Mind The Gaps

Confocal Endomicroscopy Showed Increased Density of Small Bowel Epithelial Gaps in Inflammatory Bowel Disease

Julia J. Liu, MD,* Karen L. Madsen, PhD,* Pierre Boulanger, PhD,†
Levinus A. Dieleman, MD, PhD,* Jon Meddings, MD,‡ and Richard N. Fedorak, MD*

Objectives: Confocal endomicroscopy can be used to image intestinal mucosa. Epithelial gaps resulting from shedding of epithelial cells have been reported in patients. We hypothesize that the rate of epithelial cell shedding increases in patients with Crohn's disease, leading to more epithelial gaps and barrier dysfunction. In this study, we used probe-based confocal laser endomicroscopy to quantify epithelial cells and gaps in patients with Crohn's disease compared with controls. We also determined the density of epithelial gaps in a mouse model of inflammatory bowel disease—interleukin-10-deficient (IL-10$^{-/-}$) mice, versus the background strain using rigid probe confocal endomicroscopy.

Methods: Probe-based confocal laser endomicroscopy of the terminal ileum of both patients with Crohn's disease and controls was performed by a single endoscopist during colonoscopy. In mice, sections of the small intestine were imaged using a rigid confocal probe. Gap density was defined as the number of epithelial gaps per 1000 cells counted.

Results: In this study, we examined 6 controls (2 male and 4 female; median age 59 y) and 8 patients with Crohn's disease (5 male and 3 female; median age 42 y). The mean gap densities ($\pm$ standard error) observed for the 2 groups were 17.7 ± 5.6 and 117 ± 33 gaps per 1000 cells, respectively ($P < 0.01$). For control and IL-10$^{-/-}$ mice, the gap densities were 10.5 ± 2.2 and 17.8 ± 1.4 gaps per 1000 cells, respectively ($P < 0.01$).

Conclusions: The epithelial gap density was significantly higher in patients with Crohn's disease than controls. Gap density was also elevated in the mouse model of inflammatory bowel disease.

Key Words: epithelial gaps, confocal endomicroscopy, inflammatory bowel disease, Crohn's disease

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The intestinal epithelium functions as a barrier that prevents undesirable solutes, microorganisms, and other luminal antigens in the intestinal contents from entering the body.[1,2] The epithelial barrier was traditionally thought to comprise barriers to both transcellular and paracellular trespass: transcellular flux was prevented by the apical membrane of the epithelial cells and its adherent glycocalyx, whereas paracellular flux was restricted by tight junctions between the cells.[1] Impaired barrier function has been implicated in the pathogenesis of inflammatory bowel disease (IBD); that is, Crohn's disease and ulcerative colitis.[3,4] Damage to the epithelial barrier results in loss of tolerance to intestinal microbiota and ongoing inflammation—hallmarks of IBD. Emerging evidence now suggests that discontinuities in the epithelium, or epithelial gaps that result from epithelial cell shedding, may be an unexplored cause of barrier dysfunction.[5–7] Epithelial cell shedding in the intestine can be induced by proinflammatory cytokines, and therefore may promote inflammation and epithelial ulceration under certain conditions.[4,8]

Direct observation of the shedding of epithelial cells and resulting epithelial gaps using confocal endomicroscopy (CEM) was first reported in 2007, these gaps were observed in vivo in patients undergoing colonoscopy.[7] The investigators confirmed their clinical findings using a mouse model. Epithelial cell shedding is a normal physiological phenomenon in the healthy state. Epithelial cells arise from stem cells at the base of the crypts in the small intestine; they mature and then migrate up the villi, where they are shed. This shedding process results in discontinuities or "gaps" in the epithelium. These gaps do not contain cytoplasmic or nuclear materials.[5] Quantitative analysis of these human intestinal epithelial gaps has not been earlier reported.

In this study, we used probe-based confocal laser endomicroscopy (pCLE) to evaluate the morphology of the small intestine in patients with Crohn's disease and controls. We quantitated the density of the epithelial gaps (defined as the number of epithelial gaps per 1000 epithelial cells) in the intestines of patients with Crohn's disease versus non-IBD controls. We also carried out similar determinations using a mouse model of IBD—interleukin-10-deficient (IL-10$^{-/-}$) mice against the background 129 Sv/Ev strain.

