Piroxicam Treatment of IL-10–Deficient Mice Enhances Colonic Epithelial Apoptosis and Mucosal Exposure to Intestinal Bacteria

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Abstract: Treatment with the nonsteroidal anti-inflammatory drugs piroxicam or sulindac was recently shown to accelerate the development of colitis in interleukin (IL)-10–deficient (IL-$10^{-/-}$) mice. Although NSAIDs have been hypothesized to decrease the barrier function of the intestinal epithelium, the mechanism by which this accelerates colitis in IL-$10^{-/-}$ mice is not well understood. In this study, the effects of piroxicam on the colonic mucosa of IL-$10^{-/-}$ C57BL/6 mice were evaluated histologically. The effect of piroxicam on intestinal epithelial cells in vitro was assessed using colorimetric and fluorescent assays for cell viability and apoptotic cell death. Interactions of intestinal bacteria with the colonic mucosa were evaluated by rRNA-directed fluorescence in situ hybridization. In vivo treatment of C57BL/6 IL-$10^{-/-}$ mice with oral piroxicam markedly enhanced apoptosis of colonic epithelium and resulted in focal erosion of the mucosal surface, enhanced bacterial adhesion and invasion, and accelerated the development of colitis. In vitro, piroxicam induced apoptosis of CT26 murine intestinal epithelial cells in a dose-dependent fashion. Piroxicam-induced apoptosis of CT26 cells could not be prevented by addition of exogenous IL-10; however, IL-10 did significantly enhance their rate of proliferation. Thus, exposure to piroxicam enhances intestinal epithelial apoptosis both in vitro and in vivo and facilitates adhesion and invasion of intestinal bacteria into mucosal tissues in vivo. The role of IL-10 in this process requires further study. These studies support the hypothesis that increased exposure of mucosal cells to intestinal bacteria may lead to development of intestinal inflammation in IL-$10^{-/-}$ or other genetically susceptible individuals.

Key Words: fluorescence in situ hybridization, inflammatory bowel disease, intestinal flora, nonsteroidal anti-inflammatory drugs

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A number of experimental models for inflammatory bowel disease (IBD) have been established in rodents.[1–4] These models vary in the anatomic portion of the gut affected, the depth of inflammation, and the involvement of T_H1 versus T_H2 cytokines; however, together they have provided important evidence that abnormal immune activation and cytokine production are important in the development and perpetuation of IBD. Colitis develops spontaneously when mice deficient in the T_H2 regulatory cytokine IL-10 are colonized with intestinal bacteria but not when they are kept under germ-free conditions. Marked variations in the age of onset and severity of inflammation occur because of both environmental and genetic (strain) effects.[5–7] Most of the reported studies used interleukin (IL)-10–deficient (IL-$10^{-/-}$) mice on a 129/SvEv or Balb/C background, because these strains of mice develop colitis more rapidly than do mice on the C57BL/6 background. However, Berg et al[8] recently showed that the relatively slow development of colitis in IL-$10^{-/-}$ mice on the C57BL/6 background can be accelerated by administration of the nonsteroidal anti-inflammatory drugs (NSAIDs) piroxicam or sulindac. Exposure of IL-$10^{-/-}$ mice to NSAIDs results in rapid ($<$2 wk) and uniform development of colitis that persisted long-term after the NSAIDs are discontinued. The histology of colitis and cytokine production in NSAID-treated animals were reported to be similar to what is observed when these animals spontaneously develop colitis. Colitis did not develop when wild-type mice were treated with NSAIDs. Coadministration of a prostaglandin agonist with the NSAID prevented the development of colitis, indicating that development of colitis was related to inhibition of prostaglandin synthesis.[8] However, the precise mechanisms by which NSAIDs rapidly accelerate the development of colitis in IL-$10^{-/-}$ mice are not yet well understood.

Conventional NSAIDs such as piroxicam and sulindac inhibit cyclooxygenase enzymes (COX-1 and COX-2) that are important for prostaglandin synthesis. COX-1 is constitutively expressed within the gastrointestinal tract, where it regulates the production of prostaglandins that are essential for maintaining mucosal barrier function. NSAID inhibition of COX-1

may compromise the gastrointestinal defense mechanisms by decreasing production of prostaglandins that maintain mucosal integrity. Development of colitis is currently thought to involve an aberrant immune response to bacterial antigens. Thus, decreases in the integrity of the intestinal epithelial barrier resulting from NSAID exposure may allow antigens from intestinal bacteria to contact immune cells in the lamina propria, eventually leading to an excessive inflammatory response in genetically susceptible IL-$10^{-/-}$ animals.

We used piroxicam as described by Berg et al[8] to study the mechanism by which NSAIDs accelerate the development of colitis in the relatively colitis-resistant C57BL/6 strain of IL-$10^{-/-}$ mice. We found that piroxicam enhances colonic epithelial apoptosis and increases intestinal epithelial exposure to bacteria. Increased exposure to bacteria may potentially trigger immune responses that lead to the development of colitis in these animals.

MATERIALS AND METHODS

Animal Studies

Under the conditions present in our animal facility, commercially available C57BL/6 IL-$10^{-/-}$ mice (Jackson Laboratories, Bar Harbor, Maine) develop spontaneous colitis between 6 and 7 months of age. To accelerate the development of colitis in IL-$10^{-/-}$ mice, 4- to 6-week-old female C57BL6 IL-$10^{-/-}$ mice or C57BL/6 wild-type controls were given 200 ppm piroxicam (Sigma-Aldrich, St. Louis, Mo.) in powdered rodent food for 7 days, as described previously by Berg et al.[8] Piroxicam was discontinued, and mice were either killed immediately or observed for an additional 16 days. The severity of colitis was monitored by daily observations of weight loss and stool consistency. Freshly passed stool pellets were tested for occult or gross blood on days 3, 5, and 7 of piroxicam exposure and on days 3, 6 or 7, 10, and 14 after cessation of piroxicam, using Hemoccult Sensa II cards (Beckman Coulter, Palo Alto, Calif.). Hemoccult results were scored as follows: negative, 0; trace, 1; moderate, 2; strong hemoccult reaction or grossly visible blood, 3.

