TGF-β1 gene transfer to the mouse colon leads to intestinal fibrosis

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Valiance, Bruce A., M. Imelda Gunawan, Bryan Hewlett, Premysl Bercik, Corinne Van Kampen, Francesca Galeazzi, Patricia J. Sime, Jack Gauldie, and Stephen M. Collins. TGF-β1 gene transfer to the mouse colon leads to intestinal fibrosis. Am J Physiol Gastrointest Liver Physiol 289: G116–G128, 2005.—Crohn’s disease (CD) is a chronic, relapsing inflammatory bowel disease, characterized by transmural inflammation. In CD, the recurrent inflammatory injury and tissue repair that occurs in the intestine can progress uncontrollably, leading to the proliferation of mesenchymal cells as well as fibrosis, characterized by excessive extracellular matrix deposition. These processes thicken the bowel wall, reducing flexibility, and often culminate in obstructive strictures. Because no effective measures are currently available to specifically treat or prevent intestinal strictureting, we sought to gain a better understanding of its pathogenesis by developing a mouse model of intestinal fibrosis. Because transforming growth factor (TGF)-β1 can mediate both fibrosis and mesenchymal cell proliferation; we studied the effects of delivering adenoviral vectors encoding spontaneously active TGF-β1 into the colons of mice. We first demonstrated that enema delivery of marker adenoviral vectors led to the transfection of the colonic epithelium and transient transgene expression. Histologically, control vectors caused an acute inflammatory response, involving the recruitment of neutrophils and mononuclear cells into the colonic lamina propria; however, infection caused little if any fibrosis. In contrast, the TGF-β1 vector caused a more severe and prolonged inflammatory response as well as localized collagen deposition, leading to severe and progressive fibrosis. This was accompanied by the emergence of cells with a myofibroblast phenotype. Ultimately the fibrosis resulted in many of the TGF-β1-transfected mice developing profound colonic obstruction. Through adenoviral gene transfer technology, we describe a novel mouse model of colitis and implicate TGF-β1 in the pathogenesis of obstructive intestinal fibrosis.

Although the etiologies of Crohn’s disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel disease (IBD) remain unclear; both diseases are characterized by chronic inflammation and intestinal tissue damage (11–13, 53). However, unlike UC, in which inflammation is limited to the mucosa (10, 52), the inflammation that occurs during CD is transmural, affecting not only the intestinal mucosa but also the muscularis and serosa (7, 8, 17, 18). Whereas inflammatory changes in intestinal physiology cause much of the symptomatology associated with CD (7, 12), significant morbidity results from the irreversible tissue injury and fibrosis that frequently occur in chronically inflamed bowel segments (2, 18, 46). For reasons that are currently unclear, the reparative process associated with CD can progress uncontrollably, leading to the proliferation of mesenchymal cells, and the unrestrained deposition of extracellular matrix (ECM) (17, 18). This fibrotic process thickens the wall of the gut, reducing flexibility and narrowing the bowel lumen, often resulting in obstructive strictures (2, 17, 18). Despite the frequency of stenotic lesions in CD, little therapeutic progress has been made with respect to intestinal fibrosis and stricture formation (46). Current attempts at prevention and treatment rest primarily on long-term anti-inflammatory therapy (10, 13, 23, 24); however, these approaches often prove ineffective, with surgery and stricturoplasty constituting the major therapies for intestinal fibrosis (4, 18). Even these drastic measures provide only short-term resolution, because fibrosis and strictures tend to reoccur near the original site of obstruction (18).

In marked contrast to our limited understanding and treatment options for intestinal fibrosis, significant progress has recently been made toward understanding and treating other aspects of CD. These new insights have arisen, in part, from studies of both spontaneous and induced models of intestinal inflammation in mice (11, 42, 43, 56). This research has revealed the critical role that immunoregulatory cytokines play in preventing chronic intestinal inflammation, whereas other studies have demonstrated the facilitating role that commensal bacterial flora play in initiating inflammation in the bowel (29). These findings have now begun to impact on the therapies employed in the treatment of CD and UC, including the recent targeting of TNF-α (29, 44, 50, 51) as well as the initiation of both antibiotic (20) and probiotic (48) clinical trials.

Unfortunately the majority of mouse models of IBD have so far proven of little use in studying the pathogenesis of stricture formation, because they are accompanied by only limited intestinal fibrosis (46). Whereas this observation suggests that mice are relatively resistant to fibrosis, the potential for genetic and immunological manipulation in mice has prompted continued efforts, with a recent study by Lawrance et al. (30), demonstrating that repeated induction of trinitrobenzene sulfonic acid (TNBS) colitis eventually caused colonic fibrosis in mice. Although this is an important finding, the labor involved, as well as the 8–12 wk required to induce fibrosis using this model, may limit its applicability (33). Therefore, generating additional murine models of intestinal fibrosis is highly desirable (46), not only to aid in our understanding of the mechanisms underlying stricture formation but also to help identify key mediators of intestinal fibrosis and provide a means to test potential therapies (30, 46).

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On the basis of the limited fibrosis seen in mouse colitis models, we hypothesized that a stronger and more specific fibrotic stimulus than colitis induction might be needed to alter the normal repair process and trigger significant intestinal fibrosis in mice (46, 54). Studies examining strictured tissues from CD patients have identified the elevated expression of a number of growth factors including TGF-β1 (2, 18, 34). Although large quantities of TGF-β1 are normally produced in the gastrointestinal (GI) tract, it is normally only found in a latent precursor form, requiring cleavage and dissociation for conversion to a mature bioactive dimer (36, 63). Once activated, TGF-β1 has been strongly linked to tissue fibrosis as well as to the emergence and proliferation of myofibroblasts and other mesenchymal cells in the lung and other tissues (19, 54, 61). However, the role of TGF-β1 in the intestine is potentially more complex, because TGF-β1 is also considered an important mediator of oral tolerance and a key negative regulator of mucosal inflammation (57). In fact, overexpression of TGF-β1 has been proposed as a potential therapy for IBD (14, 26, 37).