PATIENTS AND METHODS

Patients

The study protocol was reviewed and approved by the Human Ethics Research Review Board at the University of

Alberta. The study inclusion criteria were serial patients more than 18 years of age undergoing colonoscopy; the disease group consisted of patients with a known diagnosis of Crohn's disease, and the control group consisted of patients undergoing colonoscopy for non-IBD-related indications (evaluation of irritable bowel symptoms or bleeding). Exclusion criteria were allergies to fluorescein or shellfish, pregnancy or breast-feeding, and impaired renal function (creatinine level > 100 mM).

Mouse Strains

The mouse model of IBD used here was the IL-10$^{-/-}$ knockout mouse, a homozygous IL-10-deficient strain generated from a 129 Sv/Ev genetic background. These mice display normal intestinal permeability and do not develop colitis if raised under germ-free (axenic) conditions.[9] However, if they are raised under conventional (nonaxenic) conditions, these mice universally develop colitis (beginning at 8 to 12 wk of age), along with abnormal intestinal permeability.[10] Mice from the background of 129 Sv/Ev strain were used as controls for the CEM imaging experiments described below.

Confocal Endomicroscopy

Patients

pCLE was performed for all patients by a single endoscopist using Cellvizio Ultra-High Definition coloflex miniprobes (Mauna Kea Technologies, Paris, France) after the method was reported earlier by Hsiung et al.[11] The ultra-high definition coloflex confocal miniprobe has a resolution of 1.4 μm and a representative image of the healthy small intestine is shown in Figure 1A.

Conscious sedation with midazolam and fentanyl was used as part of each procedure (with standard cardiopulmonary monitoring). An antispasmodic agent (glucagon) was used whenever necessary, to reduce the creation of movement artifacts. After colonoscopic intubation of the terminal ileum, 5 mL of intravenous fluorescein (10% solution) was administered to the patient. After the administration of fluorescein, 5 to 10 endoscopically normal-looking mucosal areas in the terminal ileum were imaged with the pCLE using the confocal probe. We elected to image only normal-looking mucosa as endoscopically inflamed areas (ileitis or ulcers) had significant architectural distortions. The epithelial lining could not be adequately discerned in most images; therefore, we made continuous video recordings of CEM of normal-looking mucosa for approximately 10 minutes. To ensure minimal mechanical trauma to the mucosa, the endoscopist performing pCLE placed the confocal probe in light contact with the intestinal mucosa. The imaged areas were biopsied and sent for pathology for light microscopy evaluation.

The analysis of the confocal images was conducted in a blinded manner. Each patient had a minimum of 3000 confocal images collected and stored. The confocal endoscopy videos were reviewed and images of sufficient quality were analyzed. A sufficient quality image is defined as en face views of the villi with at least 75% of the surface visualized. Adequate images were then scrambled for counting of the epithelial gaps and epithelial cells. The images were counted by a research assistant blinded to the status of the patients. The epithelial gaps and cells counted were recorded and the epithelial gap densities were calculated.
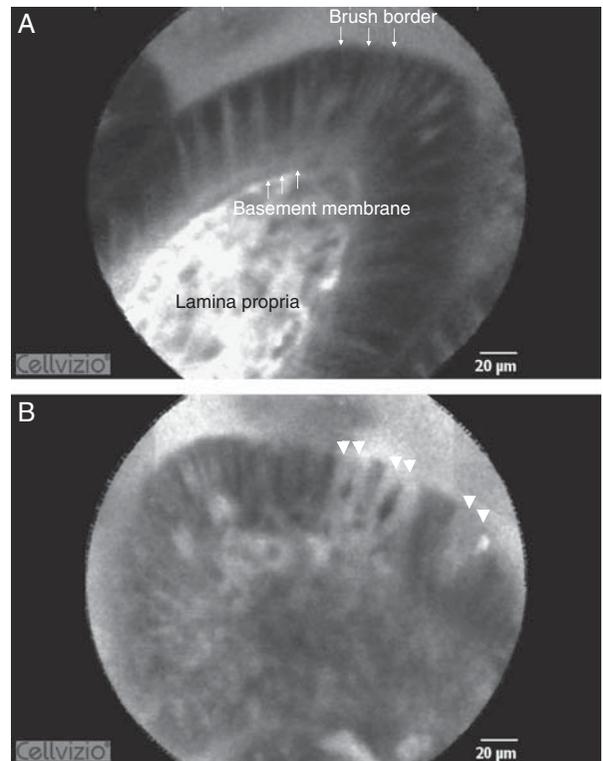

FIGURE 1. A, Representative probe-based confocal laser endomicroscopy (pCLE) image of the small intestine of a control patient (a cross-sectional view of the villus in the vertical plane). B, Representative pCLE image of the small intestine of a patient with Crohn's disease (en face view). The white arrow heads indicate several pairs of epithelial gaps in the villi.