Histological Evaluation of Colon Tissues

After death, the colon was divided into segments representing the cecum, proximal, middle, distal, and terminal colon/rectum. Colon segments were fixed in either 10% neutral buffered formalin for 18 hours or Carnoy's solution[9] for 3 hours and processed into paraffin blocks. Hematoxylin and eosin–stained sections were evaluated by 2 pathologists blinded to treatment group to determine effects of piroxicam on colon inflammation using the scoring system shown in Table 1.[10] This scale provides separate numeric evaluations for the extent of mucosal hyperplasia and inflammatory changes in the 5 colon segments examined that are summed to derive the final histological score.

Apoptosis was identified visually on hematoxylin and eosin–stained sections of colon tissue. Apoptotic cells were further highlighted in formalin-fixed, paraffin-embedded

TABLE 1. Histological Scoring of Murine Colitis

	Score
Mucosa (M)	
No significant lesions	0
Mild epithelial hyperplasia	1
Moderate epithelial hyperplasia	2
Severe epithelial hyperplasia with crypt branching or herniation	3
Inflammation (I)	
None	0
Mild, limited to mucosa	1
Moderate, in mucosa and submucosa	2
Severe, with obliteration of normal architecture, erosions, and/or crypt abcesses	3
Level 3 changes plus ulceration	4
Extent (E)—E1=% affected in any manner; E2=percent with level 3 or 4 changes	
No significant changes	0
<5% of segment affected	1
5%–30% of segment affected	2
31%–60% of segment affected	3
>60% of segment affected	4

Segment score = M + I + E1 + E2 (max = 15).

Total score is derived from summing segment scores for each segment (cecum, proximal, middle, and distal colon, and rectum) examined, with a maximum score of 75.[10]

sections by immunohistochemical staining with a rabbit monoclonal antibody 5A1 (Cell Signaling Technology, Beverly, Mass.) that is specific for activated caspase 3 (Asp175). Heat-induced epitope retrieval by microwaving in 10 mmol/L citrate buffer (pH 6.0) and overnight incubation with antibody were performed as suggested by the antibody supplier. The intensity of positively stained cells was further enhanced using catalyzed signal amplification with biotinyl-tyramine (TSA Biotin System; Perkin Elmer Life Sciences, Boston, Mass.). The number of apoptotic epithelial cells was graded according to the following scheme: 0, rare apoptosis, with fewer than 1 activated caspase-3 (aCasp3)-positive cells present per 10 consecutive ×20 fields; 1, mild apoptosis, with isolated aCasp3-positive cells present at densities higher than 1 per 10 consecutive ×20 fields; 2, frequent apoptosis, with focal clusters of aCasp3-positive cells; 3, abundant apoptosis, with clusters of aCasp3-positive cells present in each of at least 5 consecutive ×20 fields or in association with mucosal erosion or ulceration.

Cell Culture, Proliferation, and Apoptosis Assays

CT26 murine colon carcinoma cells were obtained from Dr. Lee Ellis (University of Texas M.D. Anderson Cancer Center, Houston, Tex.) and grown at 37°C, 5% CO_2 in DMEM (Gibco BRL, Grand Island, N.Y.) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were plated into 96-well flat bottom plates at 5 to 10 × 10^3 cells/well and allowed to attach before treatment. IL-10 production was measured using a Luminex bead-based fluorescent immunoassay (BioRad, Hercules, Calif.). Stock solutions of piroxicam were made in media or DMSO and used at dilutions such that final DMSO concentration was no more than 0.2%. Similar concentrations of DMSO were added to control wells. The effect of piroxicam treatment on cell survival was measured using a colorimetric tetrazolium dye reduction assay that determines the number of viable cells present (CellTiter 96 AQueous Assay, Promega, Madison, Wis.). Apoptosis was detected using the Cell Death Detection ELISA Plus kit (Roche Molecular Diagnostics, Indianapolis, Ind.). This photometric enzyme immunoassay captures cytoplasmic histone-associated DNA fragments and detects with a labeled anti-DNA antibody to provide a quantitative measure of the oligonucleosomes released from the nucleus into the cytoplasm during apoptosis. Data are expressed as the fold increase in apoptosis, as indicated by the enrichment of oligonucleosomes in the cytoplasm of piroxicam-treated cells relative to control cells treated with vehicle alone (fold increase in apoptosis = absorbance of treated cells/absorbance of control cells). The percentage of CT26 cells undergoing apoptosis after piroxicam treatment was also determined using a fluorescent annexin V assay (Immunotech, Marseilles, France) that was analyzed in the Duke Human Vaccine Institute Flow Cytometry Core Facility (supported by the National Institutes of Health award P30 AI-51445).

Detection of Bacteria Using rRNA-directed FISH

Oligonucleotide probes reactive with bacteria-specific rRNA sequences were synthesized with a Cy3, Cy5, or FITC fluorescent dye at the 5′end (MWG Biotech, Ebersberg, Germany) and applied to Carnoy's-fixed paraffin sections of colon from control or piroxicam-treated IL-$10^{-/-}$ mice. Probe sequences and variation of formamide concentration and hybridization temperature to achieve optimal stringency were described previously.[11–13] Identification of bacteria by fluorescence in situ hybridization (FISH) required hybridization signals that were clear and morphologically distinguishable as bacterial cells combined with dual reactivity with the Eub338 universal bacterial probe[11] and the 4,6-diamidino-2-phenylindole (DAPI) counterstain for DNA and absence of hybridization with the Non338 nonsense probe. Bacteria were further differentiated as belonging to the *Bacteroides fragilis* or *Eubacterium rectale-Clostridium coccoides* groups using the Bfra602 or Erec482 probes, respectively.[13] Hybridization signals were detected using a Nikon e600 fluorescence microscope (Nikon, Tokyo, Japan) and photo-documented with a Nikon DXM1200 camera and software (Nikon).

