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BASIC–ALIMENTARY TRACT

A Murine Model of Chronic Inflammation–Induced Intestinal Fibrosis Down-Regulated by Antisense NF-κB

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See editorial on page 1889.

Background & Aims: To elucidate extracellular matrix (ECM) changes underlying intestinal fibrosis, a frequent complication of inflammatory bowel disease, we developed a murine model of chronic colitis associated with intestinal fibrosis. Methods: Chronic inflammation was established by weekly intrarectal administration of trinitrobenzene sulfonic acid (TNBS). In 2 variations of the model an antisense oligonucleotide for nuclear factor κ B (NF-κB) p65 was given prophylactically or therapeutically to block chronic inflammation–associated fibrosis. Colonic inflammation and fibrosis were determined by histology. Total collagen level was estimated by hydroxyproline quantification. Colonic expression of collagens (Col1a2, Col3a2), ECM remodeling genes (matrix metalloproteinase [MMP]-1, -3, and tissue inhibitor of matrix metalloproteinase [TIMP]-1), and inflammation-modulating cytokines (tumor necrosis factor α [TNF-α], interferon γ [IFN-γ], transforming growth factor β1 [TGF-β1], and insulin-like growth factor 1 [IGF-1]) were assessed by semiquantitative reverse-transcription polymerase chain reaction. Control and TNBS-treated colonic mesenchymal cells were characterized by morphology, phenotype, and functional response to TNF-α and IFN-γ. Results: Colons of TNBS-treated mice contained acute and chronic inflammatory infiltrates, increased collagen, fibrogenic tissue architecture, and increased expression of TNF-α, TGF-β1, IGF-1, Col1a2, MMP-1, and TIMP-1. Colonic mesenchymal cells from TNBS-treated mice were also morphologically distinct from those of the control mice, with increased TIMP-1 expression in response to IFN-γ treatment. Fibrosis persisted for 2–4 weeks after cessation of the TNBS treatment. In mice given NF-κB antisense prophylactically, 67% were fibrosis-free, whereas of those treated after establishing chronic inflammation, 43% were free of fibrosis. Conclusions: Extended TNBS treatment of mice yielded chronic intestinal inflammation-associated fibrosis with extensive fibrogenic ECM changes that could be counteracted by specific blockade of NF-κB.

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are life-long, relapsing conditions affecting primarily the gastrointestinal tract of young adults. Unlike UC, intestinal inflammation in CD is transmural, often associated with luminal narrowing and stricture formation caused by excessive extracellular matrix (ECM) deposition.[1] Current therapies can relieve inflammation in CD, but do not alter the natural history of the disease or its progression to intestinal fibrosis and obstruction that often results in bowel resection.[2,3] Unfortunately, surgery does not prevent the recurrence of bowel inflammation or the ECM changes that cause fibrosis and stenosis.[4]

Animal models have greatly advanced our understanding of the mechanisms of gut inflammation.[5–15] Such models, however, have focused almost exclusively on the immune-mediated mucosal inflammation with little attention to chronic disease and intestinal fibrosis. Models that reproduce transmural inflammation and the associated chronic changes in ECM as seen in CD are therefore required.

The hapten 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), administered as an enema, originally was used in rats to induce chronic intestinal inflammation.[16] In the mouse, when mucosal epithelial integrity is disrupted by a concomitant administration of ethanol, the contact-sensitizing hapten crosses into the colonic mu-

Abbreviations used in this paper: α-SMA, α smooth muscle actin; ECM, extracellular matrix; IFN, interferon; IGF, insulin-like growth factor; MMP, matrix metalloproteinase; NF-κB, nuclear factor κ B; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinase; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

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cosa where it induces a Th1-like delayed hypersensitivity response.[14,17] This model, maintained for 2–3 weeks, is used widely to investigate immune events underlying the acute inflammatory responses in the gut, and the resulting colitis usually is maximized by the administration of sublethal doses of TNBS.[17,18]

We developed and characterized a modified TNBS-induced murine model of colitis that leads to long-term, instead of the short-term, inflammatory changes that occur in the models described earlier. Our model features chronic intestinal inflammation combined with abundant collagen deposition and fibrogenic ECM changes that are stable for 4 weeks after TNBS treatment. The analysis of selected genes at both the transcript and protein levels is consistent with enhanced deposition of ECM and tissue remodeling. We further show that the antisense oligonucleotide for p65 nuclear factor κB (NF-κB) subunit used before in the short-term models to abrogate inflammation only,[19] given prophylactically in this study, prevented extensive fibrotic connective tissue deposition and blocked moderate to severe inflammation in our long-term chronic colitis model. Additionally, we show that the p65 NF-κB antisense oligonucleotide given therapeutically to animals with established chronic colitis was able to resolve severe inflammation and fibrosis, although a small percentage of the mice still displayed moderate fibrosis.