Mice

CEM was performed in mice with the FIVE 1 (Fluorescence In Vivo Confocal Endomicroscopy System; Optiscan Pty. Ltd., Notting Hill, Victoria, Australia). The probe has a resolution of 0.7 μm with a field of view of 500 × 500 μm. For both strains, we used 8-week-old mice (before the onset of colitis in the IL-10$^{-/-}$ strain) for confocal imaging. The mice were housed in a standard animal facility with a 12-hour light/dark cycle. Fifteen IL-10$^{-/-}$ mice and 10 129 Sv/Ev mice were used. We elected to use more IL-10$^{-/-}$ mice, as we anticipated greater variations in the density of the epithelial gaps in the IBD strain mice. To completely eliminate movement artifacts, we performed rigid probe CEM on mice immediately after cervical dislocation. A midline abdominal incision (2 cm) was made at this time, and a segment of the terminal ileum was exteriorized, flushed with normal saline, and opened longitudinally along the antimesenteric border using cautery. The tissue was stained topically with 0.5 mmol/L of acriflavinee hydrochloride and then washed with normal saline. Topical acriflavine was chosen as the staining agent for the following reasons: (1) acriflavine stains the nuclei of cells in the intestine, which allows us to determine the epithelial gaps, using methods reported earlier by Kiesslich et al[7]; (2) the duration of staining using acriflavine is much longer than intravenous fluorescein (which dissipates after 30 min); (3) the intensity of the stain is relatively stable for acriflavine, making reconstruction of the 3-dimensional (3D) images possible; and (4) ease of administration for

acriflavine (topical spray) versus fluorescein (tail vein injection). Serial cross-sectional images along the z axis of the tissues (z-stacks) were obtained using the rigid probe CEM system. In each case, 3 to 5 images were taken within a $500 \times 500 \mu m$ field of view along a 2 cm segment of terminal ileum, 5 cm proximal to the cecum. Typically, the resulting z-stacks consisted of 20 to 30 cross-sectional images of tissue 0 to 70 μm below the upper tip of the villi.

These z-stacks were then digitally aligned and the features extrapolated to create a surface relief of the relevant segment of the small intestine. Interpolation of the 2D frames in each stack was done by an imaging expert (Boulanger) using feature-guided, shape-based image reconstruction software (VolView 3.0, Kitware Inc, Clifton Park, NY). The 3D relief images were then analyzed to isolate and quantify the shape, extent, and position of each of the lesions left behind in which cells had been shed (ie, the epithelial gaps). In this study, 3D images were generated only from the rigid-probe CEM mouse model data.

Statistical Analysis

We have carried out analysis of continuous variables using the Wilcoxon rank-sum test with the Statistical Analyzing System (SAS software, Cary Institute, Cary, NC). Each continuous variable was expressed as the mean value ± the standard error. A P value of less than 0.05 was considered significant; all tests were 2-tailed.

RESULTS

Patients

The control group enrolled 6 patients with a median age of 59 years (range: 42 to 71 y). The study group had 8 patients with Crohn's disease with a median age of 42 years (range: 29 to 69 y). The control group consisted of 2 male and 4 female patients, whereas the Crohn's disease group consisted of 5 male and 3 female patients. The characteristics of the patients are summarized in Table 1. In the control group, 2 patients underwent colonoscopy for colon cancer screening and 4 others had symptoms of irritable bowel syndrome. In the Crohn's disease group, 5 patients took 5-aminosalicylic acid compounds, 2 were on immunomodulators, and 1 was treated with biological agents. Of

the 8 patients with Crohn's disease, 3 patients had active disease, and the remaining 5 were in remission. The 3 active patients underwent colonoscopy for the evaluation of clinical symptoms, whereas the other 5 patients had colonoscopy for follow-up or surveillance of dysplasia. Endoscopic findings of patients with Crohn's disease were: 2 patients had ileitis, 1 patient had moderate pancolitis, 4 patients had proximal colitis, and 1 patient had distal colitis. pCLE imaging of the terminal ileum was done on only normal endoscopic appearing areas in the terminal ileum.

