. It delivers highly specific, effective knockdown with greater stability compared with unmodified siRNA molecules. Only the antisense strand can participate in RNAi, avoiding unwanted off-target effects, allowing effective knockdown only of the targeted gene. Stealth RNAi chemical modification minimizes the induction of nontarget gene expression pathways. BLOCK-iT Fluorescent Oligo (Invitrogen) as well as scrambled K8 RNAi were used as the control. BLOCK-iT is a fluorescent-labeled, double-stranded RNA duplex with the same length, charge, and configuration as standard siRNA. The sequence of the BLOCK-iT Fluorescent Oligo is not homologous to any known gene, ensuring against induction of nontarget gene expression caused by introduction of the oligo into cells (38). Cells were transfected with appropriate vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. 48 h after transfection, cells were stimulated with IL-6, and permeability to FITC dextran (4 μg) was performed as described below. Total cell lysates were collected for K8 expression.

**Confocal Microscopy**—Monolayers of Caco2-BBE cells were washed in Hanks’ balanced salt solution, fixed in buffered 3.7% paraformaldehyde for 20 min, incubated overnight with primary antibody (anti-K8/18 rabbit polyclonal antibody, 1:400) in a humidity chamber, washed with PBS, and subsequently incubated with fluoresceinated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Monolayers were also counterstained with rhodamine-phalloidin to visualize actin. Monolayers mounted in p-phenylenediamine glycerol (1:1) were analyzed by confocal microscopy (Zeiss dual laser confocal microscope; Zeiss, Oberkochen, Germany) as described previously (36). Using actin staining, the apical-most surface of the cell was marked as 0 μm, and the basolateral surface was marked at the level of actin stress fiber (18.7 μm from the top of the cell). xy sections were taken at 1.2 μm from the top (above the level of tight junction) and at the level of actin stress fiber. Quantification of confocal images was performed on unprocessed images using Metamorph Imaging System Software (Universal Imaging Corp., West Chester, PA) as described (36). The average grayscale pixel intensity +1 S.D. of a small region was measured and defined as background. To subtract background, the threshold of each channel was set at the value obtained for background. The average pixel intensity +1 S.D. was measured for the threshold images. The data are presented as the mean (±S.E.).

Colon tissue from WT and IL-6−/− mice embedded in paraffin or optimal cutting media were obtained as described by Castaneda et al. (39). The sections were rehydrated using graded alcohols. Sections were treated with 0.5% Triton X-100 + 0.08% saponin in PBS at room temperature for 35 min. The sections were rinsed in PBS and incubated with rhodamine-phalloidin (1:60) diluted in PBS for 40 min at room temperature.Sections were subsequently blocked with 2% BSA for 1 h at room temperature. Sections were incubated with primary antibody, K8/K18 (Chemicon; 15 μg/ml in 2% BSA in PBS solution) or rabbit IgG (Sigma, control) or anti-actin (Sigma; 1:50,000 in 2% BSA) for 1 h at room temperature. After washing 3 times with PBS, they were incubated with FITC-conjugated secondary antibody (Bio-Rad; 1:100 in 2% BSA in PBS solution) or rhodamine-conjugated anti-mouse secondary antibody (Bio-Rad; 1:100 in 2% BSA in PBS solution) for 45 min at room temperature and then mounted with Slow Fade (Molecular Probes, Eugene, OR) mounting medium and examined using Zeiss LSM microscope.

**Measurement of Macromolecular Permeability**—Paracellular permeability was determined by measuring apical to basolateral flux of fluoresceinated dextran (FD-4, M, 4; Sigma) using a modification of previously described method (40). Briefly, confluent epithelial monolayers on 0.33 cm² 0.4-μm pore size permeable supports were washed twice with Hanks’ balanced salt solution containing calcium chloride and magnesium sulfate (HBSS +) and maintained at 37 °C on a shaking warm plate. FD-4, 1 ng/ml, was added apically at time 0, and 50-μl samples were removed from the basolateral compartment at 30-min intervals from 0 to 120 min, inclusive. Fluorescence intensity of each sample was measured (excitation, 492 nm; emission, 525 nm; Cytofluor 2300; Millipore Corp., Waters Chromatography, Bedford, MA), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Paracellular flux was calculated by linear regression of sample fluorescence (Excel 5.0, Microsoft WA, Power Macintosh 7200).