Materials and Methods

Animals

Twelve-week-old female mice of the CD-1 strain, an outbred genetic background, and BALB/c inbred mice were used in this study (Charles River Laboratories Inc., Wilmington, MA). The outbred CD-1 strain was chosen because of their shown susceptibility to ECM changes.[20] Mice were housed and fed standard mouse chow and tap water ad libitum throughout the study following protocols approved by the Institutional Animal Care and Use Committee at Case Western Reserve University School of Medicine and the Johns Hopkins University School of Medicine.

Induction of Colonic Inflammation

BALB/c (model 1, Figure 1) and outbred CD-1 (models 1–3, Figure 1), anesthetized by an intramuscular injection of ketamine HCl 50 mg/mL, xilazine HCl 20 mg/mL, and acepromazine 10 mg/mL in sterile water (0.04 mL) were randomized into control and treatment groups. The schedule of TNBS (Sigma Chemical Co., St. Louis, MO) in 30% to 45% ethanol enemas administered as described earlier is outlined for the 3 models used here in Figure 1. Control mice were given 0.1 mL water or saline or 30% ethanol alone. All mice were examined 4 times a week for signs of colitis including weight loss, diarrhea, rectal bleeding, and prolapse as described

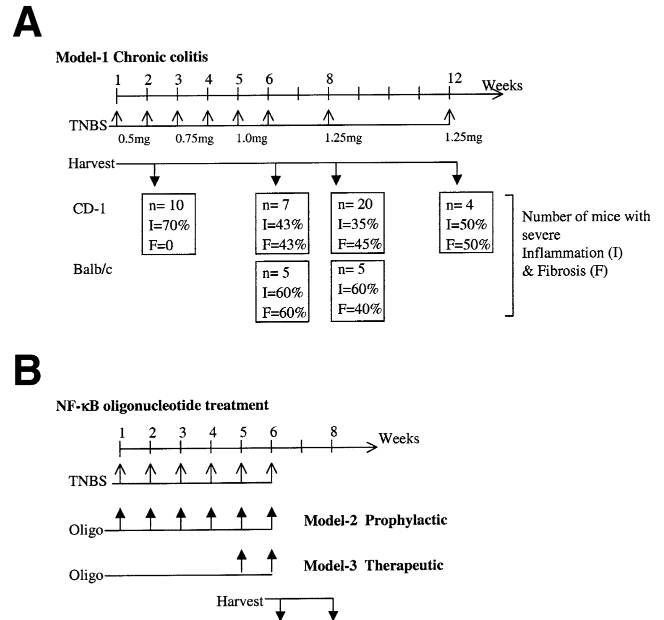

Figure 1. Overview of (*A*) model 1 and (*B*) models 2 and 3. (*A*) In model 1, CD-1 and BALB/c mice were given weekly TNBS treatments as indicated in the time scale. Mice (number of animals as shown) were harvested at weeks 2, 6, 8, and 12, 3 days after the TNBS enema for macroscopic examination of the colons (an example shown in Figure 2) and histology. Mice with moderate to severe inflammation (I) and fibrosis (F) were scored in histology sections of the colons (representative colon sections are shown in Figure 3). (*B*) In models 2 and 3, CD-1 mice were given TNBS as in Figure 1*A* for weeks 1–6 only. Mice were harvested 3 days after week 6 and at week 8. In the prophylactic model 2, an antisense oligonucleotide to the p65 NF-κB subunit (150 μg in 0.1 mL water) was given as an enema 1 day before the TNBS enema, every week for 6 weeks. A group received a control oligonucleotide in which the sequence of the antisense oligonucleotide was scrambled, another control group received saline enemas. In the therapeutic model 3, the same antisense oligonucleotide and the control oligonucleotide enemas were given on weeks 5 and 6, 1 day before the TNBS enema. Quantitative estimates of mice with mild, moderate, and severe inflammation and fibrosis for models 2 and 3 are shown in Figure 8.