To evaluate the potential role of TGF-β1 in intestinal fibrosis, we locally overexpressed spontaneously active TGF-β1 in the colons of mice by using adenoviral vectors. As seen in other systems, recombinant adenoviral vectors provide an efficient means to overexpress cytokine genes in a tissue-restricted manner (54, 66). Our laboratory and others have shown that after transient disruption of the protective mucus barrier, adenoviral vectors delivered by enema readily infect the colonic epithelium (60, 64). We found that gene transfer of active TGF-β1 led to colonic inflammation accompanied by the thickening of the smooth muscle tissues and the localized deposition of ECM within the bowel wall, along with the emergence of cells presenting with a myofibroblast phenotype. Over a 2- to 4-wk period, the fibrosis rapidly progressed, frequently leading to obstruction of the distal colon. These results indicate that overexpression of active TGF-β1 by the colonic epithelium is capable of inducing intestinal fibrosis, designating TGF-β1 as a potential therapeutic target in the prevention of fibrosis in CD.

**MATERIALS AND METHODS**

**Mice**

Specific pathogen-free, male NIH Swiss mice (6–10 wk old) were purchased from the National Cancer Institute (Frederick, MD), housed in filter topped cages in groups of four to five, and given ad libitum access to autoclaved food and water. The protocols were approved by and were in direct accordance with the guidelines of the University of British Columbia’s and the McMaster University’s Animal Care Committees and the Canadian Council on the Use of Laboratory Animals.

**Recombinant Adenovirus Vectors**

The recombinant human type 5 adenovirus AdTGF-β1,232/225 has been previously described (54). In brief, it contains the cDNA of the coding region of full-length TGF-β1 but contains mutations replacing the cysteines at positions 223 and 225 with serines, rendering the expressed TGF-β1 spontaneously biologically active (6). The control virus, AdDL70-3, as well as the AdCA35 and AdDK1 (hereafter referred to as AdLacZ and AdLuc) containing the β-galactosidase (β-gal) and firefly luciferase genes, respectively, have also been previously described (5). These genes are under the control of the mouse cytomegalovirus (CMV) immediate early promoter and terminated by the SV40 polyadenylation signal inserted into the E1 region of the Ad5 using the BHG10 backbone described by Bett et al. (5). High titers of viral constructs were generated as previously described (5). Viruses purified by CsCl gradient centrifugation were dialyzed thoroughly against four changes of autoclaved Tris-HCl buffer, pH 7.4, containing 10% glycerol, aliquoted, plaque titered in 293 cells, and stored at −70°C until use.

**Enema Administration of Recombinant Adenoviruses to Mice**

Mice were anesthetized with Enflurane (Abbott Laboratories, St. Laurent, Quebec) and then given an intrarectal enema of 50% ethanol (vol/vol) (diluted in distilled H2O) using a catheter made of PE50 polyethylene tubing attached to a 1-ml syringe. The tip of the catheter was inserted 4 cm proximal to the anus, and a total volume of 100 μl was injected (58). Mice were held in a vertical position for 30 s after the injection to ensure distribution of the enema throughout the colon. Mice were given 3 h to recover, then again anesthetized and given another enema of 100 μl of phosphate-buffered saline (PBS), pH 7.4, containing 1 × 109 adenoviral plaque-forming units (pfu). Additional experiments were performed on mice with established TNBS colitis. In these studies, mice were anesthetized as described above, after which they received an enema of 4 mg of TNBS in 100 μl of 50% ethanol. The mice were left to recover for 24 h, after which the colitic mice were again anesthetized and given a PBS enema containing 1 × 109 adenoviral pfu. The health status of all the treated mice was followed closely and all mice were weighed daily. Any mice that showed >15% weight loss or became moribund were immediately euthanized.

**Tissue Collection**

In brief, at time points after the delivery of adenoviral vectors, mice were euthanized by cervical dislocation. Depending on the virus given, the large bowel as well as various tissues including spleen, liver, and the mesenteric lymph nodes were removed. The tissues were then fixed for histological analysis, immunohistochemistry or prepared for quantification of transgene expression, as described below.

**Histology and Scoring of Inflammation**

Tissues were fixed in 10% neutral buffered formalin overnight and then transferred to 50% ethanol for 2 h, followed by storage in 70% ethanol. Formalin fixed tissues were then embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin, Masson’s trichrome or elastin Van Gieson (EVG) staining, using standard procedures. Microscopic colonic damage and mucosal inflammation were assessed in colonic tissues taken 1–2 cm from the rectal verge, in the various treatment groups over a 28-day time course, by use of a scoring system previously described (62), and expressed as the colonic damage score.

**Expression and Quantification of Marker Transgenes**

**Histochemical localization of β-gal (LacZ) product.** Histochemical staining for β-gal expression was performed using a procedure described by Mastrangeli et al. (32) with minor modifications. In brief, tissues were fixed with 2% formaldehyde in PBS at 4°C for 1 h. Tissues were then rinsed twice with PBS and immersed in staining solution containing 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, and 0.5 mg/ml of the X-gal stain (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside: Boehringer Mannheim, Indianapolis, IN) at 37°C overnight. The stained intestinal tissues were then paraffin embedded, sectioned at 6 μm, and counterstained with nuclear fast red. Photographs were taken with a Zeiss camera.

**Luciferase quantification.** To quantify luciferase transgene expression, the colon was removed, opened longitudinally, cleaned of fecal...
material, and kept on ice. Tissues were subsequently homogenized in buffer (0.1 M potassium phosphate pH 7.8, 1 mM PMSF, and 10 μg/ml aprotinin) with a tissue homogenizer and centrifuged to remove debris. The homogenates were then immediately assayed for luciferase activity as previously described (35).

**Immunohistochemical Staining for α-SMA and Desmin**

Paraffin-embedded tissue sections were immunostained using standard techniques. In brief, tissue sections were deparaffinized, and endogenous peroxidase was blocked. Sections were blocked with 5% goat serum for 30 min, and incubated overnight with the primary antibody. The monoclonal anti-α smooth muscle actin (SMA) antibody (Sigma Chemical, St. Louis, MO) was used at a dilution of 1:100 (54), and the monoclonal anti-desmin antibody was used at a dilution of 1:300. Sections were also incubated with either rabbit serum (Sigma Chemical) or control IgG as negative controls. Sections were then incubated for 15 min with biotinylated goat anti-rabbit or antimouse IgG (Histostain-SPTM Bulk Kit; Zymed Labs, San Francisco, CA) and incubated for 10 min in streptavidin peroxidase conjugate. Finally they were placed in a substrate/chromagen mixture [3-amino, 9 ethyl-carbazole (AEC) (red) for SMA, 3′,3′-diaminobenzidine (DAB) (brown) for desmin], and color was developed for 15 min before counterstaining with Mayer’s hematoxylin. Photographs were taken with a Zeiss camera.