**In vivo permeability assay to assess barrier function was performed using a FITC-labeled dextran method as described (41). Briefly, 6-8-week-old WT and IL-6−/− mice were used. Food and water were withdrawn for 4 h, and mice were gavaged with a permeability tracer (60 mg/100 g body weight of FITC-labeled dextran, FD-4, M, 4000, Sigma). Serum was collected retro-orbitally 4 h after FD-4 gavage, and fluorescence intensity of each sample was measured (excitation, 492 nm; emission, 525 nm; Cytofluor 2300; Millipore), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Permeability was calculated by linear regression of sample fluorescence (Excel 5.0, Microsoft Office).

**Data Analysis—**Results were analyzed using Student’s t test. Differences were considered significant at the p < 0.05 level.

**Experimental Animals**—The Animal Care Committee of the Emory University, Atlanta approved all procedures performed on animals and was in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States Public Health Service. IL-6−/− mice (C57/B6 background)
were purchased from Jackson Laboratories. The homozygous IL-6-deficient mice used were the progeny of heterozygous breeding pairs of C57/B6 background with disruption of the IL-6 gene that were backcrossed for more than six generations. These mice developed normally and were fertile. Age- and sex-matched WT and IL-6−/− littermates used in the study were between 6 and 8 weeks old at the beginning of the experimental protocol and were maintained under conditions as described previously (39). Mice were given DSS (ICN Biomedicals, Aurora, OH) at 3% (w/v) in tap water ad libitum for 4 days. Age-matched male and female wild type and IL-6−/− littermates receiving tap water served as controls. Mice were observed daily and evaluated for changes in body weight.

RESULTS

IL-6 Increases the Expression of K8/K18—To determine whether IL-6 modulates keratin expression, Caco2-BBE cells grown in tissue culture inserts were stimulated via the basal surface with IL-6 (100 ng/ml). Cells were lysed at various times after IL-6 stimulation, and mRNA and protein expression of K8/K18 was determined by Northern blot and Western blot, respectively, as described under “Materials and Methods.” As seen in Fig. 1A, a small amount of K8 mRNA was detected at base line. However, K8 mRNA was significantly increased at 2 h and was maximal at 4 h. Similar results were obtained with K18. Like K8, IL-6 induced mRNA expression of K18 at 2 h and was maximal at 4 h. K18 mRNA levels returned to base line at 8 h after IL-6 stimulation. Protein expression of K8 and K18 reflected mRNA expression (Fig. 1B). K8 expression was seen at 4 h, and K8 protein level was maximal 12–24 h after IL-6 stimulation. K18 protein expression also increased at 4 h and was maximal at 24 h.

We next examined the distribution of K8/K18 in Caco2-BBE cells. To localize keratin, immunofluorescence staining was performed using anti-K8/K18 antibody. Cells were treated with vehicle or IL-6 (24 h) and fixed with paraformaldehyde, and keratin distribution was examined by confocal microscopy (Fig. 2). Reticular pattern of keratin was seen at the periphery of control cells. IL-6 significantly increased the expression of K8/K18, which was most prominent in the subapical region as seen in xz computer reconstructed images. The en face (xy plane) images of Caco2-BBE epithelia were taken at the subapical region (1.2 μm from the top of the cell, at the level of apical junctional complex). To ensure adequate permeabilization under these conditions, phospho-CREB, a nuclear protein, was visualized using anti-phospho-CREB antibody under the same experimental condition (inset, Fig. 2A). To quantitate the expression of K8/K18, the pixel intensity of the confocal images taken at the subapical surface was measured as described under “Materials and Methods.” IL-6 treatment increased the expression of K8/K18-β-actin by 2.5-fold compared with vehicle treatment (p < 0.001).