earlier,[21] as well as signs of systemic inflammation such as piloerection, lethargy, and periorbital exudates.[22]

For prophylactic (model 2, Figure 1*B*) and therapeutic treatment (model 3, Figure 1*B*) of chronic inflammation and fibrosis, the antisense phosphorothioate oligonucleotide to the murine p65 NF-κB subunit was used as described earlier.[19] The CD-1 mouse strain was given weekly enemas as follows: (1) saline, (2) TNBS, (3) TNBS plus p65 NF-κB antisense 5′GAAACAGATCGTCCATGGT3′, (4) TNBS plus control scrambled sequence oligonucleotide 5′GTACTACTCTGAG-CAAGGA3′. In model 2, TNBS enemas were given for 6 weeks, as described for model 1 and the oligonucleotide enemas (150 μg in 0.1 mL water) were administered 1 day before each TNBS enema. In the therapeutic model 3, all conditions were as in model 2, except that the oligonucleotide treatments were given 1 day before the fifth and sixth TNBS enema. The mice were weighed every third day and killed for histology 2 weeks after the last TNBS and oligonucleotide treatment.

Tissue Processing

The colons were removed intact from the anus to the ileocecal junction, the length was measured, opened longitudinally, cleaned, and weighed. At macroscopic examination, the distal 5 cm of the TNBS-treated colons were indurated, edematous, thickened, with evidence of mucosal hemorrhage. Sections were taken from these involved regions for the following experiments: (1) formalin fixation and histologic examination; (2) fibroblast isolation; (3) hydroxyproline quantification; and (4) total RNA isolation.

Serial paraffin sections of the colon were stained with H&E to assess the degree of inflammation and with trichrome to detect connective tissue. A gastrointestinal pathologist with special expertise in intestinal inflammation (J.W.) examined each slide (model 1) in a blinded fashion. Models 2–3 were scored at the Johns Hopkins University (by S.C. and F.W.) in a blinded fashion. By using a modification of an established system for grading experimental colitis, the degree of inflammation was scored as absent, mild, moderate, or severe based on the density and extent of both the acute and chronic inflammatory infiltrate, loss of goblet cells, and bowel wall thickening.[21] An inflammatory infiltrate of low cellularity confined to the mucosa was scored as mild inflammation, and transmural inflammation with extension into the pericolonic adipose tissue with high cellularity was scored as severe. Intermediate changes were scored as moderate inflammation. Fibrosis was scored as mild, moderate, or severe, depending on the density and extent of trichrome-positive connective tissue staining and disruption of tissue architecture, compared with the water control mice.

Intestinal Mesenchymal Cell Isolation and Stimulation

Cells were isolated from control and TNBS-treated colonic tissue according to a previously reported technique.[23] Resulting cell monolayers were cultured in Dulbecco's minimal essential medium with 2% heat-inactivated fetal calf serum for 24 hours before stimulation, and then exposed to either recombinant human tumor necrosis factor α (TNF-α; 50 U/mL; R&D Systems, St. Paul, MN) or recombinant murine interferon γ (IFN-γ; 50 U/mL; PharMingen, San Diego, CA). The time of optimal induction, as judged by maximal messenger RNA (mRNA) expression levels, was determined by a time-response curve, ranging from 0–24 hours after stimulation with TNF-α or IFN-γ for both control and TNBS-treated mesenchymal cells. Optimal induction of mRNA expression by TNF-α and IFN-γ expression was achieved after 12 and 6 hours' incubation, respectively.