The indications for colonoscopy in the control group were symptoms of irritable bowel syndrome (abdominal pain, diarrhea, and constipation) for 3 patients, and bleeding (either rectal bleeding or a positive fecal occult stool test) for 3 other patients. In all cases, images of the terminal ileum were continuously recorded at 10 cm proximal to the ileocecal valve. We reviewed a total of 44,166 pCLE images (2D) of the terminal ileums from patients, and 1960 of these images were of sufficient quality to be analyzed. Only adequately imaged villi—defined as villi with 75% of their surface area visualized in the stacks of 2D pCLE images—were used in subsequent analysis.

Epithelial gaps appeared as hyperdense areas between the epithelial cells on pCLE images, because of leakage of the intravenously administered fluoroscein contrast material through the intercellular space. Individual epithelial gaps were counted only if they were found in at least 3 sequential 2D confocal images. Conventional light microscopy of the intestinal biopsies in all patients showed normal villous architecture. We were unable to accurately count the epithelial gaps on histologic specimens because of fixation and section artifacts. Representative control and Crohn's disease 2D pCLE images of the small intestine are shown in Figures 1A and B, respectively. To quantitate epithelial gap density, the total number of epithelial cells and epithelial gaps for each villus were manually counted for 10 villi from each patient. For each of these 10 adequately-imaged villi, the gap density was calculated as the number of gaps divided by the number of epithelial cells counted, normalized to a count of 1000 cells; thus, gap density is here defined as the number of gaps per 1000 epithelial cells. As shown in Figure 2, the gap density (mean ± standard error) for the control patients was found to be 17.7 ± 5.6 gaps per 1000 cells. In patients with Crohn's disease, the calculated gap density was significantly higher, 117 ± 33 gaps per 1000 cells ($P < 0.01$ for the Wilcoxon signed-rank test).

Mice

We carried out 3D reconstructions of the 2D cross-sectional CEM images obtained from the mouse intestine. These 3D reconstructed images allowed clear differentiation of epithelial gaps from goblet cells. The analysis was done on villi that were adequately imaged, defined as en face villi images showing 75% of villi surfaces on the CEM images. These villi images from each animal were manually counted, to determine the total number of epithelial cells and epithelial gaps. Representative epithelial gaps and goblet cells in the mouse terminal ileum seen on CEM are shown in Figure 3. Each villus was found to have a mean total of 177 ± 4 epithelial cells for the control mice and 212 ± 14 epithelial cells for the IL-10$^{-/-}$ mice. The mean gap density (mean ± standard error) was determined to be 10.5 ± 2.2 gaps per 1000 cells for the 129 Sv/Ev (normal) mice. The mean gap density of the IL-10$^{-/-}$ mice was

TABLE 1. Patient Characteristics of the Study and Control Group

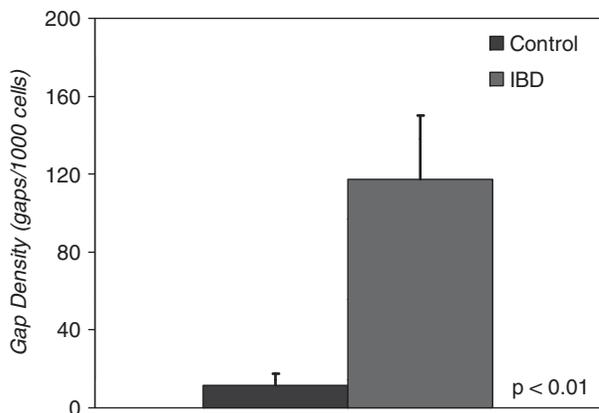|	Controls	Crohn's Disease
Age (median)	59	42
Sex		
Male	2	5
Female	4	3
Race		
White	5	8
Nonwhite	1	0
Indication for colonoscopy		
IBS	3	
Bleeding	3	
Evaluation of clinical symptoms		3
Follow-up or surveillance		5
Treatment		
5 ASA		5
Immunomodulator		2
Biologics		1

IBS indicates irritable bowel syndrome.