IL-6 Induces the Expression of Keratin in the Cytoskeletal Fraction—Most of the keratin is distributed in the detergent-insoluble fraction. However, under some conditions the solubility of keratin may be increased by post-translational modification such as phosphorylation (5). Such modification is thought to contribute to protective effects of keratin by altering its distribution or association with other proteins (33, 42–44). We, therefore, determined the distribution of keratin between detergent-soluble and -insoluble fractions after IL-6 treatment. Caco2-BBE cells were treated with IL-6 for varying times, and cell lysates were separated into Triton-X–100-soluble and -insoluble fraction as described under “Materials and Methods.” As seen in Fig. 3A, the majority of K8 was found in the insoluble fraction, and a smaller proportion of keratin was detected in the detergent-soluble fraction in the vehicle-treated controls. IL-6 treatment increased K8 expression in both the soluble (~3-fold) and the insoluble fraction (~4-fold) compared with unstimulated cells. However, the distribution of K8 was similar to the control cells in that the majority of K8/K18 were in the detergent-insoluble fraction.

IL-6 Increases Keratin Phosphorylation—An important aspect of keratin function involves phosphorylation at specific serine residues (33, 42–45). For example, phosphorylation at
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Ser-431 increases during mitosis or upon exposure to epidermal growth factor in association with filament reorganization (45). During a variety of cellular stresses, including heat and drug exposure, Ser-73 is phosphorylated, whereas under normal conditions, it remains dephosphorylated. To determine whether IL-6 induces serine phosphorylation of K8, cell were treated with vehicle or IL-6 for 24 h, and cell lysates were subjected to immunoblotting using K8 phospho-specific antibodies. As shown in Fig. 3B, IL-6 induced K8 phosphorylation at phosphoserine 431 and 73, suggesting post-translational modification of keratin in response to IL-6. Although base-line phosphorylation levels of K8-phosphoserine 431 or 73 differed, the time course of phosphorylation in response to IL-6 was similar. The IL-6-induced K8 phosphorylation was maximal at 12 h (4.5-fold increase of K8 in response to IL-6 was similar. The IL-6-induced K8 phosphorylation at phosphoserine 431 and 73, respectively, as shown are representative of two independent experiments; n = 6 per time point, significantly different from 0 h; #, p < 0.04; *, p < 0.001. IL-6 induces serine phosphorylation of K8. Caco2-BBE monolayers were pretreated with basolateral IL-6 (100 ng/ml) for 12 or 24 h and subjected to Western blotting with anti-K8/18 antibody (rabbit polyclonal, 1:400 dilution) or anti-phospho-CREB (1:500 dilution) followed by fluorescent isothiocyanate-conjugated secondary antibody. Monolayers were also stained with rhodamine/phalloidin (actin, red). Images were visualized by indirect immunofluorescence using a Zeiss confocal microscope. Vertical sections (xz) were taken off the monolayers to define the top (set at 0 μm) and bottom of the monolayer (at the level of stress fiber). En face sections (xy) were then generated from apical plane in the vertical section. Shown here are en face (xy) images taken 1.2 μm from the top at the level of apical junctional complex. Also shown are reconstructed vertical section (xz) images taken through full thickness of the monolayer. The inset in the top left panel shows phospho (p)-CREB nuclear staining. The intensity of bands from panel A was quantified as described under “Materials and Methods.” The bar graphs represent relative band intensity K8/K18-β-actin membrane, mean ± S.E. n = 4, significantly different from vehicle; ***, p < 0.001. CTL, control.
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**FIGURE 4.** K8 mediates IL-6-induced barrier protective effects. A, IL-6 decreases paracellular permeability. Paracellular flux of FD-4 was measured in confluent Caco2-BBE monolayers after basolateral exposure to IL-6 (100 ng/ml) as described under “Materials and Methods.” The graph represents % change in FD flux compared with vehicle-treated monolayers (6.4 ± 0.5 μmol/cm² h⁻¹). An average of three experiments was performed in quadruplicate with error bars representing S.E.; p < 0.01 compared with vehicle. B, IL-6 is unable to induce its barrier protective effects in the absence of K8. Caco2-BBE monolayers were transfected with scrambled or K8-specific siRNA as described. Cells were stimulated with basolateral IL-6 (100 ng/ml) for 24 h, and paracellular flux of FD-4 was measured. The graph represents % change in FD flux compared with vehicle-treated monolayer (scrambled siRNA + vehicle, 15.1 ± 1.6; K8 siRNA + vehicle, 14.9 ± 1.1 μmol/cm² h⁻¹). Values represent data from two independent experiments; n = 12; *, p < 0.04; #, p < 0.05. C, cells were lysed, and K8 expression was determined by Western blot using anti-K8/18 antibody. β-Tubulin served as a loading control.