Immunohistology

Formalin-fixed, paraffin-embedded sections were pretreated by deparaffin, antigen unmasking, and blocking with 1% H_2O_2 for 10 minutes and 1.5% goat normal serum for 60 minutes. The specimens were incubated with primary antibody for 60 minutes. The primary antibodies used included monoclonal mouse anti-human α-smooth muscle actin (α-SMA) (1:800), anti-human desmin (1:100) (both from DAKO Co., Carpinteria, CA), and anti-human vimentin (1:4000) (Zymed Laboratories Inc., South San Francisco, CA), all of which recognized the murine antigen as well. After washing, the sections were incubated with biotin-conjugated goat anti-mouse immunoglobulin G secondary antibody (1:200) for 60 minutes, then with avidin biotin enzyme reagent for 30 minutes, and then with DAB for 1–2 minutes and counterstained with Gill's (#2) hematoxylin (Sigma Chemical Co.) for 10 seconds. All steps were performed at room temperature. Between each step the sections were washed in phosphate-buffered saline (pH 7.4) 3 times, 5 minutes per wash.

mRNA Assessment by Semiquantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from fresh full-thickness colonic tissue as well as colonic mesenchymal cell monolayers by using the guanidinium thiocyanate method as described earlier.[24] One-microgram aliquots of total RNA were reverse transcribed to assay for Col1a2 and Col3a2, matrix metalloproteinase 1 (MMP-1), MMP-3, TIMP-1, transforming growth factor β1 (TGF-β1), insulin-like growth factor 1 (IGF-1), TNF-α, and IFN-γ expression by reverse-transcription polymerase chain reaction in colonic tissue, and MMP-1, MMP-3, TIMP-1, TGF-β1, and IGF-1 expression in cultured mesenchymal cells. The thermal cycle involved a 5 minute hot start at 94°C, followed by a sequence of 94°C for 30 seconds, annealing for 60 seconds, and extension at 72°C for 90 seconds. Primers, specific cycle numbers, and annealing temperatures used for each gene are shown in Table 1. The resulting products were analyzed by agarose gel electrophoresis and quantified by densitometry on a Bio-Rad Gel Doc 1000 (Bio-Rad, Hercules, CA). Results were standardized to β-actin.

Collagen Estimation by Hydroxyproline Quantification

Whole colonic tissue was hydrolyzed in 6 N HCl/0.5% phenol for 24 hours at 110°C in vacuo. After drying, appropriate aliquots were loaded into a Beckman 6300 amino acid analyzer (GMI, Inc., Albertville, MN) with the ordinary hydrolysate method altered by reducing the initial column temperature by 2°C to 48°C to enhance the separation of aspartate from hydroxyproline.[25] Hydroxyproline was expressed as a ratio to total amino acids measured in the tissue (Protein Sequencing Facility, University of Pittsburgh, Pittsburgh, PA).

Statistical Analysis

Results comparing control and TNBS-treated groups were analyzed using the analysis of variance Bonferroni-Dunn test for data not normally distributed, or the Student t test when results were consistent with normally distributed values.

Table 1. RT-PCR Primers for Selected Genes

Gene	Primers	Annealing	Product	Cycle no.
Colla2	5′-TCT CCA CTC TTC TAG TTC CT 3′-TTG GGT CAT TTC CAC ATG C	55°C	250 bp	29
Coll3a2	5′-CCC AGA ACA TTA CAT ACC A 3′-ATT AAA ACA AGA TGA ACA C	51°C	376 bp	27
MMP-1	5′-TGA ACA TCC ATC CCG TGA CC 3′-ATC TCC ACC CCA CCC CCA CC	55°C	484 bp	27
MMP-3	5′-CGA TGC TGC CAT TTC TAA TAA A 3′-TAT GTG GGT CAC TTT TTT GGC A	55°C	241 bp	27
TIMP-1	5′-TCG GGG CTC CTA GAG ACA CA 3′-ACC TGA TCC GTC CAC AAA CA	56°C	555 bp	28
IGF-1	5′-CTG CAA TAA AGA TAC ACA TCA TGTCG 3′-TCA AAT GTA CTT CCT TTC CTT CTC CT	42°C	397 bp	38
TGF-β1	5′-GCC CTG GAC ACC AAC TAT TGC 3′-AGC TGC GAC TTG CAG GAG CGC	55°C	337 bp	36
TNF-α	5′-GTT CTA TGG CCC AGA CCC TCA CA 3′-TAC CAG GGT TTG AGC TCA GC	55°C	366 bp	29
IFN-γ	5′-CAT TGA AAG CCT AGA AAG TCT G 3′-CTC ATG AAT GCA TCC TTT TTC G	55°C	267 bp	29
β-Actin	5′-GTG AAA AGA TGA CCC AGA TCA T 3′-GCT TCT CTT TGA TGT CAC GCA CGA T	57°C	295 bp	22

Possible correlations among inflammatory cytokines (IFN-γ and TNF-α), profibrogenic growth factors (TGF-β1 and IGF-1), MMPs, and TIMP expression were determined by the Pearson correlation. For all analyses, $P < 0.05$ was considered to be statistically significant.