**Image Analysis of Extracellular Matrix Content in the Colon**

The distal 4 cm of the colon of each mouse was divided into four equal sections, with section 1 representing the most distal part of the colon, closest to the rectal verge. Sections 2, 3, and 4 were sequentially further from the rectum. By use of routine histological techniques, these full-thickness sections were paraffin embedded, cut into 3-μm cross sections, and stained for ECM proteins with Masson’s trichrome staining. After the trichrome staining, sections were counterstained with Mayer’s hematoxylin for the visualization and identification of nuclei. Quantitative digital morphometric analysis of ECM was performed according to a protocol adapted from that described in detail elsewhere (22). In brief, 6–12 randomly chosen high-power fields (×400 magnification) for each cross section were photographed with a Nikon camera. The color wavelengths of the copied image were transformed into digital readings, by using the National Institutes of Health 1.56 Image Analysis Program (http://rsb.info.nih.gov/nih-image/), allowing for quantification of the various color wavelengths with pixels as the unit of measure. By using the original image for comparison, the color spectra were analyzed and those corresponding to ECM (green wavelengths) were quantified. The percentage of the tissue that was ECM was then calculated by dividing the total pixel area of the ECM by the pixel area corresponding to the total tissue in the field of view. Because the fibrosis in this model was localized to a small region of the colon, the average percentage area of ECM within a given colon section was calculated by using the mean of the percentage ECM obtained from the different high-power fields. At minimum, the colons of five to six mice were analyzed in each treatment group at each time point.

**Detection of TGF-β1 Protein by ELISA**

To quantify TGF-β1 expression in colonic tissues, mice were euthanized and their colons were removed and treated as described for the luciferase assay. The samples were assayed for TGF-β1 protein as previously described (54) with a commercial human TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN) that also detects porcine and mouse TGF-β1 protein (sensitivity 5 pg/ml). Because the ELISA is receptor based, it only detects active TGF-β1. Therefore direct measurement of samples detected only spontaneously active TGF-β1. To test for the levels of total TGF-β1 (active + latent), the latent form of TGF-β1 was activated by acidification (54) to dissociate the latency associated peptide. Biological activity of the transgene-derived protein was previously confirmed by using a murine HT-2 lymphocyte cell line as described by Tsang et al. (59).

**Data analysis**

Data were expressed as means ± SE; n refers to the number of mice tested. Statistical significance was calculated with the Student’s t-test for comparison of two means or a one-way of analysis for the comparison of three or more means. Multiple comparisons were performed by using the Newman-Keuls multiple comparison test. P < 0.05 was considered significant.

**RESULTS**

**Choosing Enemas to Deliver Adenoviruses to the Colon**

Because intravenous or intraperitoneal injection of adenoviral vectors leads to little or no transgene expression in the GI tract (21, 66), our laboratory and others have focused on enema delivery as the best means to transfect the mouse colon (60, 64). The mucus layer normally prevents infection of the colonic epithelium (not shown); therefore, achieving efficient transfection necessitates the transient disruption of this barrier, either through colonic distension and prolonged enemas (64) or, as in this study, through the application of dilute ethanol as a mild barrier breaker. The following section characterizes the location, level, and duration of expression of two reporter transgenes (β-galactosidase and luciferase) within the colons of mice after enema delivery of the AdLacZ and AdLuc adenovectors.

β-Gal Is Transiently Expressed by Transfected Colonic Epithelial Cells

After the enema delivery of the AdLacZ virus, infected mice were euthanized over an 8-day time course, and tissues were removed and stained for β-gal activity. Whereas no cells expressed β-gal in the uninfected colons of mice (Fig. 1A), examination of AdLacZ-infected colons under a dissecting microscope revealed numerous blue-stained cells on the luminal surface of colon, but no staining on the serosal surface of the colon or any staining of the small bowel, cecum, spleen, or liver. Although β-gal staining was found over the length of the large bowel, greater numbers of positive cells were observed in the last 2 cm of the colon. Histological analysis identified the majority of the β-gal-positive cells as superficial epithelial cells (Fig. 1, B and C); however, blue-stained epithelial cells were also occasionally found at the base of colonic crypts (not shown). No β-gal staining was observed in any cell types other than epithelial cells. Although distribution of β-gal expression was patchy, in some tissue cross sections up to 40% of epithelial cells expressed the β-gal transgene. Similar large numbers of transfected cells were seen for the first two days postinfection (PI), but numbers began to decrease by day 3 PI. By days 4 and 5 PI, only scattered blue cells were evident, and by day 8 PI, surface expression of β-gal was no longer detected. No β-gal-positive staining was found after infection with other adenoviral vectors, including AdLuc and AdDL70-3 (not shown).
Luciferase Transgene Is Transiently Expressed in the Transfected Colon

To quantify transgene expression in the colon, the luciferase-encoding AdLuc virus was also tested. The advantages of the luciferase reporter gene include the absence of endogenous luciferase activity in mammalian tissues, as well as a relatively short half-life providing a more accurate quantitative measure of current transgene expression. Similar to our observations with the β-gal vector, luciferase expression was found primarily in the distal colon [6.5 × 10^5 relative light units (RLU)/g of tissue] with less expression (1.2 × 10^5 RLU/g) detected in the proximal colon (see Fig. 1D). Low but detectable luciferase expression was also detected in the ileum (1.0 × 10^4 RLU/g) (Fig. 1D), but expression never rose above background in the jejunum, spleen, liver, or mesenteric or iliac lymph nodes, indicating that the luciferase transgene was selectively expressed in the distal GI tract.

As shown in Fig. 1E, luciferase activity in the distal colon was highest at day 1 PI (6.5 × 10^5 RLU/g), with expression reduced but still substantial at day 2 PI (1.1 × 10^5 RLU/g). Luciferase expression stabilized at days 3, 5, and 8 PI, at levels close to 1 × 10^5 RLU/g. Although low, the luciferase activity detected at these time points remained significantly higher than the background levels detected in tissues infected with the control AdDL70-3 virus (<1 × 10^3 RLU/g). The luciferase activity of AdLuc-infected mice euthanized at days 14 and 28 PI had reverted back to baseline levels (not shown), indicating that luciferase transgene expression was transient. Interestingly, the duration of transgene expression was similar to that found by Wirtz et al. (64), after quantitative analysis of a β-gal transgene in mouse colons.