Statistical Analysis

Statistical comparison of groups was performed using Student *t* test. A value of $P \leq 0.05$ was considered to be significant.

Ethical Considerations

All animal studies were approved by the Institutional Animal Care and Use Committee of the Duke University Medical Center.

RESULTS

Piroxicam Treatment Accelerates Development of Colitis in IL-$10^{-/-}$ Mice

Based on measured food consumption, mice received an average of 49 mg piroxicam/kg/d over the 7 days of treatment (range was 34–67 mg/kg/d over 41 cage-days). Piroxicam-treated C57BL/6 IL-$10^{-/-}$ mice lost an average of 0.5 g during treatment compared with an average weight gain of 0.5 g by control IL-$10^{-/-}$ mice who received the powdered diet without piroxicam ($P < 0.007$). Piroxicam-treated IL-$10^{-/-}$ mice also developed significant colonic bleeding, with mean bleeding scores of 1.5 ± 0.1 (n = 52 mice) for tests obtained on days 3, 5, and 7 of treatment compared with 0.3 ± 0.1 (n = 10 mice) for control mice ($P < 10^{-9}$; Fig. 1A). Histological scores of mice killed at day 7 showed corresponding differences in colonic inflammation. Age-matched (6–8 wk) control IL-$10^{-/-}$ mice had minimal inflammation (mean histological score ± SEM = 9 ± 1; n = 5). In contrast, piroxicam-exposed had moderate to severe colitis (histological score = 35 ± 3; n = 5; $P < 0.0001$; Fig. 1B). The severity of colitis in these 6- to

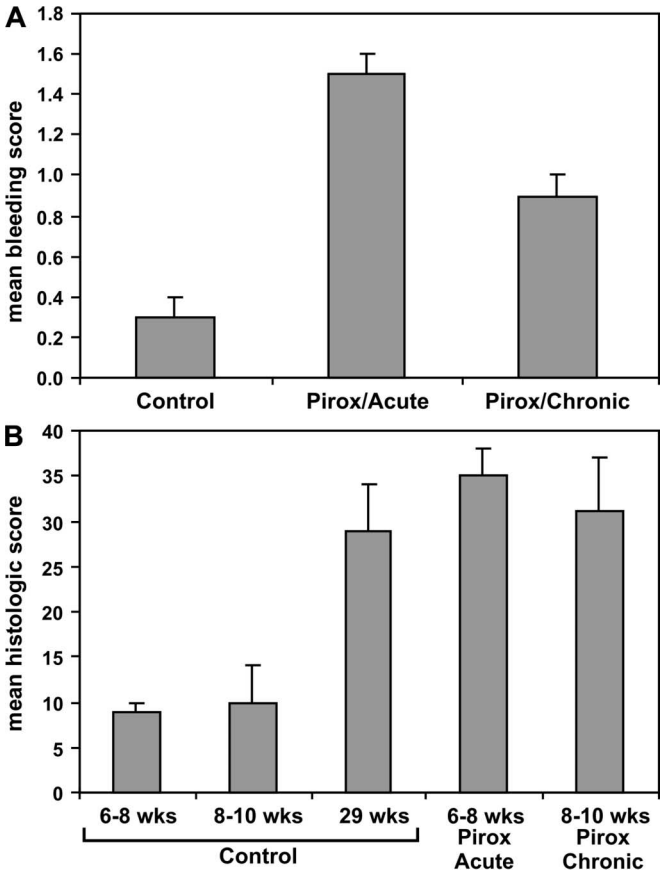

FIGURE 1. Piroxicam exposure accelerates the development of chronic colitis in IL-10$^{-/-}$ mice. A, the clinical severity of colitis was determined based on the presence of stool blood. A bleeding score was derived. The mean bleeding score shown represents the average score for tests done on days 3, 5, and 7 of piroxicam treatment of the colitis induction phase (Pirox/Acute; n = 52 mice) and on days 3, 6 or 7, 10, and 14 of established colitis after piroxicam was discontinued (Pirox/Chronic; n = 15 mice). Control IL-10$^{-/-}$ mice were age-matched but were not exposed to piroxicam (n = 10 mice). The clinical severity of colitis decreased somewhat over the 16-day observation period after the discontinuation of piroxicam but was still significantly greater than in control mice ($P = 0.0001$). B, the mean colon histological score ± SEM is shown for groups of 5 to 8 mice. Control IL-10$^{-/-}$ mice received no treatment to enhance or to prevent colitis. Their histological scores thus reflect the severity of spontaneous colon inflammation. NSAID-treated mice received 200 ppm piroxicam in their diet for 7 days and were either killed immediately (acute) or 16 days after discontinuation of piroxicam (chronic). The age of mice at death is indicated. A histological score of at least 12 is indicative of colitis.

8-week-old piroxicam-exposed mice was similar to that seen in 29-week-old IL-10$^{-/-}$ mice with spontaneous colitis (Fig. 1B). Colonic lesions in piroxicam-exposed mice included mild to moderate mucosal hyperplasia, infiltrates of mononuclear and polynuclear leukocytes into the mucosa and/or submucosa, frequent mucosal erosions in association with focally extensive apoptosis of colonic epithelial cells, and mucosal ulcerations. Focal transmural inflammation was also observed in some animals.