Results

An Overview of the Models

The 3 models of TNBS treatment developed in this study are outlined in Figure 1. The rationale for testing 3 variations of TNBS treatment and intervention with NF-κB antisense is as follows. In the first model, model 1, we gave TNBS challenges for 6 weeks to establish chronic inflammation and then again at weeks 8 and 12 to ensure sustained inflammation, harvesting animals for evaluating inflammation and fibrosis 3 days after the TNBS challenges. Approximately 70% of the CD-1 mice showed severe inflammation 3 days after the second TNBS enema, indicating that this strain is amenable to TNBS-induced intestinal inflammation (Figure 1*A*). This model also was tested in a limited way in the BALB/c strain in which 60% of the mice showed severe inflammation and fibrosis by 6 weeks. As we gained more experience with this model, we felt that TNBS enemas at weeks 8 and 12 were unnecessary. Rather, it was more important to determine if fibrosis persisted after 6 weeks of TNBS treatment without further TNBS. Therefore, with the BALB/c strain in model 1 and the CD-1 mice in models 2 and 3, TNBS enemas were stopped after the first 6 weeks. At week 8, the BALB/c mice in model 1 showed persistence of severe inflammation in 60% of the mice and although fibrosis had resolved in a subset, 40% still showed severe fibrosis. In models 2 and 3 the 6 TNBS treatments were similar to model 1. In model 2, the NF-κB antisense oligonucleotide was given as a prophylactic with every TNBS challenge, whereas in model 3 the antisense treatment was given after establishing chronic inflammation with 4 weekly TNBS treatments. The results of models 2 and 3 and representative histology from each model are discussed further later.

Macroscopic Changes

The mice lost weight 2–4 days after the initial TNBS enema, which they regained or exceeded before the next enema. Systemic manifestations included piloerection in 43%, lethargy in 50%, and diarrhea in 20% of mice after the second TNBS enema, which usually occurred on days 1–3 after an enema. At no stage were frank rectal bleeding, rectal prolapse, or periorbital exudates observed. Both strains showed 25% mortality between 3 and 6 weeks of TNBS treatment. Figure 2 shows representative examples of colons removed from water- and TNBS-treated (CD-1 strain) animals at week 8 (model 1). The TNBS-treated colons were thickened and edematous over the distal 4–6 cm. No inflammatory changes were observed in the water/saline controls, whereas a minority (17%) of ethanol-alone control colons displayed mild to moderate colonic thickening and edema (data not shown).

Increased colonic to total body weight is used as an indication of colonic inflammation.[18] In model 1 in the TNBS-treated group, from weeks 2–12, showed in-

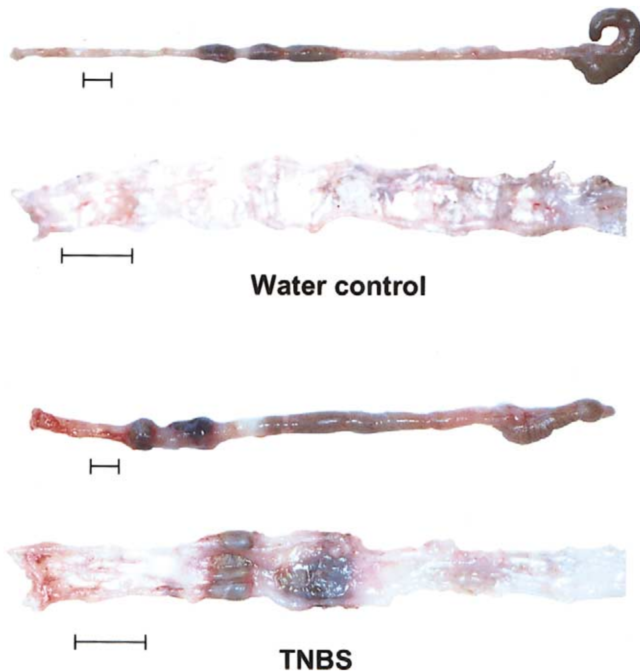

Figure 2. Macroscopic appearance of colons from water- and TNBS-treated mice killed at 8 weeks (model 1). TNBS-treated colons display thickened edematous walls and mucosal ulcerations, these changes being most remarkable over the distal 4–6 cm. *Bars* represent 1 cm.

creased colonic:body weight compared with the control groups, suggesting persistent colonic inflammation (data not shown).

