Phosphatidylinositol 3-Kinase-dependent Pathways Oppose Fas-induced Apoptosis and Limit Chloride Secretion in Human Intestinal Epithelial Cells

IMPLICATIONS FOR INFLAMMATORY DIARRHEAL STATES

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The epithelial lining of the intestine serves as a barrier to luminal bacteria and can be compromised by pathologic Fas-mediated epithelial apoptosis. Phosphatidylinositol (PI)3-kinase signaling has been described to limit apoptosis in other systems. We hypothesized that PI3-kinase-dependent pathways regulate Fas-mediated apoptosis and barrier function in intestinal epithelial cells (IEC). IEC lines (HT-29 and T84) were exposed to agonist anti-Fas antibody in the presence or absence of chemical inhibitors of PI3-kinase (LY294002 and wortmannin). Apoptosis, barrier function, changes in short circuit current (∆Isc), and expression of adhesion molecules were assessed. Inhibition of PI3-kinase strongly sensitized IEC to Fas-mediated apoptosis. Expression of constitutively active Akt, a principal downstream effector of the PI3-kinase pathway, protected against Fas-mediated apoptosis to an extent that was comparable with expression of a genetic caspase inhibitor, p35. PI3-kinase inhibition sensitized to apoptosis by increasing and accelerating Fas-mediated caspase activation. Inhibition of PI3-kinase combined with cross-linking Fas was associated with increased permeability to molecules that were <400 Da but not those that were >3,000 Da. Inhibition of PI3-kinase resulted in chloride secretion that was augmented by cross-linking Fas. Confocal analyses revealed polymerization of actin and maintenance of epithelial cell adhesion molecule-mediated interactions in monolayers exposed to anti-Fas antibody in the context of PI3-kinase inhibition. PI3-kinase-dependent pathways, especially Akt, protect IEC against Fas-mediated apoptosis. Inhibition of PI3-kinase in the context of Fas signaling results in increased chloride secretion and barrier dysfunction. These findings suggest that agonists of PI3-kinase such as growth factors may have a dual effect on intestinal inflammation by protecting epithelial cells against immune-mediated apoptosis and limiting chloride secretory diarrhea.

Animal models of inflammatory bowel disease suggest that derangements in either T cell function or epithelial barrier function play a causal role in the pathophysiology of inflammatory bowel disease (1, 2). Because the epithelial lining of the intestine serves as a critical barrier preventing luminal food antigens, bacterial products, and microorganisms from infiltrating the submucosa (3), disruption in this barrier may initiate or perpetuate intestinal inflammation. One mechanism by which intestinal barrier function may be compromised in inflammatory bowel disease is pathologic intestinal epithelial cell apoptosis. Increased intestinal epithelial cell apoptosis is observed in ulcerative colitis (4) and celiac sprue (5), although it is unclear whether this increase in apoptosis results in compromised barrier function in vivo.

We have developed an in vitro model of immune-mediated epithelial cell apoptosis that permits us to study the effect of Fas-mediated apoptosis on epithelial barrier function (6). In this model, T84 colonic epithelial cells are used because they reproduce the crypt cell phenotype with respect to transport function (7–9), barrier function (10, 11), and protein expression (12, 13). As with colonic crypt epithelial cells, T84 cells express the Fas receptor and are sensitive to Fas-mediated apoptosis (4). These cells have also been used to model crypt abscesses (14). Basolateral cross-linking of Fas on T84 monolayers induces apoptosis. Despite massive cell death, the barrier function to relatively small macromolecules remains intact. Our findings suggested that the intestinal epithelium is quite resilient in the face of apoptotic damage and is able to repair the wound created by apoptotic cell loss. Using this model, we wished to investigate the molecular mechanisms that protect against dysregulated apoptosis and perturbed barrier function in the intestine. The current study focuses on the inter-relationship between the Fas death receptor and phosphatidylinositol (PI)3-kinase signaling pathways in intestinal epithelial cells. We have chosen to study this interplay because Fas-mediated apoptosis of crypt intestinal epithelial cells is associated with human inflammatory bowel disease (4), and PI3-kinase-dependent pathways protect against Fas-mediated apoptosis in the immune system (15, 16).

Several lines of evidence support a role for PI3-kinase-dependent signaling in regulation of apoptosis in the intestine. In response to a variety of extracellular stimuli, PI3-kinase phos...
phorylated products including phosphotidylinositol 3,4,5-trisphosphate (PIP$_3$) (17–19). The generation of PIP$_3$ activates signaling pathways downstream of PI3-kinase, including Akt (also known as protein kinase B), a kinase with anti-apoptotic properties (19–21). A recently identified lipid phosphatase, PTEN, down-regulates PI3-kinase signaling by dephosphorylating PIP$_3$, thereby inhibiting the recruitment and activation of Akt (22–25). Mutations in PTEN are responsible for the genetic syndrome of Cowden’s disease, characterized by hamartomatous polyps of the gastrointestinal tract (26, 27). PTEN$^{+/−}$ mice develop intestinal polyps and dysplasia of the colonic epithelium (26, 28, 29). These mice also develop lymphomas as a result of defective Fas-mediated apoptosis (16). Mice deficient in the catalytic subunit of PI3-kinase γ develop invasive colorectal cancer (30). Recent data demonstrate that PI3-kinase opposes intestinal epithelial differentiation in vitro (31). Taken together these data suggest that PI3-kinase-dependent pathways play a role in the regulation of apoptosis in the intestinal epithelium.

PI3-kinase and Akt may also play important roles in epithelial adhesion and barrier function. Cellular adhesion to the extracellular matrix or neighboring cells results in the activation of PI3-kinase, recruitment of Akt, and protection against apoptosis (32–34). Membrane-bound Akt and PIP$_3$ accumulate at the leading edge of cells responding to chemotactic factors, suggesting that cell movement is regulated by Akt (35–38). Finally, PI3-kinase activation is involved in actin cytoskeletal rearrangements leading to cell spreading (39, 40).