Accordingly, Caco2-BBE cells plated on filters were transfected with K8-specific siRNA or scrambled siRNA. After transfection (48 h), cells were stimulated with IL-6 for 24 h. Permeability was measured with FD-4 as described under “Materials and Methods.” As seen in Fig. 4B, IL-6 treatment resulted in decreased permeability in cells transfected with scrambled siRNA (vehicle, 15.1 ± 1.6; IL-6, 9.81 ± 0.6 μmol/cm² h⁻¹, p < 0.04; 36 ± 6.1% decrease compared with vehicle). Cells transfected with K8 siRNA showed no change in base-line permeability compared with scrambled siRNA-transfected cells (14.9 ± 1.1 μmol/cm² h⁻¹). In contrast, there was a loss of IL-6-mediated barrier protection in cells with knock down of K8 (18.8 ± 1.2 μmol/cm² h⁻¹; 25% increase compared with K8 siRNA transfected and treated with vehicle). K8/K18 protein was significantly down-regulated by K8 siRNA (Fig. 4C). Together, these data suggest that K8 is required for the barrier protective function of IL-6.

**K8/K18 Is Involved in IL-6-mediated Barrier Protection in Vivo**—We used IL-6⁻/⁻ mice to determine the effect of IL-6 in mediating barrier protection in vivo. To determine paracellular permeability in vivo, mice were administered FITC-dextran by gavage, and fluorescence was quantitated in the serum as described under “Materials and Methods.” As shown in Fig. 5, there was no difference in serum FD-4 levels in untreated WT and IL-6⁻/⁻ mice (0.76 ± 0.1 and 0.78 ± 0.02 mg of FD-4/μg of protein in WT and IL-6⁻/⁻ mice, respectively). However, administration of DSS to IL-6⁻/⁻ mice resulted in significantly increased FITC translocation compared with WT mice given DSS (Fig. 5). These data demonstrate that IL-6 is barrier-protective under conditions where barrier function is compromised. To understand the role of K8/K18 in IL-6-induced barrier protection in vivo, we next determined K8/K18 expression in WT and IL-6⁻/⁻ mice. Colonic lysates and frozen tissue sections from WT or IL-6⁻/⁻ mice were obtained as described under “Materials and Methods.” Western blot and confocal imaging was performed using anti-K8/K18 antibody. STAT-3 and β-tubulin were used as loading controls in Western blot. Surprisingly and interestingly, K8 levels were significantly down-regulated in IL-6⁻/⁻ mice as compared with WT mice at base-line conditions as evidenced by Western blot and confocal imaging (Fig. 6, A and B). Together, these data are in agreement with our in vitro observation that IL-6 is barrier-protective and K8/K18 mediates IL-6-induced barrier protection under conditions of intestinal stress known to cause barrier dysfunction.