Changes in Colonic Tissue Architecture and Total Collagen

Figure 3 shows cross-sections of the colon at distances 1–7 cm from the anus in CD-1 mice treated with TNBS at week 8 (model 1). Maximal, full-thickness inflammation was evident between the 4- to 6-cm segment: the mucosa and muscularis propria were focally destroyed and hyperplastic lymphoid follicles were prominent. The lumen was noticeably dilated beyond 7 cm, indicating a functional obstruction of the colon in the distal areas of inflammation and fibrosis. There were trichrome-positive diffused collagen deposits in the mucosa and submucosa in the 1- to 6-cm segment. Most importantly, in mice given weekly TNBS treatments for 6 weeks, without additional TNBS treatment, trichrome-stained collagen deposits were still evident 4 weeks after the sixth TNBS enema (Figure 4*A* and *B*). Total hydroxyproline was increased significantly in the week 8 TNBS-treated colons compared with the water controls (mean ± SEM, 0.0069 ± 0.00048 vs. 0.0055 ± 0.00034; $P = 0.021$; n = 8), supporting the histologic observations that the level of collagen was increased in the TNBS-treated colons.

To determine if this model of chronic colitis and fibrosis could be developed in other mouse strains as well, we administered TNBS weekly to BALB/c mice (n = 20) for 6 weeks, and examined these macroscopically and by histology after 2 additional weeks without further TNBS treatment (model 1, Figure 1). As seen in the CD-1 outbred strain, 2 weeks after the last TNBS enema, the BALB/c continued to show extensive trichrome-stained collagen deposits in the submucosa

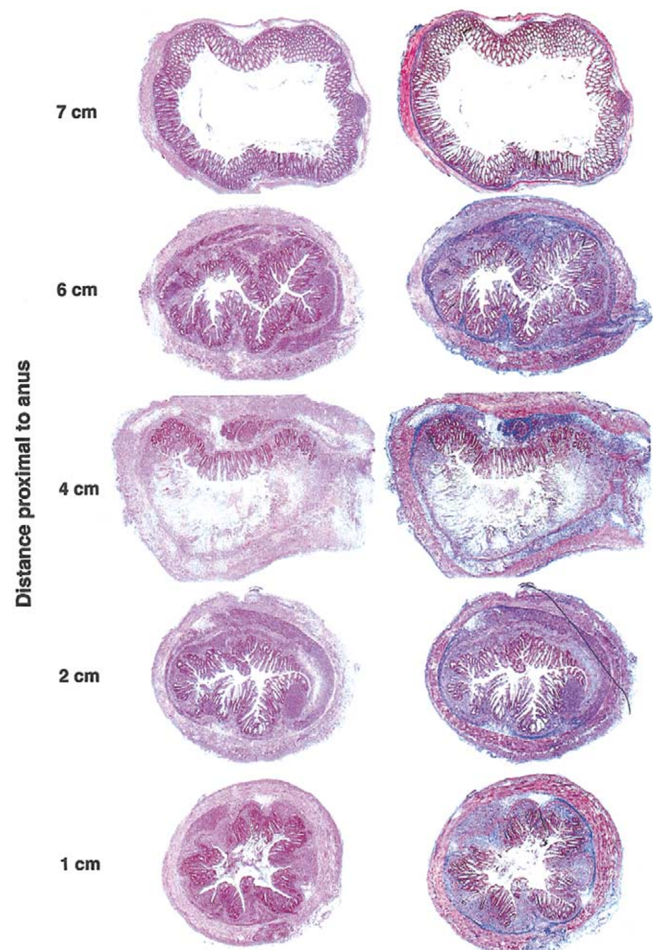

Figure 3. Microscopic appearance of colon at 8 weeks in a TNBS-treated mouse (model 1). Serial (1–7 cm from the anus) paraffin-embedded sections were stained with H&E (*left panels*) to assess the degree of inflammation, or trichrome (*right panels*) to determine the level of connective tissue deposition. At 7 cm, the colon is markedly dilated but free of inflammation and without increase in collagen deposition. At 6 cm, moderate, predominately chronic, full-thickness inflammatory infiltrates are noted, which are associated with increased collagen deposits located predominantly in the superficial layers. At 4 cm, moderate to severe acute and chronic inflammation involving all layers of the bowel wall results in disruption of the muscularis propria and is associated with hyperplastic lymphoid follicles. At 2 cm, findings are similar to those observed at 6 cm. At 1 cm, less marked, chronic inflammatory infiltrates predominate in the superficial layers in association with moderately increased collagen deposits. All sections are shown at 20× magnification.