FIGURE 2. Measured intestinal epithelial gap densities for human controls and patient with Crohn's disease. Epithelial gap density is expressed as the number of gaps per 1000 cells. The *P* value is from a comparison of means using a Wilcoxon signed-rank test. IBD indicates inflammatory bowel disease.

significantly higher, 17.8 ± 1.4 gaps per 1000 cells ($P < 0.01$ for the Wilcoxon signed-rank test).

Representative 3D CEM images of the small intestines of control and IL-10$^{-/-}$ mice are shown in Figures 4A and B, respectively. The gap densities for the 2 mouse strains are compared graphically in Figure 5.

DISCUSSION

This study examined the density of epithelial gaps in patients with Crohn's disease and controls using pCLE. We also performed CEM in a mouse model of IBD and its background strain to quantitate the epithelial gap densities. The densities of epithelial gaps were analyzed from the confocal images (both pCLE in patients and CEM in mice) and manual counting of epithelial cells and gaps. Our data
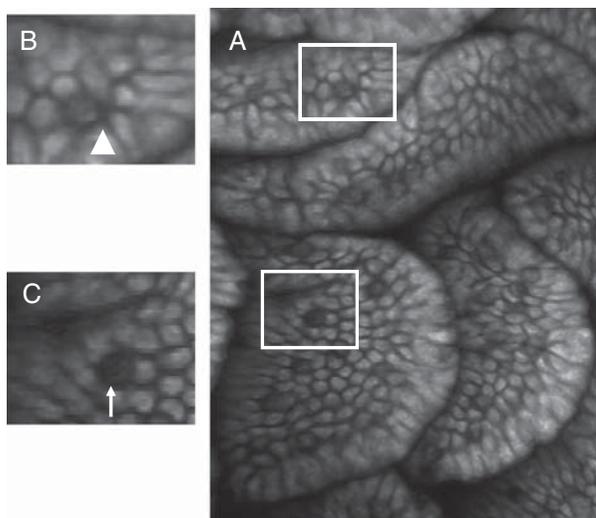

FIGURE 3. Representative epithelial gap and goblet cell in the mouse terminal ileum seen on confocal endomicroscopy. A is a 3D reconstruction image from a series of 25 z-stacks of the mouse terminal ileum. The boxed areas in (A) are shown at higher magnification in (B) and (C). White arrow head in (B) indicates epithelial gap whereas white arrow in box (C) indicates a goblet cell.

indicate that the epithelial gap density is significantly increased for patients with Crohn's disease. Elevated epithelial gap densities were also observed in the IL-10$^{-/-}$ (IBD model) mice, compared with background 129 Sv/Ev mouse-strain.

This study is the first quantitative analysis of epithelial gap density, an indicator of epithelial cell shedding in the small intestine. We found that the epithelial gap density is significantly elevated in the intestinal epithelium of patients with Crohn's disease compared with controls. This increased epithelial cell shedding in Crohn's disease is believed to be cytokine-mediated. In particular, tumor necrosis factor-α (TNF-α) is an important inflammatory mediator of IBD and has been shown to induce a significant rise in epithelial cell shedding in a mouse model,[7] and increase intestinal permeability.[12–16] In addition, an anti-TNF monoclonal antibody, infliximab, has been widely used to induce and maintain remission for patients with Crohn's disease, and was shown to normalize intestinal permeability.[17] Other therapeutic evidence provides further links between proinflammatory cytokines and alterations in intestinal barrier function.[12–16]

In pCLE images of patients' intestines, epithelial gaps seem to be hyperdense because of leakage of the intravenously administered fluoroscein through the intercellular spaces. Thus, the appearances of epithelial gaps are quite different than epithelial cells or goblet cells. However, in CEM images of mice, differentiating epithelial gaps from goblet cells in the acriflavine-stained 2D confocal images can be challenging. As acriflavine is a nuclear stain, goblet cells seem to be hypodense in 1 plane of the 2D confocal images because their nuclei are usually located more basally than the surrounding epithelial cells. Two criteria that allow for easier differentiation in the mouse model have been described by Kiesslich et al.[7] First, goblet cells have a distinctive architecture, a rather flat apical surface, a mucin granule at the center of the cell, and a nucleus near the basal pole. Second, the goblet cells resemble a target (concentric circles) in the en face view. In contrast, epithelial gaps do not contain a nucleus and seems relatively uniform from all angles of view. The confirmation of these criteria to differentiate epithelial gaps from goblet cells is done in samples from Math1$^{\Delta intestine}$ mice, a strain with an intestine-specific mosaic deletion of Math1 that results in the elimination of 90% of goblet cells from the terminal ileum.[18]