Wild-type mice similarly treated with piroxicam did not lose weight during treatment. However, they did experience significantly increased colonic bleeding (mean bleeding score = 1.4 ± 0.2; n = 5) compared with wild-type mice receiving powdered food without piroxicam (mean bleeding score = 0.1 ± 0.1; n = 5; $P = 5 \times 10^{-5}$). Although capsule enteroscopy has shown erythema, breaks, and denudation of the small intestinal mucosa of healthy humans after NSAID treatment,[14] no such lesions were identified grossly or microscopically in a randomly selected section of small intestine from each of 5 piroxicam-treated wild-type mice. Despite their increased incidence of occult bleeding and histological evidence of focal inflammation, the severity of colitis on day 7 of piroxicam treatment (mean histological score = 17 ± 5) did not differ statistically from that in wild-type animals that were not exposed to piroxicam (mean histological score = 11 ± 2; $P = 0.36$).

The mean bleeding score in IL-10$^{-/-}$ mice decreased slightly to 0.9 ± 0.1 during the 16-day observation period after the discontinuation of piroxicam (Fig. 1A). However, histological examination of colon tissues from these mice showed continued moderate to severe inflammation (mean histological score = 32 ± 6; n = 5) including mucosal hyperplasia, leukocytic infiltration into the mucosa, submucosa, and/or muscularis externa, and ulceration. Histological signs of chronic inflammation such as crypt branching and herniation were also present focally in 3 of 5 animals.

Piroxicam Treatment Enhances Colon Epithelial Apoptosis in IL-10$^{-/-}$ Mice

The high frequency of mucosal erosions associated with apoptosis of colon epithelial cells in mice examined immediately after 7 days of piroxicam treatment led us to specifically examine the effects of in vivo piroxicam treatment on epithelial apoptosis in these mice. Marked epithelial apoptosis was identified visually by hematoxylin and eosin staining of colon tissue sections from piroxicam-treated IL-10$^{-/-}$ mice (data not shown), using the established histological criteria of cell shrinkage, nuclear condensation, and formation of apoptotic bodies. Immunohistochemical staining for activated caspase 3, an enzyme present specifically in apoptotic cells, showed a clear trend toward increased apoptosis with piroxicam treatment. The mean apoptosis score was 2.4 ± 0.2 for piroxicam-treated IL-10$^{-/-}$ mice compared with 1.4 ± 0.4 for control IL-10$^{-/-}$ mice ($P = 0.07$ for n = 5 per group; Fig. 2). Intestinal epithelial apoptosis in piroxicam-treated mice was not increased when evaluated 16 days after discontinuation of piroxicam compared with control IL-10$^{-/-}$ mice who were never exposed to piroxicam (data not shown). Similarly,

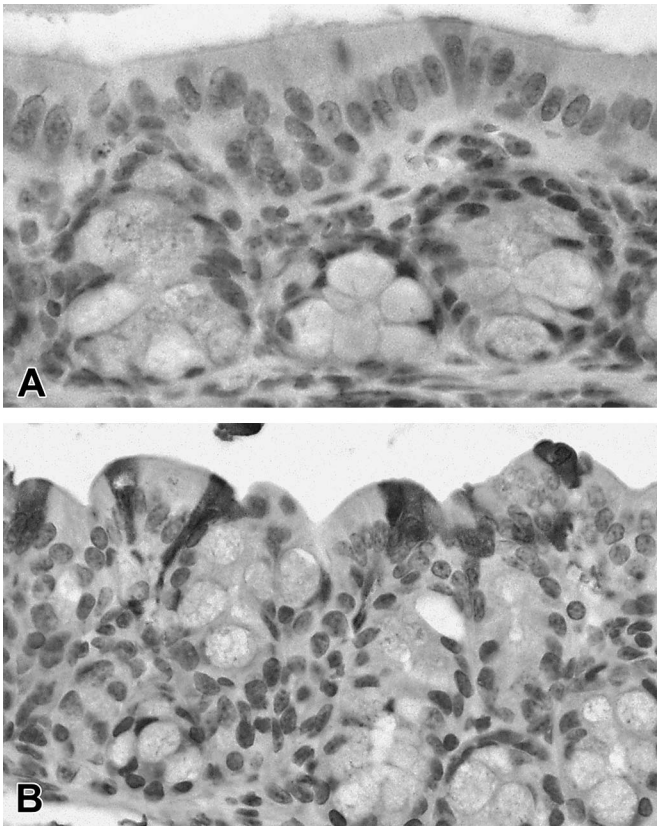

FIGURE 2. Increased colon epithelial apoptosis in piroxicam-exposed IL-10$^{-/-}$ mice. Immunohistochemical staining for activated caspase-3 (brown color) shows markedly increased apoptosis in (B) piroxicam-exposed versus (A) control IL-10$^{-/-}$ mice.

increased apoptosis was not observed in wild-type mice exposed to piroxicam.

Piroxicam Induces Apoptosis of Colon Epithelial Cells In Vitro

To directly determine the effects of piroxicam on intestinal epithelial cells, CN26 murine intestinal epithelial cells were treated in vitro with a range of piroxicam concentrations. Piroxicam treatment decreased cell survival in a dose-dependent fashion (Fig. 3A). The decrease in cell number was due almost entirely to apoptosis, as indicated by detection of oligonucleosomes within the cytoplasm of piroxicam-treated cells (Fig. 3B) as well as increased percentages of annexin V-positive cells (Fig. 3C). Although CT26 cells are potentially capable of making IL-10, a Luminex bead-based fluorescent immunoassay showed that no IL-10 is detectable in spent media from CT26 cultures. To determine whether the addition of exogenous IL-10 could protect intestinal epithelial cells against piroxicam-induced apoptosis, a dose range of 1, 10, and 100 ng/mL recombinant IL-10 was added to CT26 cultures 2 hours before the addition of 1 mg/mL piroxicam. No change in apoptosis was detected at 18 hours, indicating that these IL-10 doses alone are insufficient to protect monocultures of intestinal epithelial cells from piroxicam-induced apoptosis (Fig. 4A). Although addition of exogenous IL-10 did not prevent piroxicam-induced apoptosis, a small but statistically significant, dose-dependent increase in cellular proliferation was observed 18 hours after treatment of CT26 cells with IL-10 (Fig. 4B).