In this study, we tested the hypothesis that PI3-kinase-dependent mechanisms protect intestinal epithelial cells from Fas-mediated apoptosis and barrier dysfunction. We found that inhibition of PI3-kinase sensitizes intestinal epithelial cells to Fas-mediated apoptosis and exacerbates the barrier dysfunction associated with Fas-mediated apoptosis. Caspase inhibition protects against both apoptosis and increased monolayer permeability. Inhibition of PI3-kinase results in increased epithelial conductance and chloride secretion, which is increased further by activation of Fas. Despite marked apoptosis, the intestinal epithelial monolayer is able to maintain a relatively impermeable barrier by undergoing cytoskeletal changes and maintaining cell-cell adhesion. Our studies identify molecular mechanisms by which peptide growth factors may exert a beneficial clinical effect in patients with inflammatory diarrheal states.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis—HT-29 cells were grown in modified McCoy’s medium with 5% penicillin/streptomycin supplemented with 10% fetal bovine serum. Confluent monolayers of the human colon cell line T84 were grown on 12-mm Transwells, polycarbonate membranes (Costar 3401) and maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with 5% penicillin/streptomycin, 5% l-glutamine, supplemented with 5% fetal bovine serum. The cells were kept in a humidified incubator at 37°C with 5% CO$_2$. Apoptosis was induced by the addition of agonist Ab to the Fas receptor (Upstate Biotechnology, Inc., Lake Placid, NY). The caspase inhibitor Z-VAD-FMK (Biomol Research Labs) or an irrelevant caspase inhibitor CBZ-Phe-Ala-FMK was added at a final concentration of 50 µM for 2 h before addition of anti-Fas (Enzyme Systems Products, Livermore, CA). The cells were cotransfected with the PI3-kinase inhibitor LY294002 (Calbiochem) or wortmannin (Biomol Research Labs) for 2 h prior to addition of anti-Fas Ab unless otherwise stated. LY294002 was used at a concentration of 25 µM. Anti-Fas Ab was used at a concentration of 250 ng/ml added to the basolateral well of T84 culture inserts. Apoptosis was quantified by counting the number of nuclei demonstrating chromatin condensation per high power field or, in the case of M30 staining (below), by counting the number of M30-positive cells. Nuclear debris was not counted. An average of 10 fields/experiment were counted, and the observer was blinded to the treatment.

Conductance, Resistance, and Permeability Measurements—Transepithelial electrical resistance (TER) was measured using a Millipore Millicell-ERS Voltmeter. The experiments were performed when monolayers achieved a TEER $>$500 Ω cm$^2$ (at 200–250 µA/cm$^2$). Chloride secretion was measured as short circuit current ($I_{sc}$) across monolayers of T84 cells, mounted in Ussing chambers (window area, 0.6 cm$^2$) modified for use with cultured cells (41, 42). $I_{sc}$ (normalized to µA/cm$^2$) was used to quantitate both basal transepithelial chloride secretion and that induced by inhibition of PI3-kinase and cross-linking Fas. T84 cells were preincubated with the PI3-kinase inhibitors and were challenged with anti-Fas Ab (Enzyme Systems Products, Livermore, CA), followed by fluorescein-conjugated anti-mouse-IgG-biotin (Molecular Probes, Eugene, OR) and FITC (molecular weight, 3,000) (Molecular Probes, Eugene, OR) were utilized as described previously (44). Briefly, 10 µg/ml of FITC or dextran-FITC was added to the apical well; the samples (50 µl) were removed from the basolateral well at indicated times and quantitated with a PerSeptive Biosystems CytoFluor® Multi-Well Plate Reader, Series 4000. The sensitivity of this assay is 5 ng/ml. Some monolayers were exposed to UV irradiation, which led to $>$90% cell death and served as a positive control for flux. UV irradiation of monolayers was accomplished by delivering 200 mJ/cm$^2$ using a Stratalinker 1800 (Stratagene).

Gene Expression Assays—T84 cells were seeded in 12-mm transwells at 200,000 cells/well on the day before transfection. Eugene 6 (Roche Molecular Biochemicals) reagent was used as per the manufacturer’s instructions to transfect 1 µg of total DNA/well. To identify transfected cells, 0.3 µg of green fluorescent protein (GFP) plasmid (CLONTECH) was cotransfected with 0.7 µg of pcDNA3 (control plasmid) (Invitrogen), myristylated Akt, kinase-inactive Akt (45), or p35 (46, 47). Flow cytometry demonstrated a transfection efficiency ranging from 10 to 20% (data not shown).

Immunofluorescence Studies—For staining of caspase-cleaved cytokeratin 18, M30 Cytodeath monoclonal Ab and anti-mouse-IgG-biotin were purchased from Roche Molecular Biochemicals and used as per the manufacturer’s instructions. Unless otherwise stated, all incubation steps were performed at room temperature. For actin staining, membranes were washed twice with PBS and fixed with 3% paraformaldehyde in PBS for 20 min, then permeabilized with cold acetone for 7 min at 20°C. Then, permeabilized membranes were washed twice with PBS and fixed with 3% paraformaldehyde (Hanks’ buffered saline solution with 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$, fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized with 3% Triton X-100 in PBS for 5 min, incubated with 5 µM glycine in PBS for 20 min, blocked in PBS with 2% goat serum and 1% bovine serum albumin fraction V for 1 h, washed with PBS-GS (0.2% goat serum, 0.1% bovine serum albumin fraction V, 5 µM glycine in PBS), incubated with 5 µg/ml anti-EpCAM for 1 h, washed with PBS-GS, and incubated with 5 µg/ml rhodamine-conjugated anti-mouse IgG (Chemicon, Temecula, CA) for 1 h. The nuclei were counterstained with 2.5 µg/ml Hoechst 33258 (Molecular Probes) for 15 min. For EpCAM staining membranes were first washed with warmed Hanks’ buffered saline solution with 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$, fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized with 3% Triton X-100 in PBS for 5 min, incubated with 5 µM glycine in PBS for 20 min, blocked in PBS with 2% goat serum and 1% bovine serum albumin fraction V for 1 h, washed with PBS-GS (0.2% goat serum, 0.1% bovine serum albumin fraction V, 5 µM glycine in PBS), incubated with 5 µg/ml anti-EpCAM for 1 h, washed with PBS-GS, and incubated with 5 µg/ml rhodamine-conjugated anti-mouse (Chemicon, Temecula, CA) for 1 h. The nuclei were counterstained with 2.5 µg/ml Hoechst 33258 as described above.

