The intestinal epithelium functions as a barrier that prevents undesirable solutes, microorganisms, and other luminal antigens in the intestinal contents from entering the body. The epithelial barrier was traditionally thought to comprise barriers to both transepithelial and paracellular transport: transepithelial 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. Impaired barrier function has been implicated in the pathogenesis of inflammatory bowel disease (IBD); that is, Crohn’s disease and ulcerative colitis. 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. Epithelial cell shedding in the intestine can be induced by proinflammatory cytokines, and therefore may promote inflammation and epithelial ulceration under certain conditions.

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. 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. 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 (creatine 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 129Sv/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 129Sv/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.

Cautery. The tissue was stained topically with 0.5 mmol/L of acriflavine 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 epithelial cells, which allows us to determine 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 129Sv/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 acriflavine 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 al7; (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
acrilavine (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 × 500 μm 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 the 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 fluorescent 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 quantify 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 | 3 |
| Bleeding | | |
| Evaluation of clinical symptoms | 3 | 3 |
| Follow-up or surveillance | 5 | - |
| Treatment | | |
| 5 ASA | 5 | - |
| Immunomodulator | 2 | - |
| Biologics | 1 | - |

IBS indicates irritable bowel syndrome.
In addition, an anti-rand 12–16 Measured intestinal epithelial gap densities for www.jcge.com First, goblet cells have a mice had / /C0 2010 Lippincott Williams & Wilkins /C0 2010 J Clin Gastroenterol /C0 /C15 have reported that TNF- have shown in Figures 4A and B, / /C0 mice are shown in Figures 4A and B, / /C0 mouse terminal ileum. The boxed areas in (A) are shown at higher / /C0 3D reconstruction image from a series of 25 z-stacks of the mouse / /C0 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. / /C0 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 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, 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. 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 fluorescein 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. 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 [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 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
abnormal intestinal permeability seen in IL-10−/− mice raised under nonaxenic conditions. 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.

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