Piroxicam Treatment Increases Adhesion and Infiltration of Bacteria Into the Colonic Mucosa of IL-10$^{-/-}$ Mice

Increased apoptosis of colon epithelial cells may lead to erosion of the mucosal surface if the proliferation of replacement epithelial cells is not correspondingly increased. Loss of mucosal integrity may increase exposure of colon epithelial cells and immune cells in the underlying lamina propria to luminal bacteria and their antigens. Exposure to intestinal bacteria has been previously hypothesized to induce or enhance colonic inflammation in genetically susceptible individuals. Therefore, FISH assays were performed to determine how piroxicam treatment affected exposure of the colonic mucosa to intestinal bacteria. Control wild-type (n = 6) and IL-10$^{-/-}$ mice not exposed to piroxicam (n = 6) had a distinct mucus-filled gap between the epithelial surface and fecal compartment, starting with the middle colon and becoming thicker distally (Fig. 5, A–C). Bacteria were not observed within or below this mucus gap in any of the wild-type or control IL-10$^{-/-}$ mice that were not exposed to piroxicam. A mucus gap was also observed in IL-10$^{-/-}$ mice killed on day 7 of piroxicam treatment. However, in this group, bacteria were additionally present beneath the mucus layer of all colonic segments, directly covering the epithelial surface (Fig. 5, D–F). Foci of invasive bacteria could also be detected within epithelial cells and potentially deeper in the mucosal tissue (Fig. 6). The adherent and invasive bacteria hybridized primarily with the Bfra602 probe that recognizes organisms belonging to the *Bacteroides fragilis* group (Figs. 5 and 6).

DISCUSSION

Colitis is known to be a potential side effect of NSAID treatment in both in humans and rodents. NSAIDs inhibit the cyclooxygenase enzymes that are responsible for synthesis of the prostaglandins that regulate mucosal integrity and the proinflammatory prostaglandins. However, the precise mechanisms by which NSAID treatment induce colitis have not previously been established. Our data show that exposure to piroxicam induces apoptosis of colon epithelial cells, as evidenced by decreased cell survival, increased annexin V positivity, and the appearance of histone-associated oliconucleosomes within the cytoplasm of cells treated with piroxicam in vitro. Mice treated with piroxicam in vivo showed classic morphological evidence of colon epithelial apoptosis, confirmed by immunodetection of enhanced activation of caspase-3 in

colon epithelial cells. The data presented further show a link between NSAID (piroxicam)-induced apoptosis, enhanced bacterial adhesion and invasion, and accelerated development of colitis in genetically susceptible IL-$10^{-/-}$ mice.

IL-10 is a regulatory cytokine that is primarily considered to have immunosuppressive properties. IL-10 normally decreases the production of T_H1 cytokines by T cells and can also suppress macrophage activity. Loss of the regulatory functions of IL-10 with regard to immune responses against colonic bacteria has been suggested to be responsible for the development of spontaneous colitis in IL-$10^{-/-}$ mice. Genetic lack of IL-10 or its inhibition by neutralizing antibodies to the IL-10 receptor (IL-10R) have been shown to enhance autoreactive proliferative responses of T cells in response to staphylococcal enterotoxin B (SEB).[15] IL-10 expression was shown to protect syngeneic intestinal epithelial cells from apoptosis induced by SEB-primed spleen or colonic T cells, although this was attributed to the regulatory effects of IL-10 on activated T cells.[15] However, it is possible that IL-10 may also affect epithelial cells directly. Intestinal epithelial cells have been shown to express mRNA for both subunits of the IL-10 receptor-signaling complex.[16] Furthermore, both epithelial cell lines and freshly isolated murine intestinal epithelial cells derived from small or large intestine demonstrate specific saturable binding of IL-10.[16] IL-10 has previously been shown to protect thyroid epithelial cells against apoptosis by up-regulating expression of the antiapoptotic cellular FLICE inhibitory protein (cFLIP) and Bcl-xL.[17,18] Autocrine production or exogenous addition of IL-10 protected normal and malignant thyroid epithelial cells against apoptosis induced by chemotherapeutic agents. Exposure of these cells to neutralizing antibodies against IL-10 down-modulated Bcl-2 and Bcl-xL, induced apoptotic cell death, and enhanced susceptibility of the remaining population to chemotherapeutic agents.[19] However, in contrast to what is seen in the thyroid, in our studies, addition of exogenous IL-10 to isolated intestinal epithelial cells in vitro failed to increase their resistance to piroxicam-induced apoptosis. We hypothesize that, by