Transfection Increases TGF-β1 Levels in the Colon

Because enema delivery of marker adenoviruses achieved substantial, albeit transient transgene expression in the colon, we next tested the impact of overexpressing TGF-β1 in the colons of mice. NIH Swiss mice were divided into two groups and given enemas of either the AdTGF-β1 virus or the control AdDL70-3 virus. Unlike the β-gal or luciferase transgenes for which there was little or no background expression, the colonic mucosa normally contains high levels of endogenous TGF-β1, albeit predominantly in the inactive precursor form. Therefore, to examine the expression of spontaneously active TGF-β1, colonic tissues were analyzed for both active and total levels...
TGF-β1, by performing ELISA analysis without activation (for measurement of spontaneously active TGF-β1) as well as by activating the samples to measure total (both spontaneously active and latent) TGF-β1.

Uninfected mouse colons contained an average of 738 ± 89 pg of total TGF-β1/g of tissue (see Fig. 2A), although most of the TGF-β1 was inactive because spontaneously active TGF-β1 was below the limit of detection (5 pg of TGF-β1/g). By day 1 post-AdDL70-3 infection, levels of total TGF-β1 in the colon had increased threefold (2,790 ± 365 pg/g); however, only a small amount (40 pg/g) of active TGF-β1 was detected (see Fig. 2A). The increase of predominantly inactive TGF-β1 in the control virus infected colon probably reflects endogenous expression by inflammatory and immune cells involved in the host response to the adenovector. Over the next 7 days, total levels of TGF-β1 fell from their day 1 peak, such that by day 3 PI they were no longer significantly elevated over the levels found in uninfected colons. Throughout this time course, levels of active TGF-β1 remained low and were usually below the limits of detection.

When the AdTGF-β1 virus was administered, we detected an impressive 453 ± 41 pg/g of active TGF-β1 in the colonic lysates taken on day 1 PI, whereas total levels of TGF-β1 reached 5,380 ± 745 pg/g (Fig. 2B). On day 2 PI, spontaneously active TGF-β1 levels rose to more than 800 pg/g of tissue despite the total level of TGF-β1 falling slightly to 3,450 pg/g. Over the following 6 days, both active and total levels of TGF-β1 decreased from their peak levels in a time-dependent manner, with 213 ± 64 pg of active TGF-β1/g found at day 3 PI and by day 5 PI, levels of active TGF-β1 were 97 ± 15 pg/g. Active TGF-β1 levels were low (20 pg/g) but still detectable at day 8 PI (see Fig. 2B). Over the same time course, total levels of TGF-β1 slowly declined but remained significantly elevated over control levels at day 3 and day 5 PI, until at day 8 PI when levels were 1,224 ± 250 pg/g and no longer significantly elevated over control levels. Compared with the TGF-β1 levels found in the control virus-infected mice, the AdTGF-β1 virus mice had active levels of TGF-β1 that were significantly higher on all days tested, whereas total levels of TGF-β1 were significantly greater from days 1 through 5 PI inclusive.

**TGF-β1 Gene Transfer Induces Severe Macroscopic Changes in the Colon**

After the successful induction of TGF-β1 transgene expression in the colonic mucosa, we followed the progress of the transfected mice over the next month. We found that the transfection protocol was associated with a limited mortality (<5% with control virus, <10% with the TGF-β1 virus), occurring within the first 3 days PI. During this early time frame, both the AdDL70-3- and the AdTGF-β1-infected mice lost ~10% of their body weight (not shown) and exhibited signs of lethargy, but between days 3 and 5 PI, both groups of mice showed signs of recovery, regaining their lost weight and resuming their normal behavior. This recovery was maintained in the AdDL70-3 mice until the end of the study (day 28 PI). In contrast, more than 55% (17 of 30) of the AdTGF-β1-transfected mice adopted a hunched posture and exhibited a distended and hard abdomen between days 14 and 28 PI. The affected mice were euthanized, and, upon necropsy, the cause of the abdominal swelling was determined to be a grossly enlarged colon (see Fig. 3B). Specifically, we observed a considerable distension of the proximal colon, with this dilated region filled with stool pellets, suggesting that the colon was obstructed. Longitudinal dissection of the distended colon invariably revealed the distal colon to be thickened and stiff to the touch, indicative of severe fibrosis. In comparison, assessment of the colons from AdDL70-3-infected mice (between days 14 and 28 PI) revealed a normal appearance, similar to the colons of mice receiving only the ethanol pretreatment. We observed no signs (0/45) of any dilatation or bowel thickening in the AdDL70-3-infected mice (see Fig. 3A). To rule out the possibility that the AdDL70-3 virus could also induce fibrosis and obstruction, but at a slower pace than the AdTGF-β1 virus, an additional 10 mice were infected with AdDL70-3 and housed for a total of 2 mo PI. Over this extended time course, they showed no signs of disease, and after euthanization, their colons were normal in appearance, with no signs of obstruction or other abnormalities (not shown).

**Following the Time Course of Pathology and Fibrosis**

On the basis of the significant pathology observed after TGF-β1 transfection, we were interested in studying the time course of the inflammation and pathology that followed.
Ethanol Pretreatment Plus PBS Induces Masson’s trichrome with intestinal pathology we observed, by staining colonic tissues previously described (62). The damage was ex-inflammatory in the distal colons of mice in the various treatments and mucosal injury was more severe than seen with the control virus, the inflammatory infiltrate and ulcerations observed in the AdTGF-β1-transfected mice were significantly larger and more numerous than those seen with the control virus (Fig. 5). The resulting inflammatory infiltrate was occasionally tranmural but still primarily comprised of neutrophils as well as mononuclear inflammatory cells. Pathology also included a loss of goblet cells as well as epithelial sloughing. Although the acute injury was more severe than seen with the control virus, the tissue injury and inflammation resolved over the next week, such that by days 10-14 PI, the colonic mucosa had returned to almost normal condition. However, in some sections, a chronic inflammatory infiltrate was still seen in the submucosa or in the subserosa. Colonic tissues stained with Masson’s trichrome exhibited little evidence of fibrosis over this time course, although an occasional small area of ECM deposition could be seen associated with the submucosal inflammation in the AdTGF-β1-transfected mice (see Fig. 4F). In addition, the serosa as well as the subserosal region (between the serosa and muscularis propria) of AdTGF-β1-transfected mice occasionally appeared thicker than normal, in association with an increased cellularity of this region at days 10-14 PI.