**FIGURE 5.** IL-6 mediates barrier-protective effects in vivo. WT and IL-6⁻/⁻ mice (C57/B6) were given FITC-dextran (M, 4000) orally as described under “Materials and Methods.” Serum was obtained retro-orbitally and processed for fluorescence. Data are represented as mg of FITC/μg of protein. Each bar represents the mean ± S.E.; n = 4 a versus b or c, p < 0.01; c versus b, p < 0.05.

decrease compared with vehicle-treated monolayer, p < 0.01). To study the role of IL-6-induced K8 in its barrier protective function, we used a RNAi strategy to achieve down-regulation of K8 expression. We chose K8 because it is the only type II keratin present in Caco2-BBE cells (9). Because keratins are obligate heterodimers requiring one type II partner, the down-regulation of K8 would have a more complete effect on the overall amount of keratins than the manipulation of either type I keratin (K18 or K19).
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In this study we demonstrate that K8 and K18 intermediate filaments of intestinal epithelial cells are regulated by IL-6. Our data show that IL-6 induces up-regulation of these keratins in the colonic epithelial cell line, Caco2-BBE, whereas IL-6 null mice show decreased expression of K8/K18. With the exception of tumor necrosis factor-α, which has been shown to induce K6 expression in the epidermal tissue in the context of cell proliferation (46), to our knowledge our study is the first demonstration of transcriptional/translational-mediated induction of epithelial keratin by an immunoregulatory cytokine. Identification and characterization of cis and trans regulatory elements of keratin genes and transcription factors that bind to regulatory factors in the keratin promoter have been examined for some keratin genes including K8, K18, and K19. Among the transcriptional factors, Sp1 and KLF-4 regulate the expression of K8 and K18 (47–50). Keratin overexpression under some conditions such as cerulein-induced pancreatitis was shown to be associated with NF-κB activation, suggesting a role for NF-κB in the transcriptional regulation of keratin expression (51). Although the signaling pathway of keratin induction by IL-6 is not known, IL-6 is known to directly or indirectly induce Sp1 (52, 53) as well as activate NF-κB (29), and hence, activation of Sp1 and/or NF-κB may potentially be involved in IL-6-mediated keratin expression in Caco2-BBE cells.

Interestingly, there was a dramatic decrease in K8/K18 expression in IL-6<sup>−/−</sup> mice, suggesting that IL-6 may regulate base-line expression of K8/K18 in colonic epithelial cells. This finding was rather surprising as base-line expression of IL-6 is low. Nevertheless, we and others have demonstrated that human intestinal epithelial cells secrete IL-6, albeit small quantities (26), and possess IL-6 receptor at the same density or higher than monocytes under basal physiological conditions (27–29). Additionally, pericryptal fibroblasts that are in intimate contact with intestinal epithelial cells secrete IL-6 at base-line physiological conditions (28). Because IL-6 receptors are abundantly expressed by intestinal epithelial cells, they can respond to IL-6 in autocrine or paracrine manner and regulate gene expression. It is also possible that other factors in IL-6<sup>−/−</sup> mice contribute indirectly to decreased K8/K18 expression. Regardless, together our in vitro and in vivo data demonstrate that IL-6 plays an important role in the regulation of K8/K18 expression. Our observation opens new avenues for further investigation of keratin expression in the colon and other tissues that contain simple-type epithelia which express K8 and K18.

We show that keratin intermediate filaments exhibit strong peripheral staining in the intestinal epithelial cell line, native human as well as mice colonic epithelia. In response to IL-6, K8/K18 is expression is induced and is concentrated under the apical domain in cells treated with IL-6. The subapical localization of keratins at the level of the apical junctional complex prompted us to examine the role of keratins in epithelial barrier function. In this context we observed that IL-6 decreases paracellular permeability of FD-4, suggesting a barrier-protective function by IL-6. Among the factors that mediate barrier dysfunction during inflammatory conditions are nitric oxide, platelet-activating factor, and importantly, T-lymphocyte-generated cytokines such as interferon-γ and tumor necrosis factor-α, all of which decrease epithelial barrier function. An additional cytokine that has been proposed to cause alterations in permeability is IL-6. The role of IL-6 in the regulation of barrier function is less clear than that for other cytokines. Some studies have shown that intestinal permeability is preserved in IL-6 knockout mice, indeed, had worse inflammation compared with their wild type counterparts (54, 55). These data were taken to suggest that IL-6 may contribute to the decrease in barrier function under some conditions. In contrast, several other studies have shown a barrier protective function of IL-6. For example, IL-6 has been shown to be required for maintenance of blood brain barrier function during and after injury, and IL-6 has been shown to decrease vascular permeability in bacterial meningitis (56–58). Results from some experiments even suggest that IL-6 may protect the intestinal mucosa from the consequences of systemic inflammation including permeability alterations. For example, oral administration of IL-6 to mice decreased permeability and reduced bacterial invasion through the gastrointestinal...
tinal tract (59–62). Our data are consistent with the latter studies that demonstrate barrier-protective function for IL-6.