For zona occludens-1 (ZO-1) staining, the membranes were preincubated with a buffer containing 0.2% Triton-X, 100 mM KCl, 3 mM MgCl$_2$, 1 mM CaCl$_2$, 200 mM sucrose, and 10 mM HEPES (pH 7.1) for 2 min on ice, followed by fixation in PBS with 4% paraformaldehyde, and incubated with primary anti-ZO-1 Ab (1:200 dilution) (Zymed Laboratories Inc., San Francisco, CA), followed by fluorescein-conjugated anti-rabbit Ab (1:100 dilution) (Vector Laboratories, Burlingame, CA). For desmoplakin staining, the membranes were fixed in methanol at $−20°C$, blocked in PBS with 1% bovine serum albumin, followed by incubation with primary anti-desmoplakin I + II Ab (ICN Biomedicals, Inc., Costa Mesa, CA) (1:100 dilution) and rhodamine-conjugated anti-mouse IgG (1:200 dilution) (Chemicon, Temecula, CA) for 2 h.

For GFIP-transfected cells, cells were fixed in 4% paraformaldehyde. The nuclei were counterstained with 50 µg/ml RNase A (Sigma) for 30 min, followed by an incubation with 1 µg/ml propidium iodide in PBS for 15 min. Immunofluorescence was visualized with a Leica TCS SP laser scanning inverted confocal microscope.

Immunoblot Analysis—To analyze protein expression, Western blotting was performed for the presence of phosphorylated Akt, total Akt,
and PTEN. Freshly isolated human colon epithelial cells were obtained from colonic resections after detachment in EDTA. Briefly, $1 \times 10^6$
intestinal epithelial cells (T84 or freshly isolated human colonic epithelial cells) were lysed in 150 μl of 2× SSB (100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), sonicated, and boiled at 95 °C for 5 min, and 12 μl of boiled lysate (10 μg of protein) was separated in a 10% Tris-HCl polyacrylamide gel (Bio-Rad). Proteins were transferred to nitrocellulose membranes and stained with Ponceau S to verify equal protein loading. For phosphorylated Western blots, the membranes were blocked in 5% milk, 0.1% Tween 20 in TBS for 2–3 h at 4 °C, incubated overnight at 4 °C with anti-phosphorylated Akt (Ser473) (Cell Signaling Technology, Beverly, MA) followed by a 1-h incubation at room temperature with anti-rabbit horse-radish peroxidase, developed by Lumiglo (Cell Signaling Technology), and exposed to radiographic film. For total Akt Western blots, the membranes were probed with an anti-Akt Ab as above (Cell Signaling Technology). For PTEN Western blots, the membranes were blocked in 5% milk in TBS for 2 h, incubated with 1 μg/ml anti-PTEN in TBS containing 5% bovine serum albumin and 1% Triton X-100 for 2 h, washed with 0.1% Tween 20 in TBS, incubated with 1:1000 anti-mouse horseradish peroxidase (Chemicon) in blocking buffer for 1 h, washed, and developed by Lumiglo followed by exposure to radiographic film.

Statistical Analysis—Student’s t tests, standard deviation, and standard errors were performed using the statistics package within Microsoft Excel. Two-way analysis of variance was performed for the changes of $I_a$ and G using Graph Pad Prizm (San Diego, CA). $P$ values were considered statistically significant when <0.05.

RESULTS

Inhibition of PI3-Kinase Sensitizes Intestinal Epithelial Cells to Fas-mediated Apoptosis—Intestinal epithelial cells depend on trophic signals for survival, and these survival signals may mediate their effect through activation of PI3-kinase. To constitute a relevant survival pathway in the gut, the PI3-kinase-Akt pathway must be present in human intestinal epithelial cells. We first wished to establish that the PI3-kinase-Akt pathway is functional in human intestinal epithelial cells and intestinal epithelial cell lines. We found that both freshly isolated colonic epithelial cells and intestinal epithelial cell lines express Akt protein (Fig. 1A). We also wished to determine whether the intestinal epithelial cell lines used in our studies expressed the lipid phosphatase PTEN. PTEN mutations are associated with increased levels of phosphorylated Akt and may interfere with studying PI3-kinase in these cells. Fig. 1B demonstrates that both cell lines express PTEN at a level comparable with freshly isolated colonic epithelial cells. Sequencing of the PTEN gene in T84 cells also did not reveal any mutations.2 Thus, intestinal epithelial cell lines are a valid model for studying both Fas-mediated apoptosis and PI3-kinase signaling in the gut.