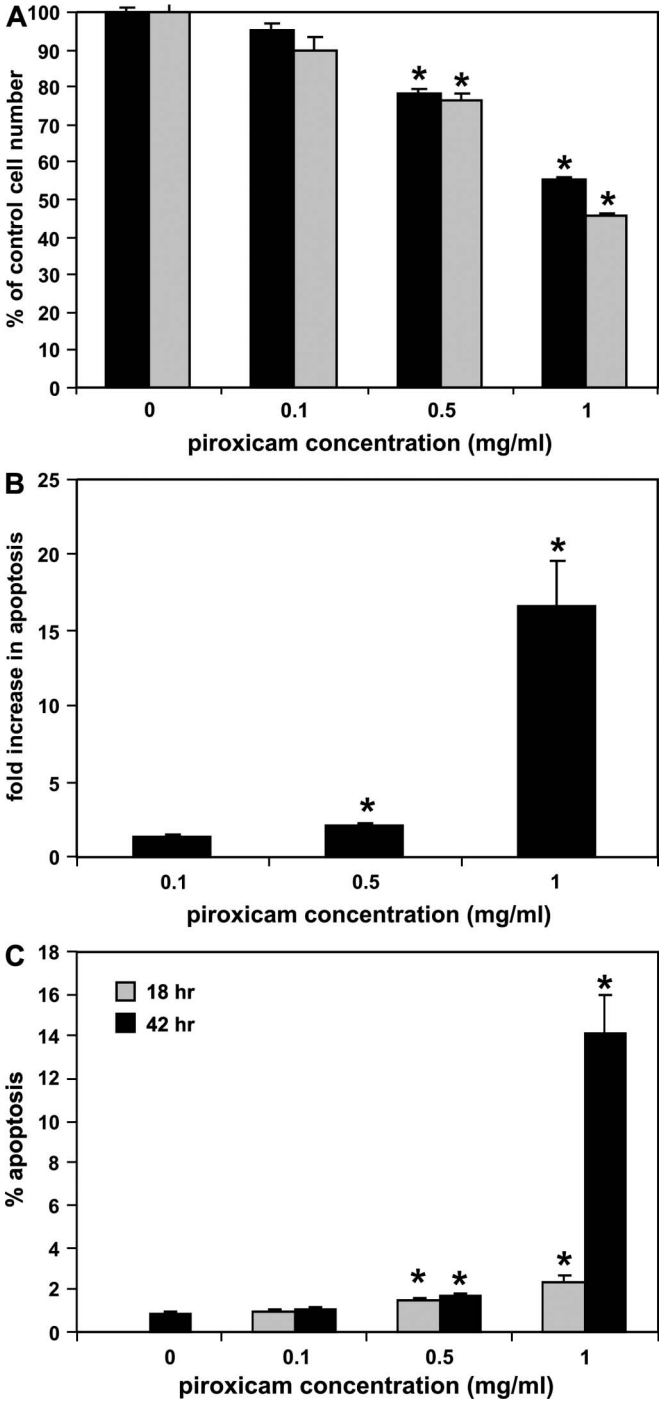

FIGURE 3. Piroxicam stimulates apoptosis of CT26 colon epithelial cells in vitro. A, CT26 cells were plated at 10^4 cells/well, allowed to attach overnight, and treated with the indicated concentration of piroxicam for 18 hours. Cell survival at 4 (black bars) and 18 hours (gray bars) after addition of piroxicam was determined using a colorimetric tetrazolium dye reduction assay. Data shown represent mean ± SEM from 3 replications. Piroxicam treatment resulted in a dose-dependent decrease in number of metabolically active cells in each culture (*, $P < 0.05$ versus control). B, apoptosis of CT26 cells after piroxicam treatment was monitored using an ELISA assay that measures histone-associated oligonucleosomes that are released into the cytoplasm. Data shown is the mean ± SEM from 3 independent experiments, measured at 18 hours after addition of piroxicam. Dose-dependent trends toward increased apoptosis were seen beginning at 4 hours after addition of piroxicam (data not shown) and were statistically significant at 18 hours (*P* versus control for 0.1, 0.5, and 1 mg/mL piroxicam is 0.06, 0.02, and 0.03, respectively). C, percentage of CT26 cells undergoing apoptosis after piroxicam treatment was also determined by flow cytometric detection of annexin V staining. Data shown is the mean ± SEM from 3 independent experiments, measured at 18 and 42 hours after addition of piroxicam.

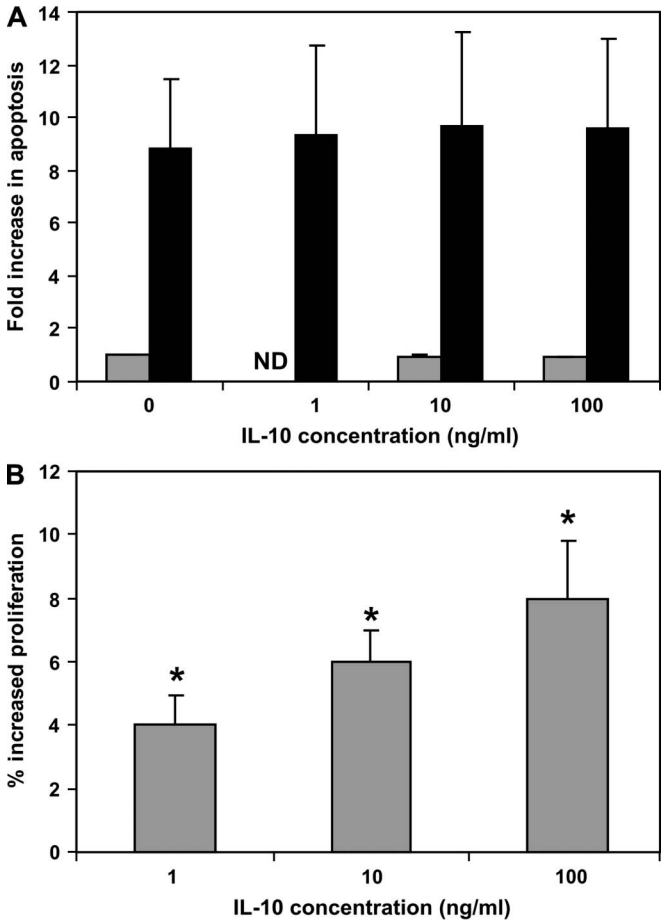

FIGURE 4. Addition of exogenous IL-10 does not protect cultured colon epithelial cells from piroxicam-induced apoptosis in vitro but does increase their proliferation. A, CT26 cells were plated at 10^4 cells/well, allowed to attach overnight, and treated with the indicated concentration of IL-10, beginning 2 hours before addition of 1 mg/mL piroxicam. Apoptosis at 18 hours of exposure to piroxicam was determined using an ELISA assay that detects histone-associated oligonucleosomes in the cytoplasm of cells undergoing apoptosis. Data shown are the mean ± SEM from 3 independent experiments. IL-10 had no significant effect on apoptosis of CT26 cells in the presence (black bars) or absence of piroxicam treatment (gray bars). B, effect of IL-10 on CT26 survival and proliferation was determined using a colorimetric tetrazolium dye reduction assay. Data shown represent the mean ± SEM increase in signal at 18 hours after addition of the indicated concentration of IL-10 compared with that seen in absence of added IL-10 for 3 independent experiments. IL-10 induced a small but statistically significant dose-dependent increase in proliferation of CT26 cells (*, $P \leq 0.04$).

increasing the proliferation of colonic epithelial cells (Fig. 4), IL-10 may also enhance epithelial regeneration and repair and thus potentially mitigate negative effects of piroxicam-induced apoptosis in the colon in vivo. This can be tested in future studies designed to clarify the role of IL-10 in colonocyte survival, including the possibility of indirect effects through other cell types that are present in the colonic microenvironment.