The role that epithelial gaps play in relation to maintenance of the intestinal mucosal integrity and the ways in which increased gap density might contribute to barrier dysfunction are being investigated. For instance, Kiesslich et al[7] have reported that TNF-α infusion induced a significant rise in epithelial cell shedding in a mouse model. The loss of barrier function is likely to be significant, as epithelial gaps can account for 3% of the surface area in the intestine. In this study, we examined IL-10$^{-/-}$ mice at 8 weeks of age, just before the development of colitis under nonaxenic conditions. The elevated epithelial gap density that we observed in these mice indicates that epithelial cell shedding may be another mechanism contributing to altered intestinal permeability.

Our observation that these IL-10$^{-/-}$ mice had significantly higher epithelial gap densities suggests that a higher rate of epithelial cell shedding and turnover was occurring, likely mediated by cytokine-induced apoptosis. The increased gap density certainly could contribute to the
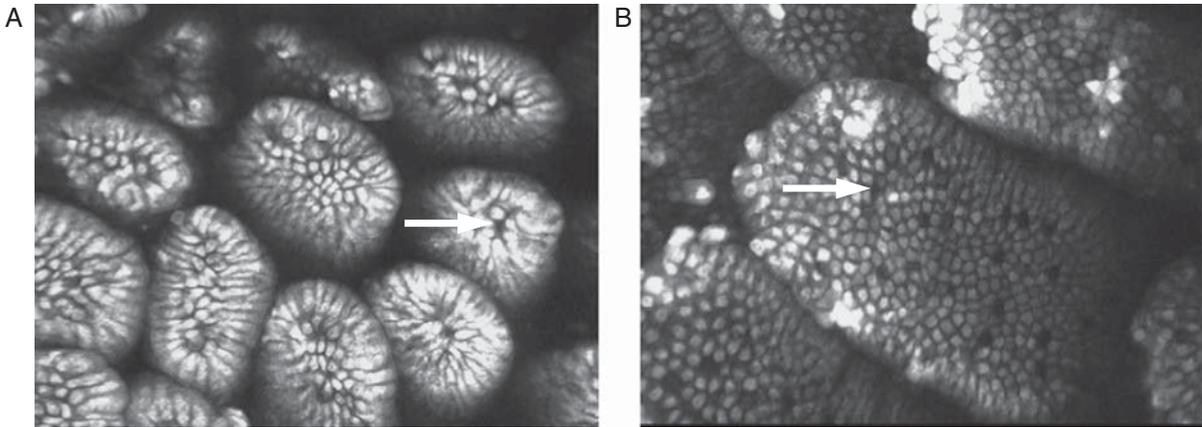
FIGURE 4. A, Representative 3-dimensional (3D) rendered confocal endomicroscopy (CEM) image of the small intestine in control 129 Sv/Ev mice. The white arrow indicates an epithelial gap, with the bar=20 μm. B, Representative 3D rendered CEM image of the small intestine in interleukin-10-deficient (IL-10$^{-/-}$) mice (a mouse model of inflammatory bowel disease). The white arrow indicates an epithelial gap, with the bar=20 μm.

abnormal intestinal permeability seen in IL-10$^{-/-}$ mice raised under nonaxenic conditions.[10] Future studies are needed to determine the correlation between abnormal epithelial gap density and intestinal permeability and to investigate the role increased epithelial gap density plays in the pathogenesis of IBD.

In our study, we found a less pronounced difference in the epithelial gap density in the mouse model of IBD than in patients with Crohn's disease. This could be due to several factors: first, we performed CEM on the mouse model at an earlier age (8 wk), before the onset of colitis, whereas all the patients with Crohn's disease had endoscopic evidence of colitis. Hence, the patients were further along in the disease course than was the mouse model. Second, IL-10$^{-/-}$ mice do not develop ileitis, whereas patients with Crohn's disease do. Third, the patient population with Crohn's disease is heterogenous, with significant variations in genetic predispositions and environmental exposures, whereas IL-10$^{-/-}$ mice are all housed in standard housing condition in cages within the same facility. Finally, acriflavine staining may have also contributed to some of the differences in gap density, as areas

without nuclei (hypodense areas) may be more difficult to visualize compared with the appearance of gap stained with intravenous. fluorescein (hyperdense areas).