Based on recent data that PI3-kinase-dependent pathways protect against apoptosis in other systems, we hypothesized that PI3-kinase-dependent signals protect intestinal epithelial cells from Fas-mediated apoptosis. To test this hypothesis, we used two models of Fas-mediated intestinal epithelial cell apoptosis: HT-29 and T84 cell monolayers (6, 48, 49). Both T84 cells and HT-29 cells express Fas receptor, and cross-linking of the receptor with agonist Ab induces apoptosis. HT-29 cells, however, require preincubation with interferon γ to sensitize cells to Fas-mediated apoptosis (48). To inhibit class I PI3-kinases we used the highly specific chemical inhibitors LY294002 and wortmannin. We determined the minimum concentration of LY294002 or wortmannin required to block insulin-stimulated phosphorylation of Akt, a target of PI3-kinase (Fig. 1C). Using the minimum concentration of these inhibitors required to block PI3-kinase, we found that inhibition of PI3-kinase strongly sensitized both HT-29 and T84 cells to Fas-mediated apoptosis (Fig. 2). Indeed, inhibition of PI3-kinase in HT-29 cells obviated the need for interferon γ sensitization for Fas-mediated apoptosis. Inhibition of PI3-kinase alone did not result in significant apoptosis. Similar results were found with wortmannin (data not shown). These results suggest that PI3-kinase-dependent pathways constitutively protect intestinal epithelial cells from immune-mediated apoptosis.

Akt, a PI3-Kinase-dependent Kinase, Mediates Protection from Fas-mediated Apoptosis in Intestinal Epithelial Cells—The data above suggest that PI3-kinase-dependent mechanisms protect intestinal epithelial cells from Fas-mediated apoptosis. We wished to test the hypothesis that Akt is the principal downstream effector mediating PI3-kinase-dependent protection from Fas-mediated apoptosis. To test this hypothesis we utilized a transient gene expression strategy to introduce a constitutively active allele of Akt. Akt is made constitutively active by the addition of a myristylation signal (myr-Akt) to the

2 J.-Y. Chow, K. E. Huang, S. Carethers, and J. Barrett, unpublished observations.
PI3-Kinase-dependent Sensitization to Fas-mediated Apoptosis Is Due to Enhanced Caspase Activation—Caspases are cysteine proteases that are responsible for the morphologic and biochemical features of apoptosis (50–52). In certain cell types, however, caspase inhibition does not prevent all of the morphologic changes of apoptosis in response to Fas stimulation (53–55). PI3-kinase-dependent activation of Akt protects against apoptosis in neuronal, hematopoetic, and epithelial cells by limiting mitochondrial release of pro-apoptotic factors and thus limiting caspase activation (19, 56–60). We hypothesized that inhibition of PI3-kinase sensitizes to Fas-mediated apoptosis in a caspase-dependent fashion. To test this hypothesis we used a biochemical strategy to inhibit caspase activation with the cell-permeable, broad specificity caspase inhibitor Z-VAD-fmk. Z-VAD-fmk inhibited apoptosis in response to cross-linking Fas in the presence or absence of PI3-kinase inhibitors (Fig. 4A). These data support the conclusion that caspase activation is required for the synergistic apoptosis seen in response to cross-linking Fas in the context of PI3-kinase inhibition.

We next wished to understand the mechanism by which inhibition of PI3-kinase sensitized to Fas-mediated apoptosis. Cross-linking Fas results in cleavage of caspase 8, which in turn leads to the cleavage and activation of the effector caspase 3. Because caspase inhibition prevented the apoptosis from PI3-kinase inhibition with cross-linking Fas, we reasoned that PI3-kinase inhibition may enhance caspase activation in response to Fas stimulation. Activation of caspase 3 and 7 in epithelial cells is associated with cleavage of cytokeratin 18 (61). We have previously shown that Fas-mediated apoptosis of T84 cells results in cleavage of cytokeratin 18 detectable 18 h following cross-linking of Fas (6). To determine whether inhibition of PI3-kinase leads to increased caspase activation in Fas-stimulated cells, T84 monolayers were exposed to anti-Fas Ab in the presence or absence of the PI3-kinase inhibitor LY294002, and the cells were stained with an Ab to the caspase cleavage product of cytokeratin 18, a marker of effector caspase 3 and 7 activation. Our data demonstrate that inhibition of PI3-kinase does not by itself result in caspase activation (Fig. 4B). In contrast, inhibition of PI3-kinase followed by cross-linking Fas results in accelerated caspase activation detectable within 6 h following cross-linking of Fas (Fig. 4B). In addition to the accelerated kinetics, inhibition of PI3-kinase followed by cross-linking Fas results in more cells demonstrating caspase activation by 18 h. These data suggest that inhibition of PI3-kinase may increase caspase substrate availability. In response to the Fas signal, a caspase amplification loop may be activated resulting in earlier and greater caspase activation compared with cells stimulated with anti-Fas Ab alone.

Inhibition of PI3-Kinase Sensitizes Intestinal Epithelial Cells to Fas-mediated Barrier Dysfunction—We have previously demonstrated that Fas-mediated apoptosis of T84 monolayers results in increased permeability to small molecules such as mannitol but not to larger macromolecules (>3,000 Da). Inhibition of PI3-kinase dramatically sensitizes to Fas-mediated apoptosis (Figs. 2 and 4B). We wished to determine the functional consequence of this synergistic apoptosis on the permeability of monolayers T84 cells, a well established model of intestinal epithelial barrier function. T84 monolayers were cultured until they attained stable electrical resistance (>2,000 ohms/cm²) and exposed to agonist anti-Fas Ab in the presence or absence of PI3-kinase inhibitors. Our data demonstrate that inhibition of PI3-kinase results in decreased transepithelial resistance in T84 monolayers exposed to anti-Fas Ab (Fig. 5A). Inhibition of PI3-kinase in combination with cross-linking Fas results in a 75% decrease in TER compared with a 50% decrease in TER in monolayers exposed to anti-Fas alone. Inhibition of PI3-kinase alone is associated with diminished transepithelial resistance (average 25% below base line) compared with control monolayers, although this decrease in transepithelial resistance is not associated with morphologic apoptosis (Fig. 2). These data suggest that PI3-kinase-dependent pathways may have at least two effects with respect to transepithelial resistance: one that is associated with Fas-mediated apoptosis and another that is independent of apoptosis.