The marked changes in distribution of bacteria within the colon after piroxicam treatment are of considerable interest. Evidence that colonic bacteria, particularly *Bacteroides* species, may participate in the pathogenesis of IBD has been presented for both rodents and humans.[20–24] Increased numbers of mucosa-associated *Bacteroides* organisms in human patients with IBD have also been detected by culture and by FISH.[25] The presence of bacteria adherent to and within colon epithelial cells in piroxicam-exposed but not control mice clearly implicates these organisms in the pathogenesis of the colitis that develops after piroxicam exposure. We hypothesize that increased immune system exposure to antigens from these bacteria results in development of chronic bowel inflammation in susceptible individuals. However, the data presented here do not allow direct effects of piroxicam on mucosal integrity or bacterial growth to be distinguished from those that arise as a result of inflammation. Further studies including time-courses that relate changes in intestinal bacteria–mucosal interactions to the onset of inflammation will be required to address these issues.

NSAIDs have been previously documented to increase the incidence of apoptosis in colonic cells. Administration of sulindac and the selective COX-2 inhibitor nimesulide to mice enhanced apoptosis in colonic epithelium of mice with azoxymethane and dextran sulfate sodium–induced colitis. NSAID-treated mice had decreased numbers of aberrant crypt foci and tumors, which was attributed to the increased level of apoptosis.[26] Nonselective NSAIDs such as sulindac and piroxicam inhibit both the COX-1 and/or COX-2 cyclooxygenase enzymes. COX-2 expression is stimulated by inflammatory mediators and results in preferential synthesis of the prostanoids (PG) PGI2 and PGE2.[27] Treatment with PGE2 or with agents that induce its synthesis inhibits apoptosis induced by camptothecin in T24 bladder epithelial cells.[28] Overexpression of COX-2 has been proposed to enhance neoplastic transformation by increasing cellular proliferation and decreasing apoptosis.[29,30] Although these studies seem to implicate COX-2 in regulating apoptosis, simultaneous inhibition of both COX-1 and COX-2 seems to be required for acceleration of colitis in IL-$10^{-/-}$ mice, because use of either COX-1– or COX-2–selective inhibitors alone does not induce colonic inflammation.[8]

Given these data, it is of interest to examine the potential role of conventional and COX-selective NSAIDs in the pathogenesis of human colitis. A recent study of 105 cases of newly diagnosed colitis and 105 age- and sex-matched controls showed increased incidence of NSAIDs or salicylate exposure in colitis patients before disease onset (odds ratio, 9.1; 95% confidence limits, 4.5–21.9; $P < 0.001$).[31] Results of retrospective studies of the effects of NSAIDs on IBD activity have been mixed.[32] A relapse rate of 25% was recorded in