Intermediate filaments including keratins have traditionally been regarded as purely mechanical components of the cytoskeleton. However, recent observations of spontaneous mucosal inflammation in K8 null mice in an FVB/N background and placental barrier dysfunction in C57B/6 K8 null mice (15, 17, 63) challenge this notion and highlight the existence of poorly understood mechanisms in the regulation of intestinal function by keratin. Based on these results, we hypothesized that K8/K18 may have an effect on the epithelial barrier, the loss of which forms the basis of the inflammatory response in colitis. Both our in vitro (K8 down-regulation using siRNA did not affect base-line permeability in Caco2-BBE cells) as well as our in vivo data (decreased K8 expression in IL-6 null mice did not affect base-line permeability) demonstrate that K8 may be dispensable for the maintenance of normal barrier function. These observations are similar to those made in Caco2BBE cells using antisense K18 as well as in vivo in K8 null mice (9, 18). On the other hand, our data demonstrate that K8 is required to mediate barrier protection under conditions where the epithelial barrier function is challenged. This is consistent with the data on K8 null mice in C57B/6 background where the absence of K8 affected placental barrier function only in the presence of tumor necrosis factor-α, a pro-inflammatory cytokine known to decrease barrier function (17). Together with the data that K8 is required for IL-6-mediated barrier protection, these data underscore the importance of K8 in maintaining barrier function in the presence of luminal insults such as DSS, which initiates inflammatory response through direct damage to epithelial barrier.

There are many possibilities by which K8/K18 may regulate barrier function during stress. The localization of K8 in the subapical region of intestinal epithelial cells in response to IL-6 raises the possibility of association of K8 with proteins in the apical junctional complex (tight junction or adherens junction), thereby stabilizing the junctional complex. Such a scenario is described in breast cancer cells wherein the direct or indirect association of K18 with desmosomal protein and/or E-cadherin was thought to contribute to cell adhesion and inhibition of invasive potential of K18-overexpressing breast cancer cells (64). Another possible mechanism by which K8 may regulate barrier function is through modulation of signal transduction pathways by phospho-K8. Phosphorylation is the major post-translational modification of keratins, and it plays a role in regulating keratin filament organization and solubility as well as its function (5, 43). Keratins are phosphorylated preferentially in the serine residues in the head and tail region of the subunits, and three human K8 (Ser-73, Ser-431, Ser-23) and two human K18 (Ser-33, Ser-52) major in vivo phosphorylation sites have been characterized (5, 43). K8/K18 phosphorylation regulates several keratin functions in a site-specific fashion, including binding to other proteins (43, 45, 65–67) or serving as a phosphate “sponge” for some stress-activated kinases (68). IL-6 induces serine phosphorylation of K8, which might be involved in the modulation of kinases that regulate the apical junctional complex but which will require further testing.

In summary, we demonstrate that IL-6 is an important regulator of K8/18 expression in colonic epithelial cells. Exogenous IL-6 induces the expression of K8/K18, whereas lack of IL-6 results in diminished base-line expression of K8/18 in the colonic epithelia. Although K8 is dispensable to maintain normal barrier function, it is required to mediate barrier protection by cytokines such as IL-6 during challenges that compromise barrier function. Thus, our data have implications for understanding the role of keratin in barrier function and suggest a potential novel role for these abundant cytoskeletal proteins that extend to intestinal disorders such as inflammatory bowel disease wherein barrier dysfunction underlies the inflammatory response.

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