![Fig. 2. PI3-kinase inhibition sensitizes T84 and HT-29 cells to Fas-mediated apoptosis.](http://www.jbc.org/content/110/38/15097/F1.large.jpg)
Based on the diminished transepithelial resistance and marked apoptosis of T84 monolayers exposed to anti-Fas Ab and PI3-kinase inhibitors, we hypothesized that this combination would increase monolayer permeability to small and large molecules. To determine the degree of leakiness of T84 monolayers undergoing apoptosis and to estimate the molecular size range of substances now able to cross the monolayer, we used FITC (FW 389.4) as the smallest probe of flux and FITC-conjugated to dextran of 3,000 Da as the largest probe of flux (44, 62). FITC flux was measured 24 h after cross-linking of Fas in the context of PI3-kinase inhibition and demonstrated greater than 10-fold increased flux compared with intact monolayers and greater than 3-fold increased flux compared with monolayers exposed to anti-Fas alone (Fig. 5B, left panel). We then used dextran-FITC of 3,000 Da to probe the magnitude of the defect caused by apoptosis of T84 monolayers. As above, we assayed for dextran-FITC flux during the peak of apoptosis 24 h following addition of anti-Fas Ab and inhibition of PI3-kinase (Fig. 5B, right panel). Despite the drop in TER and increased FITC flux, no increase in transcellular flux of dextran-FITC of 3,000 Da was detected in monolayers treated with anti-Fas Ab in the presence or absence of PI3-kinase inhibitors. UV-irradiated T84 monolayers that undergo massive apoptosis with 80% cell loss by 12 h were used as positive controls for dextran-FITC flux (Fig. 5B). These data demonstrate that an intestinal epithelial monolayer is made leaky to small molecules by apoptosis but remains impermeable to relatively small macromolecules (>3,000 Da). Inhibition of PI3-kinase alone is sufficient to decrease transepithelial resistance but is not associated with increased permeability to FITC or larger molecules. This finding of relatively well maintained barrier function in the face of apoptosis was similar to that seen in response to Fas-mediated apoptosis. These data suggest that, even when lacking the function of PI3-kinase, an intestinal epithelial cell monolayer retains its ability to compensate for apoptotic cell loss and to serve as a barrier to lumenal antigens of >3,000 Da.

Inhibition of PI3-Kinase Increases Chloride Secretion in T84 Monolayers—In addition to their roles in protection against apoptosis, PI3-kinase and Akt may play an important role in epithelial barrier function, because data demonstrate that inhibition of PI3-kinase in the presence or absence of cross-linking Fas results in diminished TER (Fig. 5A). TER is determined by paracellular permeability as well as an inverse measure of electrical conductance across a monolayer (43). We wished to determine whether the increased paracellular permeability occurring during apoptosis provided the basis for the observed decrease in TER. To address this question, T84 monolayers were treated with the PI3-kinase inhibitor LY294002 and anti-Fas Ab in the presence or absence of the caspase inhibitor Z-VAD-fmk. We have shown above that Z-VAD-fmk inhibits apoptosis in this model (Fig. 4A). Consistent with our previously published data, Z-VAD-fmk inhibits the decrease in TER that occurs in response to cross-linking Fas (Fig. 6A) (6). However, caspase inhibition only partially inhibited the decreased TER seen with PI3-kinase inhibition and cross-linking Fas. The extent to which caspase inhibition protected against diminished TER could be attributable to its effect on Fas-mediated barrier dysfunction, because caspase inhibition did not prevent the drop in TER associated with PI3-kinase inhibition alone. This latter observation is consistent with our finding that PI3-kinase inhibition is associated with diminished transepithelial resistance in the absence of significant apoptosis. These findings demonstrate that inhibition of caspases prevents the diminished TER that is secondary to apoptosis. The data further suggest that inhibition of PI3-kinase results in diminished TER that is independent of apoptosis.

PI3-kinase-dependent pathways have been implicated in regulation of chloride secretion in intestinal epithelial cells, and PI3-kinase is present in the brush border of rabbit ileum (63, 64). In T84 monolayers, epidermal growth factor inhibits calcium-dependent chloride secretion (65). The inhibitory effect of epidermal growth factor can be blocked by PI3-kinase inhibition, suggesting that PI3-kinase-dependent pathways are involved in regulation of chloride transport. To address whether inhibition of PI3-kinase has an effect on intestinal epithelial cell conductance and chloride secretion, T84 monolayers were exposed to agonist Ab to Fas or medium for 24 h and then mounted in Ussing chambers and challenged immediately with LY294002. This treatment with LY294002 results in increased chloride secretion, as assessed by an increase in short circuit current (Isc), which was followed by an increase in conductance of T84 monolayers (Fig. 6, B and C). Whereas cross-linking Fas alone had no effect on either chloride secretion or conductance,
when LY294002 was given after anti-Fas, there was a significantly greater increase in chloride secretion than with LY294002 treatment alone. There was also a much greater increase in monolayer conductance under these conditions. These data demonstrate that the diminished TER observed in T84 monolayers exposed to LY294002 is likely due to active chloride secretion, resulting in an increase in transcellular conductance across T84 monolayers. We further show that pathways downstream of Fas can potentiate chloride secretion in the setting of PI3-kinase inhibition, but not independently. These data suggest that PI3-kinase-dependent pathways tonically inhibit chloride secretion in intestinal epithelial cells.