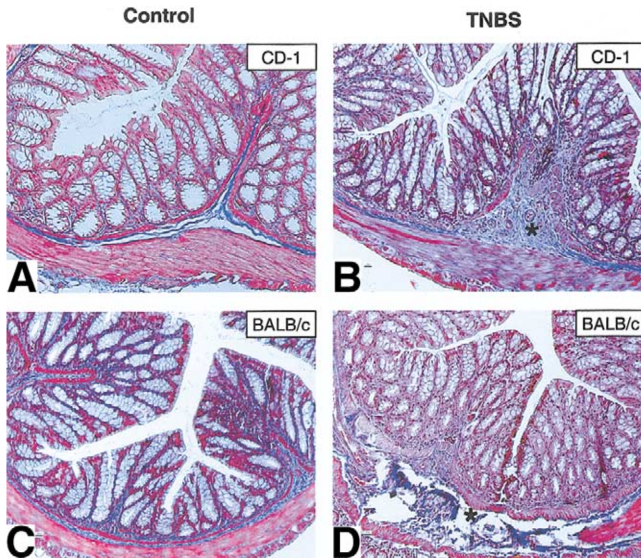

Figure 4. Trichrome-stained colonic sections showing connective tissue. The (*A* and *B*) CD-1 outbred and the (*C* and *D*) inbred BALB/c strains of mice were given (*A* and *C*) 6 weekly 30% ethanol enemas or (*B* and *D*) TNBS in ethanol as described in the Materials and Methods section. The CD-1 and the BALB/c mice were euthanized 4 and 2 weeks after the last enema. Note considerable thickening of the submucosa with disorganized tissue architecture and trichrome-stained collagen in the TNBS-treated mice in *B* and *D* (*asterisk*). Notably, fibrosis persisted for 4 and 2 weeks in the CD-1 and BALB/c strains, respectively, after cessation of TNBS enemas.

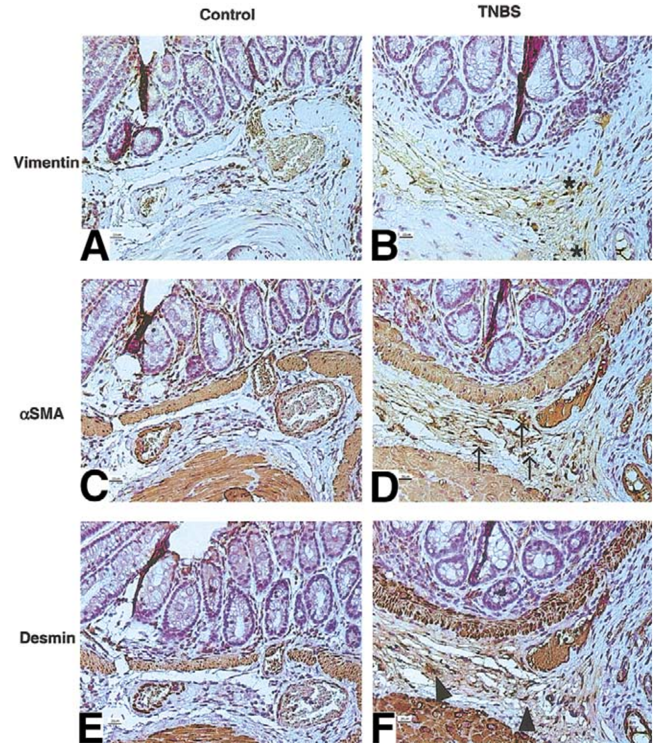

Figure 5. Immunolocalization of vimentin, α-SMA, and desmin in control and week 8 TNBS-treated CD-1 mice (model 1). Weak staining for vimentin was noted in the submucosa of the TNBS colon (*asterisks*). Anti–α-SMA stained myofibroblasts in the submucosa of the week 8 TNBS colon (*arrows*) and the muscle layers and the muscularis mucosa in both control and TNBS colons. Antidesmin stained the muscle layers in the control and TNBS colons and isolated cells in the submucosa of the TNBS colon only (*arrowhead*).