Over the next 2 wk (days 14-28 PI), more than 50% of the AdTGF-β1-transfected mice developed colonic obstructions along with a dilatation of the affected colon (Fig. 3). These deformations were invariably associated with a dramatic thickening of both the muscularis mucosa (Fig. 4G) and the muscularis externa (Fig. 4H) in affected regions of the colon. Although the thickening of the deeper layers in these intestinal segments may have involved the hypertrophy or hyperplasia of mesenchymal cells, Masson’s trichrome staining revealed significant fibrosis in these tissues as well. EVG staining was used to differentiate between different forms of ECM and identified collagen as the predominant form of ECM in the affected tissues (not shown). Interestingly, the presence of overt fibrosis was not uniform throughout the entire colon but instead was focal, affecting regions of between 0.5 and 2 cm in length in

transfection with the AdTGF-β1 virus as well as the control AdDL70-3 virus, or after the ethanol pretreatment alone. As such, we assessed the microscopic damage and mucosal inflammation in the distal colons of mice in the various treatments and mucosal injury was observed in the colons of the infection and inflammation were gone, except for the presence of enlarged colonic cryptopatches. Similarly, colonic tissues assessed at day 28 post-AdDL70-3 infection showed no signs of overt pathology. Although staining for ECM did occasionally identify small areas of collagen deposition, such areas were rare and limited exclusively to the colonic submucosa (Fig. 4E).

TGF-β1 Virus Induces Inflammation and Progressive Fibrosis

In contrast to the control virus, the administration of the AdTGF-β1 virus caused significant acute inflammation and mucosal ulcerations over the first 3 days PI (see Fig. 4C). The inflammatory infiltrate and ulcerations observed in the AdTGF-β1-transfected mice were significantly larger and more numerous than those seen with the control virus (Fig. 5). The resulting inflammatory infiltrate was occasionally tranmural but still primarily comprised of neutrophils as well as mononuclear inflammatory cells. Pathology also included a loss of goblet cells as well as epithelial sloughing. Although the acute injury was more severe than seen with the control virus, the tissue injury and inflammation resolved over the next week, such that by days 10-14 PI, the colonic mucosa had returned to almost normal condition. However, in some sections, a chronic inflammatory infiltrate was still seen in the submucosa or in the subserosa. Colonic tissues stained with Masson’s trichrome exhibited little evidence of fibrosis over this time course, although an occasional small area of ECM deposition could be seen associated with the submucosal inflammation in the AdTGF-β1-transfected mice (see Fig. 4F). In addition, the serosa as well as the subserosal region (between the serosa and muscularis propria) of AdTGF-β1-transfected mice occasionally appeared thicker than normal, in association with an increased cellularity of this region at days 10-14 PI.

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A mild mucosal inflammation was observed in the colons of ethanol (sham)-treated mice, with focal erosions and a low-grade and patchy inflammatory infiltrate limited to the mucosa observed at days 1 to 3 postenema (see Fig. 4A). Macroscopically, the colon appeared slightly hyperemic and stool pellets appeared more watery and were softer than normal. This response was transient, however, and all overt signs of the inflammation and pathology had resolved by day 5 as assessed by our scoring system (Fig. 5). Along with those mice killed over the first week, several mice were kept for 28 days postethanol treatment before death. Routine histology of these tissues proved to be identical to naive colonic tissue, whereas Masson’s trichrome staining for ECM revealed no identifiable fibrosis (Fig. 4D).

Control AdDL70-3 Virus Induces Inflammation but Little Fibrosis

Hyperemia and other macroscopic changes in the colon after exposure to the control AdDL70-3 vector were similar to those seen after ethanol pretreatment alone but were slightly aggravated and longer lasting (Fig. 5). Histologically, the acute inflammation seen with the ethanol pretreatment was moderately aggravated after infection with AdDL70-3, but still limited to the colonic mucosa. Ulceration was occasionally observed, but more frequently small erosions as well as epithelial sloughing were identified, usually proximal to neutrophil and mononuclear cell accumulations. Aside from the mucosal erosions and acute inflammatory infiltrate, occasional lymphocytic infiltrates were also seen in the colonic mucosa between days 3 and 10 PI (Fig. 4B). By days 10 to 14 PI, all obvious signs of the infection and inflammation were gone, except for the presence of enlarged colonic cryptopatches. Similarly, colonic tissues assessed at day 28 post-AdDL70-3 infection showed no signs of overt pathology. Although staining for ECM did occasionally identify small areas of collagen deposition, such areas were rare and limited exclusively to the colonic submucosa (Fig. 4E).
the distal colon. In some tissue sections, the observed fibrosis was circumferential, whereas in other areas, only part of the cross-sectioned tissues showed overt ECM deposition.

Closer examination of the affected tissues revealed that, despite the early mucosal inflammation seen in the first week after AdTGF-β1 transfection, the condition of the colonic mucosa in these affected mice between days 14 and 28 PI was similar to that seen in uninfected controls, with little evidence of mucosal inflammation or fibrosis (Fig. 5). This was striking considering the overt abnormalities seen in the deeper layers beneath the mucosa. The submucosa showed signs of chronic inflammation, including the accumulation of fibroblasts, granulocytes, and mononuclear inflammatory cells. Moreover, there was moderate collagen deposition observed in the submucosa, and significant collagen deposition was also observed in the external muscle layers (Fig. 4H). The overexpression of active TGF-β1 may have also affected the muscle cells themselves because under high magnification collagen deposition could be seen surrounding individual muscle bundles (see Fig. 4I). Along with the significant fibrosis, in most cases there was histopathological evidence of low-grade chronic inflammation in the muscle layers. Perhaps the most dramatic change seen in these fibrotic regions was the substantial thickening of the serosa and subserosa. Although normally minute in thickness, in affected regions the serosa and subserosa were substantially thickened because of fibroblast accumulation and collagen deposition (Fig. 4H). Despite these changes, the subserosa showed few signs of inflammation.