Intestinal Epithelial Monolayers Repair the Wound Created by Apoptotic Cell Loss through Actin Polymerization and Preserved Cell-Cell Adhesion—Despite dramatic apoptosis in intestinal epithelial cell monolayers exposed to agonist anti-Fas and to the PI3-kinase inhibitor LY294002, the monolayers remain relatively impermeable even to small macromolecules. We have previously shown that T84 monolayers undergoing Fas-mediated apoptosis demonstrate dramatic cell flattening to maintain E-cadherin-mediated junctions and to re-establish tight junctions between the remaining viable cells (6). We wished to determine the mechanisms by which intestinal epithelial monolayers accomplish this repair, especially in the face of PI3-kinase inhibition. PI3-kinase-dependent pathways have been implicated in the regulation of cell morphology and actin cytoskeletal rearrangements (39, 40). We began by examining the role of F-actin in apoptotic T84 monolayers and the effect of PI3-kinase inhibition on stress fiber formation. Confluent T84 monolayers express F-actin in a perijunctional apical distribution and basal distribution (Fig. 7A, Control) (66–68). Monolayers exposed to agonist Ab to Fas undergo apoptosis and the remaining viable cells polymerize actin as demonstrated by actin stress fibers seen 24 h after cross-linking Fas (Fig. 7A, anti-Fas). Inhibition of PI3-kinase causes subtle changes in the actin cytoskeleton (Fig. 7A, LY294002). Cross-linking Fas in the context of PI3-kinase inhibition leads to greater apoptosis (nuclear stain) and increased stress fiber formation compared with exposure to anti-Fas or LY294002 individually (Fig. 7A, LY294002 plus anti-Fas compared with anti-Fas alone at 6 h and 18 h). The number in the lower right corner of each panel represents the average number of M30-positive cells/high powered field ± S.D. Magnification is 600×.
PI3-kinase and Akt are associated with extracellular matrix attachment sites and points of cell-cell contact and may therefore be involved in the regulation of cell adhesion (32). We wished to look at the effect of PI3-kinase inhibition on T84 cell adhesion and junctional protein expression. Specifically, we wished to understand how T84 monolayers preserved a relatively intact barrier in the face of PI3-kinase inhibition and Fas-mediated apoptosis. We chose to examine the effect of PI3-kinase inhibition and Fas-mediated apoptosis on expression of the epithelium-specific cell-cell adhesion molecule EpCAM. EpCAM interacts with the actin cytoskeleton through its cytoplasmic tail (71). Confluent T84 monolayers express EpCAM on their lateral and basal surfaces at points of cell-cell and cell-matrix contact (Fig. 7B, Control). In apoptotic monolayers, individual apoptotic bodies are also lined by EpCAM (see arrows). T84 monolayers exposed to agonist anti-Fas Ab followed by inhibition of PI3-kinase for an additional 24 h have a marked increase in apoptosis, and the apoptotic bodies appear rimmed by EpCAM. However, there are no overt gaps in apoptotic monolayers stained for EpCAM expression. Thus, the adhesion between intact cells and apoptotic cells is maintained by EpCAM-mediated interactions.

Tight junctions and desmosomes maintain intestinal barrier function, and these structures can be perturbed in inflammatory bowel disease (72, 73) and infectious colitides (74–76). We had previously demonstrated that Fas-mediated apoptosis resulted in structural rearrangement of tight junctions and desmosomes such that the remaining cells created a bridge that excluded apoptotic cells (6). We next addressed whether inhibition of PI3-kinase has an effect on the junctional structure of T84 monolayers. T84 monolayers undergoing Fas-mediated apoptosis were exposed to LY294002 and stained for the tight junction protein ZO-1 (Fig. 8A) or the desmosomal proteins desmoplakin 1 and 2 (Fig. 8B). Consistent with our previous studies, our data demonstrate that the junctional structure is characterized by wide tight junctional outlines in areas of cell drop out (Fig. 8A, anti-Fas and anti-Fas + LY294002). Similar wide outlines are present in the desmosomal pattern in apoptotic monolayers (Fig. 8B, anti-Fas and anti-Fas + LY294002). In addition, cytoplasmic staining of desmoplakin proteins is increased in monolayers exposed to anti-Fas and LY294002, suggesting that these monolayers are undergoing rapid remodeling (77). PI3-kinase inhibition by itself does not result in changes in junctional protein distribution. These findings help to explain the relatively well preserved barrier function despite marked cell loss.

**DISCUSSION**

In health, the intestinal epithelium is replaced every 3–5 days suggesting balanced processes of cell death and proliferation. Thus, the intestinal epithelium must integrate a variety of growth and death signals while maintaining an uninterrupted barrier. Inflammatory bowel diseases, nonsteroidal anti-inflammatory drug-induced injury, and celiac disease are associated with an increased rate of intestinal epithelial cell apoptosis. This study examines two inter-related intestinal...
epithelial responses: regulation of apoptosis and repair of the defect created by apoptotic cell loss. In human and murine studies, colonic epithelial cell apoptosis occurs at the base of the crypt near the dividing stem cell and at the luminal surface (4, 78). We have previously described a model of intestinal epithelial apoptosis using T84 cells. In this model, basolateral cross-linking of the Fas receptor results in apoptotic cell loss (6). Although T84 cells reproduce the crypt cell phenotype in many respects, a homogenous cell line cannot replicate all stages of colonic epithelial cell differentiation. Because we use T84 cells that have been cultured on semi-permeable supports to confluence, little cell division is occurring during the time of our experiments. We speculate, therefore, that our model represents an intermediate stage between the dividing stem cell and the most differentiated luminal colonocytes. Using this system, we found that loss of cells through apoptosis resulted in dramatic cell flattening and maintenance of E-cadherin-mediated cell-cell interactions of the remaining viable cells. The outcome is a remarkable preservation of barrier function despite apoptotic cell loss. Our studies suggested that this model epithelium undergoes a process similar to epithelial restitution to repair the wound created by apoptotic cell loss.