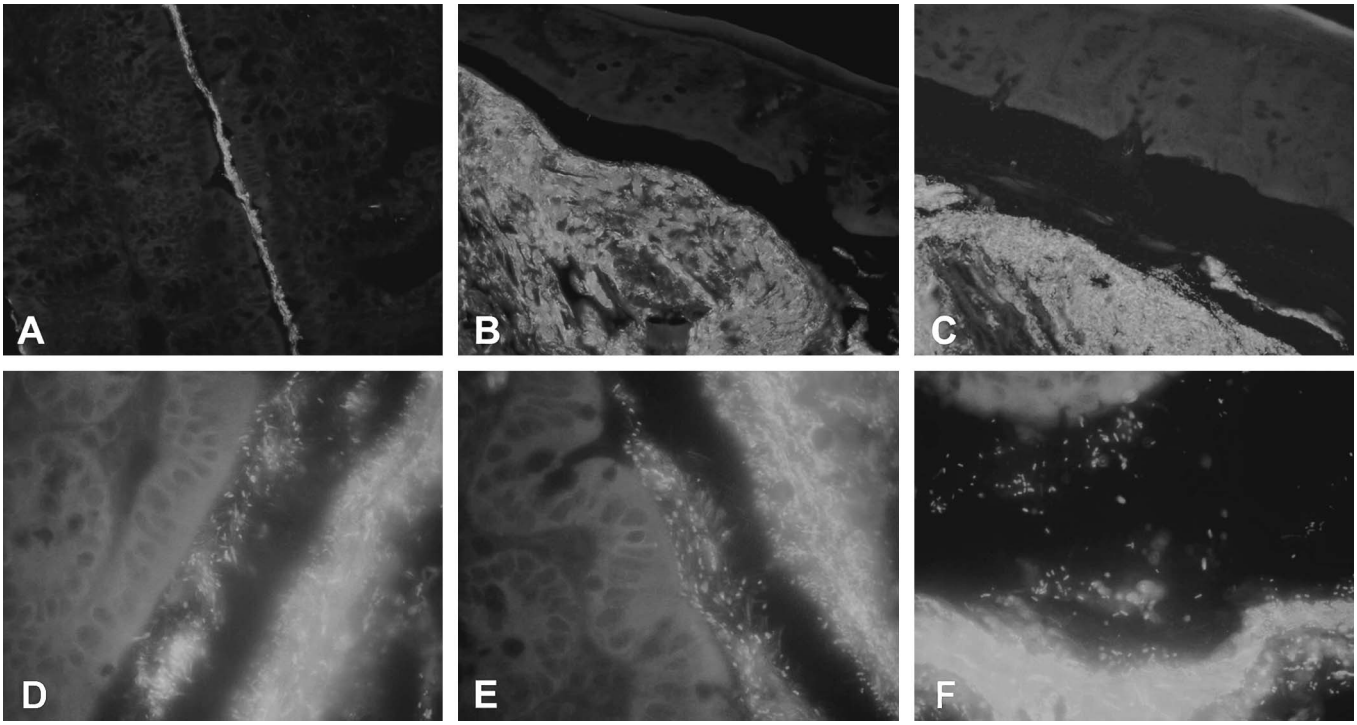

FIGURE 5. FISH detects altered bacteria-mucosal interactions in IL-10$^{-/-}$ mice treated with piroxicam. A–C, colon sections were simultaneously hybridized with Eub338-Cy3 and Eub338- FITC probes that recognize virtually all bacteria in the kingdom (Eu)Bacteria and the Erec482-Cy5 probe that recognizes bacteria of the *Eubacterium rectale-Clostridium coccoides* group. Combined Eub338-Cy3 (appears slightly green to pale orange) and Erec482 (appears darker orange to red) fluorescence is shown. The epithelium can also be seen faintly because of autofluorescence. The middle (A), distal (B), and terminal (C) segments of the colon of wild-type mice without colitis show a gradually increasing layer of mucus (nonfluorescent, seen as a black stripe) devoid of bacteria directly overlying the mucosal epithelium. The mucus layer prevents the bacteria present in the feces from contacting the mucosal surface. Hybridization with the Brfa602 (*Bacteroides fragilis* group) probe was minimal in these sections (data not shown). Original magnification, ×400. D–F, in contrast, combined FISH images of the middle (D), distal (E), and terminal (F) colon segments using the Bfra602-Cy3 and Erec482-Cy5 probes in piroxicam-treated IL-10$^{-/-}$ mice show bacteria present below the mucus layer, directly adherent to the epithelial surface. The majority of these bacteria hybridize with the Bfra602 probe (appears slightly green to pale orange). Original magnification, ×1000.

a cohort of patients with quiescent IBD with arthritis or arthralgias who were prospectively exposed to NSAIDs.[33] In a group of 33 patients with IBD who were prescribed the COX-2–selective NSAIDs celecoxib or rofecoxib, 13 (39%) had a flare of IBD within 6 weeks of initiating COX-2 therapy.[34] However, a prospective open-label monocentric trial of 32 patients with clinically inactive or mild IBD using the COX-2–selective NSAID rofecoxib showed no significant exacerbation of IBD.[32]

Regular intake of NSAIDs has been shown to be beneficial in reducing the risk of colorectal cancer in the general population and in patients with inherited mutations that place them at increased risk for colorectal cancer.[35] Use of the NSAID-like drugs sulfasalazine and its metabolite mesalamine (5-aminosalicylic acid) for therapy for colitis has been shown to protect against development of colorectal carcinoma in patients with IBD. Treatment with 5-aminosalicylic acid was associated with increased apoptosis in tumors or abnormal mucosa, without long-term effects on normal mucosa in human, mouse, and in vitro studies.[35,36] Thus treatments that moderately increase apoptosis of (possibly mutant) colon epithelial cells may be beneficial for chemoprevention of colorectal cancer, as long as such treatment does not affect mucosal integrity.

In summary, the NSAID piroxicam enhances apoptosis of intestinal epithelial cells both in vitro and in vivo, leading to the development of epithelial erosions, direct adherence of bacteria to the mucosal surface, and bacterial invasion of epithelial cells. We hypothesize that the increased prevalence and accessibility of intestinal bacterial antigens to immune cells that results from piroxicam-induced apoptosis of the colonic epithelium facilitates the development of acute and chronic colonic inflammation in IL-10$^{-/-}$ mice. Published data suggest that NSAIDs may similarly increase apoptosis

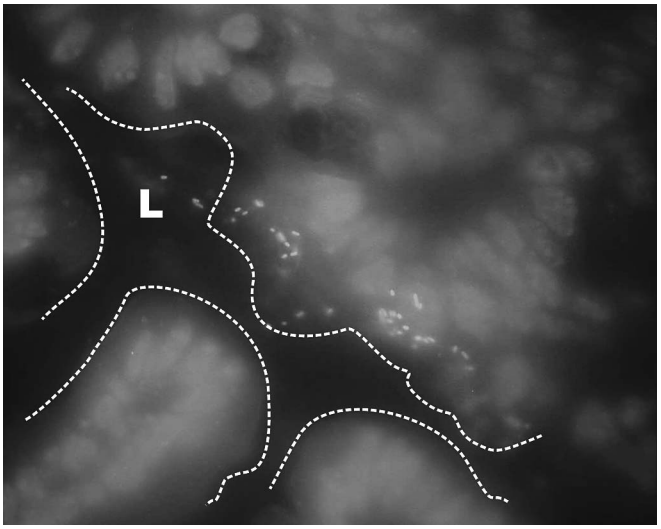

FIGURE 6. Mucosal invasion by *Bacteroides* in vivo. The nuclei of the mucosal epithelial cells are DAPI-positive (blue color), and their apical surface, as indicated by the dotted line, is visible by autofluorescence. Hybridization with the Bfra602 probe (slightly green to orange color, arrows) detects focally extensive bacterial invasion of the mucosal epithelial cells in piroxicam-treated IL-10$^{-/-}$ mice. L indicates lumen.

in the human colon. Further studies should be undertaken to determine whether NSAID use is harmful for individuals whose genetic makeup makes them particularly susceptible to NSAID-induced colonic apoptosis or who may be at increased risk for developing prolonged or inappropriate immune responses in response to mucosal contact with intestinal bacteria.

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