**AdTGF-β1 Induces ECM Deposition as Quantified by Image Analysis**

Despite the strong fibrotic response seen in the colon after AdTGF-β1 transfection, quantification of the resulting fibrosis was problematic. Fibrosis in the lung and liver is usually widely spread through these organs, allowing one to measure changes in total hydroxyproline levels in the whole organ (54, 67). In contrast, the fibrosis seen within the colon using this model was focal and varied in location. Because the site of fibrosis was not always macroscopically apparent, combining

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**Fig. 4. Histological consequences of adeno-viral infection.** Haematoxylin and eosin staining showing the histological appearance of colons from mice previously given ethanol pretreatment 3 days previous (A), given the control AdDL70-3 virus 3 days previous (B), or given the AdTGF-β1 virus, again, 3 days previous (C). In D–F, Masson’s trichrome staining shows the extent of fibrosis [extracellular matrix (ECM) stained green] seen at day 28 postethanol treatment alone (D), day 28 post-AdDL70-3 virus infection (E), and day 10 post-AdTGF-β1 virus infection (F). Note the submucosal inflammatory infiltrate in the tissue from day 10 AdTGF-β1 virus treated mouse (arrow). Between day 14 and day 28 PI, the AdTGF-β1 virus-infected mice developed a significant thickening of the muscularis mucosae (MM) (G) and of the circular muscle (CM) layers (H). In addition these 2 different mice (G and H) with colonic obstruction showed evidence of significant collagen deposition (green) in their muscle layers. A through H were taken at ×400 magnification. At higher magnification (×400) (I), one can see some fibroblast-like cells within the thickened muscle layers of a heavily fibrotic region of the bowel.
affected tissues with unaffected regions of colon would minimize any locally significant increases in hydroxyproline levels, as was found in the study by Lawrance et al. (30). Therefore we quantified the area of collagen within tissue sections using image analysis.

As described earlier, the colon of each mouse was divided into four 1-cm sections, and each was stained for ECM with Masson’s trichrome and analyzed for the percentage area of ECM in that sections. ECM levels in control, untreated colonic tissues in all four colon sections ranged from 4.5 to 5.2% (see Fig. 6). Ethanol pretreatment led to no significant increase in collagen levels at days 14 or 28 posttreatment in any of the colon sections, with ECM in the sections ranging from 4.8 to 5.5% (not shown). Similarly, AdDL70-3 infection led to only slightly higher levels of ECM in the first and second colon sections at both day 14 PI (5.5%) and day 28 PI (6.5%), and these increases did not reach statistical significance. In contrast, the AdTGF-β1 treatment led to a significant increase in ECM levels in section 2 at day 14 PI (9.0%) and a significant increase in sections 1, 2, and 3 of the colon of mice killed between days 14 and 28 PI because of the colonic obstruction. These findings confirmed our histological assessment, that AdTGF-β1 infection caused a significant yet focal fibrotic response in the transplanted colon.

AdTGF-β1 Induces Cells With a Myofibroblast Phenotype

To further characterize the putative role of mesenchymal cells in the fibrosis exhibited by AdTGF-β1-transfected mice, tissues were examined for the presence of myofibroblasts. Myofibroblasts are thought to be important contributors to fibrogenesis and wound healing in a variety of tissues including the intestine and are characterized by the expression of α-SMA and vimentin, but not the smooth muscle marker desmin (2, 16, 46). Our approach was to immunostain colonic sections for α-SMA as well as for the smooth muscle marker desmin, identifying myofibroblasts as those cells strongly positive for α-SMA but expressing little if any desmin (45, 46).

As expected, we observed desmin and α-SMA immunoreactivity around blood vessels as well as in the muscularis propria and muscularis externa in both control (not shown) and fibrotic tissues (Fig. 7, A and B). The pattern of immunoreactivity matched that expected for smooth muscle cells in these tissues. Although the muscle layers may have contained myofibroblasts, the numerous smooth muscle cells obscured their presence. In addition, a small number of α-SMA-positive but desmin-negative cells were seen in the colonic mucosa (not shown). These cells were probably fibroblasts and were seen in both control tissues and tissues taken from AdTGF-β1-transfected mice. Interestingly, under higher magnification, we observed the presence of numerous α-SMA-positive cells in the heavily fibrotic serosal and subserosal areas of colons taken from AdTGF-β1-transfected mice (Fig. 7D), but not in control virus-infected tissues (not shown). Desmin staining of serial sections revealed only a few weakly positive cells in these same subserosal regions (Fig. 7C), putatively identifying the majority of these subserosal cells as myofibroblasts and not smooth muscle cells. Staining with control antibodies showed no immunostaining (not shown). These findings suggest that myofibroblast induction in the subserosa may contribute to the ECM deposition seen in this region in AdTGF-β1-transfected mice.

Colitis Facilitates Gene Transfer and the Development of Colonic Fibrosis

Because stricture formation in CD is usually found at sites of chronic inflammation, we examined whether established colitis would facilitate TGF-β1 gene transfer and the resulting fibrosis. We as well as others (64) found that mild TNBS colitis aided in the transfection of cells in the colon, as assessed by infecting mice with the AdLacZ virus and then staining for...
Fig. 7. α-Smooth muscle actin (SMA) and desmin staining putatively identifies the presence of myofibroblasts. Immunostaining for desmin and α-SMA was performed on colonic tissue sections taken from a mouse transfected with AdTGF-β1 18 days previous and euthanized because of colonic obstruction. A: low-magnification (×100) micrograph of desmin staining (in brown) identifies both the circular (CM) and longitudinal muscle (LM) layers. B: α-SMA staining (in red) also shows both the circular and longitudinal muscle layers (×100). C: enlargement of the desmin staining shows that the region below the serosa (S) is extremely thickened and fibrotic. Aside from ECM, this region contains a number of fibroblast-like cells, few of which show any desmin staining. D: in contrast, in this enlarged panel, many of these fibroblast-like cells in the subserosal region are strongly positive for α-SMA (arrows). On the basis of their α-SMA reactivity and their low or absent staining for desmin, we putatively identified these cells as myofibroblasts.

β-gal gene transfer (see Fig. 8A). Although this transfection approach resulted in very effective transfection of the colon, the resulting β-gal expression was still localized only to epithelial cells.