The main limitation of this study was the relatively small sample size of patients. However, this study was intended to explore the density of epithelial gaps in IBD. We have ongoing clinical studies examining the relationship between epithelial cell shedding, cytokines, and intestinal permeability in patients with Crohn's disease compared with controls.

In conclusion, this study found that epithelial gap density in patients with Crohn's disease is significantly increased over that seen for non-IBD controls. This increased gap density is evidence of increased epithelial cell shedding. We obtained similar results in a mouse model of IBD. It seems likely, given these results, that increased epithelial gap density may contribute to increased intestinal permeability and ultimately, to dysfunction of the intestinal barrier in IBD.

FIGURE 5. Measured gap densities for control and interleukin-10-deficient (IL-10$^{-/-}$) mice (mean values ± standard error). The *P* value is from a comparison of means using a Wilcoxon signed-rank test.

REFERENCES

1. Montrose MH. The future of GI and liver research: editorial perspectives: I. Visions of epithelial research. *Am J Physiol Gastrointest Liver Physiol*. 2003;284:G547–G550.
2. Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest*. 2004;84:282–291.
3. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. *Gut*. 2006;55:1512–1520.
4. Garrett WS, Lord GM, Punit S, et al. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*. 2007;131:33–45.
5. Watson AJ, Chu S, Sieck L, et al. Epithelial barrier function in vivo is sustained despite gaps in epithelial layers. *Gastroenterology*. 2005;129:902–912.
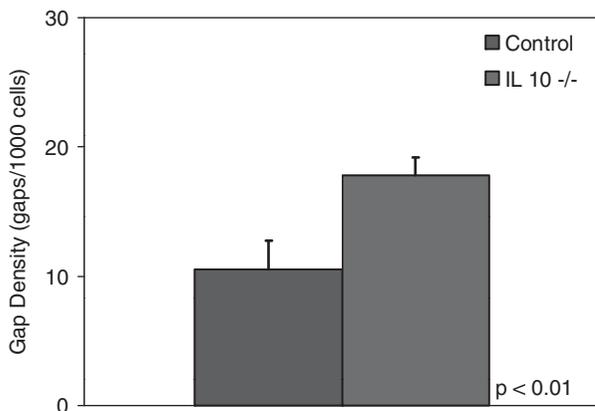
6. Bullen TF, Forrest S, Campbell F, et al. Characterization of epithelial cell shedding from human small intestine. *Lab Invest*. 2006;86:1052–1063.

7. Kiesslich R, Goetz M, Angus EM, et al. Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. *Gastroenterology*. 2007;133:1769–1778.

8. Nenci A, Becker C, Wullaert A, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*. 2007;446:557–561.

9. Madsen KL, Malfair D, Gray D, et al. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis*. 1999; 5:262–270.

10. Arrieta MC, Madsen K, Doyle J, et al. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut*. 2009;58:41–48.

11. Hsiung PL, Hardy J, Friedland S, et al. Detection of colonic dysplasia in vivo using a targeted heptapeptide and confocal microendoscopy. *Nat Med*. 2008;14:454–458.

12. Brown GR, Lindberg G, Meddings J, et al. Tumor necrosis factor inhibitor ameliorates murine intestinal graft-versus-host disease. *Gastroenterology*. 1999;116:593–601.

13. Clayburgh DR, Barrett TA, Tang Y, et al. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. *J Clin Invest*. 2005; 115:2702–2715.

14. Clayburgh DR, Musch MW, Leitges M, et al. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo (see comment). *J Clin Invest*. 2006;116:2682–2694.

15. Ferran C, Sheehan K, Dy M, et al. Cytokine-related syndrome following injection of anti-CD3 monoclonal antibody: further evidence for transient in vivo T cell activation. *Eur J Immunol*. 1990;20:509–515.

16. Musch MW, Clarke LL, Mamah D, et al. T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na^+/K^+-ATPase. *J Clin Invest*. 2002;110: 1739–1747.

17. Hanauer SB, Feagan BG, Lichtenstein GR, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet*. 2002;359:1541–1549.

18. Shroyer N, Helmrath M, Wang V, et al. Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. *Gastroenterology*. 2007;132:2478–2488.