In an effort to understand the signaling pathways by which intestinal epithelial cells limit apoptosis and promote epithelial restitution, we examined the role of PI3-kinase. Our study suggests that signaling via the PI3-kinase pathway tonically inhibits intestinal epithelial cells to Fas-mediated apoptosis remains to be elucidated fully. HT-29 colon cancer cells are susceptible to Fas-mediated apoptosis but are partially protected against apoptosis by interleukin-13 through its effect on PI3-kinase (79). Studies have shown that PI3-kinase, acting through downstream kinases, especially Akt, exerts an anti-apoptotic effect through inhibition of mitochondrial permeability, caspase 8, and FADD availability and pro-caspase 9 protease activity (21, 56, 59, 80, 81). Our study demonstrates that inhibition of PI3-kinase sensitizes cells to Fas-mediated apoptosis in a caspase-dependent fashion. We have also shown that inhibition of PI3-kinase accelerates caspase activation in

![Fig. 6. A, caspase inhibition prevents the drop in transepithelial electrical resistance in response to Fas-mediated apoptosis but not in response to PI3-kinase inhibition. T84 cells were cultured on solid supports to achieve electrically resistant monolayers. Monolayers were treated with anti-Fas Ab, LY294002, or a combination of LY294002 and anti-Fas for 24 h in the presence or absence of the caspase inhibitor Z-VAD-fmk, and TER was measured. Z-VAD-fmk prevents the drop in TER in monolayers exposed to agonist Ab to Fas and partially inhibits the drop in TER in monolayers exposed to both anti-Fas Ab and LY294002. Caspase inhibition does not block the drop in TER associated with LY294002. This graph depicts one experiment representative of three with similar results and was performed in triplicate. The error bars indicate the standard deviation. A statistically significant difference (p < 0.01) in TER compared with controls was found in anti-Fas-, LY294002-, LY294002 + anti-Fas, Z-VAD + LY294002-, and Z-VAD + LY294002 + anti-Fas-treated monolayers but not in monolayers treated with Z-VAD alone or Z-VAD + anti-Fas. B and C, inhibition of PI3-kinase increases chloride secretion and conductance in T84 monolayers. T84 cells were cultured to achieve electrically resistant monolayers. Monolayers were treated with anti-Fas Ab medium alone for 24 h and then mounted in Ussing chambers and LY294002 added as indicated. B demonstrates changes in short circuit current (ΔIsc) over 2 h after mounting in Ussing chambers, and C represents changes in conductance (ΔG). The increase in Isc precedes the increase in conductance. The control and LY294002 conductance curves begin to diverge within 15 min after the addition of LY294002. These early time points are relatively obscured by the scale of the graph. The separation in the two curves begins to achieve statistical significance 45 min after addition of LY294002 (p < 0.05). All data are the means ± S.E. from three experiments. Starting values of Isc and G (in UA/cm² and MS/cm², respectively) were as follows: control, 1.7 ± 0.3 and 1.3 ± 0.4; anti-Fas, 1.5 ± 0.5 and 1–7 ± 0.4; LY294002, 1.7 ± 0.3 and 0 ± 1; and anti-Fas plus LY294002, 1.8 ± 0.5 and 2.4 ± 0.8 (no significant differences).]
FIG. 7. A, effect of PI3-kinase inhibition on F-actin in T84 monolayers undergoing Fas-mediated apoptosis. T84 cells were cultured on permeable supports to achieve electrically resistant monolayers. Monolayers were treated with anti-Fas Ab, LY294002, or a combination of LY294002 and anti-Fas for 24 h and then stained with FITC-conjugated phalloidin to detect F-actin. The nuclei were counterstained with propidium iodide (red). A series of confocal images was taken every 0.5 μm through the monolayers and projected to visualize the actin stress fibers (top row). The bottom row demonstrates the relationship between stress fibers and nuclei in T84 monolayers. Apoptosis results in stress fiber formation in the remaining viable cells. B, T84 cell monolayer continuity is maintained through preserved intercellular adhesion mediated by EpCAM. T84 cells were cultured on permeable supports to achieve electrically resistant monolayers. Monolayers were treated with anti-Fas Ab, LY294002, or a combination of LY294002 and anti-Fas for 48 h and then stained with an antibody that recognizes EpCAM (blue), and nuclei were counterstained with propidium iodide (red). A series of confocal images was taken every 0.5 μm through the monolayers and projected to visualize the relationship between nuclei or nuclear debris and EpCAM-lined cytoplasm in apoptotic monolayers. EpCAM staining surrounds individual cells and apoptotic bodies.

response to cross-linking Fas. Our data suggest a model in which PI3-kinase-dependent pathways, by limiting caspase availability, dampen an apoptotic stimulus, such as Fas receptor engagement in intestinal epithelial cells. Future studies will examine the caspase activation sequence in intestinal epithelial cells and the effect of PI3-kinase on its dynamics.