To test the effect of preexisting colitis on fibrosis development, day 1 postcolitic mice were given an intrarectal enema of 1 × 10⁹ pfu of Ad DL70-3 or AdTGF-β1 virus. The mice receiving the control virus went on to develop significant colitis that resulted in some mortality (15%), but with no signs of colonic obstruction or significant fibrosis (Fig. 8B). In contrast, mice that received the AdTGF-β1 virus developed a very severe transmural pancolitis. As a result, the resulting inflammation necessitated the euthanization of 60% of the infected mice over the next 5 days PI. Interestingly, all those mice that survived past day 5 (4 of 10) went on to develop a severe and accelerated transmural fibrosis (see Fig. 8C), occurring as early as day 10 PI. The resulting fibrosis very rapidly led to colonic obstruction that necessitated the euthanization of these mice by day 14. Although the rapid and aggressive fibrosis exhibited by the mice undergoing this protocol suggests that colitis both facilitates adenoviral gene transfer to the colonic epithelium and accelerates the development of colonic fibrosis, the resulting high mortality prevented further study of the mechanisms involved.

DISCUSSION

Although the pathogenesis of CD is unclear, stricture formation in particular is poorly understood, despite being one of the major causes of hospitalization for CD patients (2, 18). Whereas excessive ECM deposition as well as the proliferation of mesenchymal cells are thought to be contributory factors to stricture development, studying the underlying mechanisms of intestinal fibrosis has been hampered by the lack of relevant animal models (18, 46). As observed in a recent review by Pucilowska et al. (46), there is a growing consensus that mice are more resistant to fibrosis than other species. In fact, despite the number of murine models of IBD currently available, only the repeated induction of colitis (30) has been described as causing significant intestinal fibrosis, and no mouse models have thus far exhibited overt bowel stricturing or obstruction. However, the number of research tools available for use with mice makes the mouse the preeminent animal species to model human disease and test potential therapies, and thus the development of additional mouse models of intestinal fibrosis is a priority for IBD researchers (46). In this study, we describe a novel gene transfer based model of intestinal fibrosis in mice, involving the transfection of the colonic mucosa by adenoviral vectors encoding spontaneously active TGF-β1.

Achieving local overexpression of proteins of interest within the GI tract has proven a difficult task, in part because of the natural barriers that protect the gut, as well as the relative inaccessibility of intestinal tissues to manipulation. These difficulties have thus limited the assessment of what roles specific mediators, such as TGF-β1, play in pathological events in the intestine. Although we examined the utility of injecting recombinant TGF-β1 into the bowel wall through surgeries, the short half-life of these proteins and the difficulties of repeated injections and delivery to the intestine made this approach impracticable. Other researchers have used a systemically delivered plasmid-based gene transfer approach to study the immunosuppressive effects of TGF-β1 on colitis development (26). Unfortunately this approach was not specific to the GI tract and appeared to induce a generalized immunosuppression rather than acting locally at the site of inflammation (26). Additional studies have utilized transgenic mice that overexpress TGF-β1 in a tissue-specific or systemic fashion. Although these mice exhibited fibrosis in their liver and kidneys (7), no overt changes in the GI tract were observed, suggesting that systemic overexpression of TGF-β1 is insufficient to cause
Gene transfer has been used in a variety of tissues (15, 27, 31, 54, 55, 66), the transfection of the intestine has proven extremely difficult. Few studies have been able to efficiently transfect intestinal tissues (21, 47, 64, 65), presumably because of the obstructing mucus and epithelial barriers. To overcome these obstacles, we used dilute ethanol pretreatment of the colon to transiently disrupt these barriers as a means to improve transfection efficiency. Using β-gal- and luciferase-encoding adenoviral vectors, we initially demonstrated that intrarectal administration of adenoviral vectors led to the infection and subsequent expression of these transgenes by superficial colonic epithelial cells, predominantly in the distal colon. Moreover, overexpression of these transgenic proteins was compartmentalized to the lower GI tract, with no apparent spill over into the systemic circulation (not shown). This localized expression likely reflects the route of administration and the well-characterized tropism of adenoviruses for epithelial cells (40, 41).

Our studies demonstrated that high levels of transgene expression in the colon lasted only a few days, with peak levels of active TGF-β1 protein found at day 2 PI. Compared with other tissues such as the lung, the rapid turnover of the gut epithelium (25) appeared to be the limiting factor controlling the duration of adenoviral-based transgene expression in this model. Although active TGF-β1 levels rapidly declined over the next 6 days, they still remained significantly above baseline levels at days 3 and 8 PI. This low-level but prolonged expression of active TGF-β1 may reflect the infrequent transfection of the longer-lived stem cells found at the base of the colonic crypts. The rapid turnover of transfected colonocytes, as well as the relative resistance of the colon to transfection, necessitated the high doses of adenoviruses used in this study. Although mice receiving 10⁸ pfu of AdTGF-β1 did occasionally develop intestinal fibrosis, the induction of fibrosis at this dose was less frequent and more variable (not shown). We saw no evidence of significant fibrosis induction in mice transfected with lower doses of the AdTGF-β1 virus. Another factor that influenced the development of the fibrosis in this model was the genetic background of the mice used. This study focused on NIH Swiss mice because they were relatively easy to transfect by using adenoviral vectors and because they developed significant intestinal fibrosis. Other mouse strains were tested, including the inbred BALB/c mouse strain. Whereas BALB/c mice were as readily transfectable by enema as the NIH Swiss, and did develop intestinal fibrosis (not shown), their responses were less overt than those observed in the NIH Swiss mice, in keeping with previous studies classifying BALB/c mice as being “fibrosis resistant” (27).