In this study, we hypothesized that PI3-kinase-dependent pathways might play a role in intestinal epithelial barrier function, either as a result of modulating cellular sensitivity to an apoptotic signal or through an effect on epithelial cell-matrix and cell-cell signaling pathways (32, 33). We were surprised to find that when marked apoptosis was induced during inhibition of PI3-kinase, relatively small macromolecules (3,000 Da) were still unable to traverse the injured barrier. Thus, whole bacteria or lipopolysaccharide (10,000–20,000 Da) would be excluded, but deficient barrier function to smaller molecules could explain the increased permeability to disaccharides in patients with Crohn’s disease (82). We have also previously shown that the barrier function of this model intestinal epithelium is quite resilient in the face of Fas-mediated apoptotic injury. The data presented in the current study demonstrate that repair of apoptotic injury even in the case of monolayers undergoing >90% apoptosis is a process that is dependent on PI3-kinase. Furthermore, PI3-kinase-dependent pathways do not seem to be required for repair of apoptotic injury because actin polymerization and dramatic cell flattening are even more pronounced when Fas is cross-linked in combination with PI3-kinase inhibitors than when cells are treated with anti-Fas alone. Although we have not directly examined the role of actin polymerization in repair of the apoptotic monolayer, studies in which the actin cytoskeleton has been disrupted have demonstrated diminished epithelial barrier function, suggesting that actin plays a major role in maintenance of an impermeable epithelial barrier (83–85). Our model of T84 cell apoptosis parallels some of the findings seen during epithelial restitution with respect to actin polymerization and cell flattening (66, 68, 86). The signal(s) required for an intact epithelial cell to sense and respond to the death of its neighbor are not understood. However, our data suggest that intestinal epithelial cells do not require PI3-kinase for the cytoskeletal changes involved in repairing the defect created by apoptotic cell loss.

Our study is the first to examine the expression of the adhesion molecule EpCAM in a model intestinal epithelium in the intact and apoptotic states. EpCAM, also known as the KSA antigen, was first described as a cell surface antigen present on the majority of epithelial-derived tumors. Characterization of EpCAM revealed that it is involved in homophilic cell-cell interactions and that its cytosolic tail interacts with the actin cytoskeleton (71, 87, 88). A monoclonal Ab to EpCAM is currently being evaluated as treatment for patients with advanced colon cancer (89). We have examined the spatial expression pattern of EpCAM in T84 cells and found that in intact monolayers, expression of EpCAM is basal and lateral (Fig. 7B), suggesting that EpCAM is involved in both cell-cell as well as cell-matrix interactions in the intestine. We have previously shown that apoptotic monolayers maintain E-cadherin-mediated junctions between the remaining intact cells (6). Our current studies with EpCAM demonstrate that apoptotic bodies continue to be lined by EpCAM and are in continuity with the intact cells. This finding suggests that apoptotic and intact cells continue to interact even as the apoptotic cell is fragmenting and lifting away from the remainder of the monolayer. The
finding of EpCAM-lined apoptotic bodies in continuity with intact cells may help to explain why the apoptotic cell does not leave a hole as it separates from the monolayer and may provide a signal for the neighboring intact cells to fill an imminent void. The interaction of EpCAM with the actin cytoskeleton may integrate the signals required for an intact cell to recognize the retraction of its apoptotic neighbor, polymerize actin, and change its morphology. The function of EpCAM in intestinal epithelial barrier function deserves further investigation.

In addition to exploring the role of PI3-kinase in barrier function, we have examined the role of PI3-kinase in chloride secretion. We have previously shown that inhibition of PI3-kinase in T84 cells blocks epidermal growth factor-mediated inhibition of chloride secretion, suggesting that PI3-kinase-dependent pathways play a role in the regulation of this transport process (65). Our studies extend this observation by suggesting that PI3-kinase-dependent pathways may tonically inhibit chloride secretion in intestinal epithelial cells, at least under certain circumstances. Our data further suggest that in the response to infection, cross-linking Fas may play a coregulatory role in chloride secretion in the intestinal epithelium. Recent studies in an animal model of pseudomonal pneumonia demonstrate that Fas-mediated death of lung epithelial cells controls the primary infection, whereas Fas mutant mice succumb to disseminated bacterial infection (90). Enteroinvasive bacteria have been shown to induce apoptosis in intestinal epithelial cells in vitro, supporting the notion that apoptosis may be a means to prevent the spread of infection (91). Fas-mediated signaling in the intestinal epithelium may play a similar role, with infected intestinal epithelial cells undergoing apoptosis and the remaining cells secreting chloride in an effort to expel the remaining organisms. These hypotheses await testing in animal models.

The combined results of our studies have several implications for the medical therapy of inflammatory bowel diseases. In the setting of inflammation, diminished growth factor signals may render intestinal epithelial cells susceptible to immune-mediated apoptosis. Studies performed in animal models of colitis demonstrate that growth factors, such as growth hormone, epidermal growth factor, and keratinocyte growth factor, ameliorate the ulceration of the mucosa in colitic animals (92, 93). A recent study also demonstrated clinical benefit from growth hormone therapy in the treatment of Crohn’s disease (94). These trophic hormones are agonists of PI3-kinase (95–97). Our studies suggest that one benefit of these growth factors is in protecting intestinal epithelial cells from immune-mediated apoptosis. Another potential benefit may be the inhibition of chloride secretion by intestinal epithelial cells. Indeed, Crohn’s disease patients treated with growth hormone

FIG. 8. Distribution of the junctional proteins ZO-1 and desmoplakins 1 and 2 in apoptotic T84 monolayers. T84 cells were cultured on permeable supports to achieve electrically resistant monolayers. Monolayers were treated with anti-Fas Ab for 24 h followed by 2 h of LY294002 where indicated. The monolayers were stained with an antibody (green) that recognizes the tight junction protein ZO-1 (A) or desmosomal proteins desmoplakin 1 and 2 (B), and nuclei were counterstained with propidium iodide (red). A series of confocal images was taken every 0.5 μm through the monolayers and projected to visualize the relationship between nuclei or nuclear debris and these proteins (bottom panels in both A and B). In apoptotic monolayers, there are wide outlines of ZO-1 and cytoplasmic staining of desmoplakin. These changes are not seen in monolayers exposed to PI3-kinase inhibitors alone.
had a marked lessening of diarrhea (94). An improved understanding of the signaling pathways utilized by intestinal epithelial cells to protect against immune-mediated apoptosis may result in the development of highly specific agonists that may be used to protect intestinal epithelial cells against apoptosis and increased chloride secretion in inflammatory bowel diseases.

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PI3-Kinase and Fas Signaling in the Intestine

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