To understand how transfection of intestinal epithelial cells with AdTGF-β1 could contribute to colonic fibrosis, it is important to note that intestinal epithelial monolayers infected in vitro with AdTGF-β1 release transgenic TGF-β1 protein predominantly on their basolateral side. Thus epithelial cells infected in our model should release transgenic TGF-β1 protein into the colonic mucosa. This finding helps to explain the dramatic pathology seen in the AdTGF-β1-transfected mice. However, it should be noted that in vivo infection with the active AdTGF-β1 not only resulted in the production of spontaneously active protein, but increased levels of latent protein in the mouse colon as well. Previous studies have demonstrated that the protein produced by the AdTGF-β1 virus is largely spontaneously active. Considering the distance between the

Fig. 8. Colitis facilitates gene transfer and fibrosis generation. To determine what effect preexisting colonic inflammation had on the development of intestinal fibrosis, trinitrobenzene sulfonic acid (TNBS) colitis was induced in NIH Swiss mice, and 1 day later they were subsequently infected with AdLacZ or AdDL70-3 control viruses or the AdTGF-β1 virus. A: colonic mucosa from a mouse given TNBS colitis and then infected with 10⁹ plaque-forming units of AdLacZ virus 1 day later. The tissue was subsequently collected 1 day later and stained for β-gal activity. Note the numerous blue-stained epithelial cells (arrows) on the mucosal surface (×40). The transfection and β-gal staining is substantially more widespread than occurred in noncolitic mice [see Fig. 1, B and C (×40 as well)]. B: cross section from a colitic mouse given the control virus (AdDL70-3) 14 days previous. Staining with Masson’s trichrome reveals mild fibrosis (green), and a colon showing a relatively normal appearance, although some inflammatory infiltrate can still be observed in the mucosa. In contrast, C shows a colonic section from a colitic mouse given the AdTGF-β1 virus that was euthanized at day 10 after colitis due to colonic obstruction. Staining with Masson’s trichrome reveals impressive fibrosis (green) reaching from the submucosa through to the serosa. The massive ECM deposition seen after this procedure was matched by the observed presence of many fibroblasts and inflammatory cells within the muscularis mucosa and muscularis externa.
transfected epithelium and the fibrosis seen in the deeper layers, the latent protein is likely derived by a process of autocrine stimulation of the endogenous TGF-β1 gene by the active transgenic protein. As previously stated, the GI tract is the site of substantial endogenous TGF-β1 production (3, 34), therefore resident immune and stromal cells or recruited inflammatory cells may well be the source of this latent TGF-β1; however, the high degree of homology between the viral-encoded porcine TGF-β1 and mouse TGF-β1 precluded determination of the origin of the TGF-β1 protein. These results suggest that both viral-based and endogenous TGF-β1 may contribute to the fibrosis seen in this model. In addition, TGF-β1 is unlikely to be the only fibrogenic cytokine active in this model, as TGF-β1 can stimulate macrophages and other cells to produce a variety of fibrogenic mediators (49).

The emergence of αSMA-expressing fibroblast-like cells found in areas of heavy fibrosis and putatively identified as myofibroblasts suggests that overexpression of TGF-β1 in the gut contributed directly or indirectly to the development of this cell type (34, 38). Myofibroblasts have been demonstrated in a variety of fibrotic and healing tissues, although what role they play in intestinal fibrosis is unclear. The origin of the myofibroblast is uncertain, although fibroblasts are clearly a potential source. Considering the widespread interest in this cell type (39), the identification of numerous myofibroblasts in the colonic subserosa of AdTGF-β1-infected mice may offer the opportunity to better characterize the development and function of these cells. Although myofibroblasts may be important in the development of intestinal fibrosis, the presence of collagen surrounding muscle bundles in the muscularis externa may indicate that smooth muscle cells also contributed to the collagen deposition seen in this model. Interestingly, although previous studies transfecting the AdTGF-β1 virus into the lung found widespread deposition of the matrix proteins collagen, elastin, and fibronectin (54), EVG staining of fibrotic colonic tissues found that collagen was the predominant matrix protein deposited in this model. The prevalence of collagen in this study may reflect factors intrinsic to the colon, or it may indicate a difference in the relative roles of muscle cells compared with myofibroblasts in the deposition of ECM in intestinal fibrosis.

In the study by Lawrance et al. (30), the colonic fibrosis that occurred after the repeated induction of TNBS colitis was inhibited by treatment with antisense oligonucleotides against NF-κB, indicating that inflammation was required to induce fibrosis in their model. Similarly, CD strictures usually occur at sites of chronic inflammation, and in our study, AdTGF-β1 delivery as well as the ethanol pretreatment in our model also induced an inflammatory response. In fact, although the mucosa rapidly healed from these events and returned to a histologically normal appearance, there was evidence of prolonged mononuclear cell infiltrate in the submucosa and muscle layers of the AdTGF-β1-infected mice. Thus the host inflammatory response that occurs in this study may be a necessary prerequisite for the development of intestinal fibrosis. However, it should be noted that only a subset of CD patients develop intestinal strictures, and, in fact, anti-inflammatory treatments of CD patients are often not effective in preventing strictures, suggesting that a more complex process is involved. Considering the recent discovery that defects in NOD2/CARD15-dependent innate immunity predispose CD patients to developing intestinal fibrosis (1), enteric microflora leaking into the intestinal mucosa may prove to be an important contributor to the process of fibrosis and stricture formation in CD. Taken together with our demonstration of TGF-β1-induced strictures, future studies investigating a link between enteric microflora, innate immunity, and the activation of TGF-β1 may prove useful in defining the underlying mechanisms that lead to bowel fibrosis.

In summary, we describe a mouse model that mimics many of the features of CD-induced intestinal fibrosis including the development of intestinal obstructions. This study not only provides a model that can aid in elucidating the mechanisms that lead to intestinal fibrosis, but it also validates the use of intestinal gene transfer for modeling pathological events in the GI tract. Our results also identified both smooth muscle cells and myofibroblasts as potential contributors to the fibrosis seen in this model. On the basis of the widespread prevalence of stricture formation in CD (2), determining the respective roles of these cell types as well as the mediators involved in GI fibrogenesis is extremely important. Because a complex pathway of cytokines and growth factors involved in pulmonary fibrosis has already been elucidated (16, 28), testing the roles of these mediators in our model will help to determine to what extent the pathways of fibrosis are conserved between the lung and the bowel. Because current attempts at prevention and treatment of bowel strictures so often prove ineffective, this model may also prove useful in testing and developing new therapies that target key inflammatory mediators of relevance to CD, such as TNF-α (44, 53), or those therapies that specifically target fibrosis, such as the use of decorin, a proteoglycan inhibitor of TGF-β1 that can suppress lung fibrosis (68). Other more selective anti-fibrotic approaches, such as the targeting of myofibroblasts, may provide a distinct advantage over the side effects and risks associated with nonspecifically suppressing the inflammation seen in IBD.

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