(Figure 4*D*, asterisk) compared with minimal trichrome staining of the ethanol control (Figure 4*C*).

Expression of TNF-α, IFN-γ, TGF-β1, IGF-1, Col1a2, Col3a2, MMP-1, MMP-3, and TIMP-1 mRNA in Colonic Tissue

To define mechanisms underlying the fibrogenic process, we elucidated by RT-PCR, changes in gene expression for collagens (Col1a2 and Col3a2), inflammation-mediating cytokines/growth factors (TNF-α, IFN-γ, TGF-β1, and IGF-1), and enzymes responsible for tissue destruction and remodeling (MMP-1, MMP-3, and TIMP-1). Compared with water control colons, TNF-α mRNA, TGF-β1, and IGF-1 mRNA were increased significantly in the TNBS-treated colons (Table 2). Although IFN-γ mRNA was increased by 1.6-fold in the TNBS-treated colons, this change did not reach statistical significance (Table 2). Of the ECM-related

Table 2. Gene Expression in Mouse Colon Tissues Detected by RT-PCR

Gene	No. of experiment	Control	TNBS	$P < 0.05$
Col 1α2	16	0.423[a] (0.035–0.81)[b]	0.999[a] (0.122–1.875)[b]	[c]
Col 3α2	21	4.56 (1.11–8.01)	5.76 (1.54–9.98)	
MMP-1	18	0.04 (0–0.08)	0.62 (0–1.24)	[c]
MMP-3	16	0.405 (0.11–0.7)	1.735 (0.08–3.39)	
TIMP-1	16	0.084 (0.012–0.473)	0.207 (0.0074–0.407)	[c]
TNF-α	16	0.017 (0.004–0.034)	0.059 (0.003–0.115)	[c]
IFN-γ	16	0.0028 (0–0.0055)	0.007 (0–0.014)	
TGF-β1	10	0.5 (0.16–0.84)	1.205 (0.57–1.84)	[c]
IGF-1	10	0.105 (0.02–0.19)	0.29 (0.09–0.49)	[c]

[a]Median expression normalized to the expression of β-actin.
[b]Range in minimum to maximum.
[c]Statistically significant difference between TNBS and control determined by Bonferroni-Dunn analysis of variance.

genes, Col1a2, MMP-1, and TIMP-1 mRNA levels were increased significantly in the TNBS-treated mice (Table 2).

We investigated possible correlations between the differentially expressed genes in the control vs. TNBS colon. No significant correlation was found between the levels of TNF-α with TGF-β or IGF-1 mRNA, or IFN-γ with IGF-1 mRNA in control or colitic animals. Instead, there was a statistically significant positive correlation between IFN-γ and TGF-β mRNA ($r = 0.755$; $P = 0.019$) in control animals, which was lost in the TNBS mice. Significant correlations between Col1a2 and MMP-3 mRNA ($r = 0.779$; $P = 0.039$), and Col3a2 and TIMP-1 mRNA ($r = 0.782$; $P = 0.038$) observed in control animals also were lost in colitic TNBS mice, which showed a statistically significant correlation between MMP-3 and TIMP-1 expression ($r = 0.586$; $P = 0.017$).

Phenotype of Colonic Mesenchymal Cells In Situ in TNBS-Treated Mice

Intestinal mesenchymal cells express multiple cytoskeletal proteins whose type and amount vary depending on the cell type, state of differentiation, and exposure to inflammatory mediators.[26,27] Therefore, we investigated the expression of α-SMA, desmin, and vimentin to determine the cellular origin of the connective tissue. Compared with controls (Figure 5*A*), the submucosa in colonic cross-sections of the week 8 TNBS-treated mice from model 1 were weakly vimentin positive (Figure 5*B*, asterisks). Immunostaining for α-SMA and desmin showed strong staining of the muscle layers in both the control and TNBS sections, as expected (Figure 5*C–F*). However, only sections from TNBS-treated colons showed α-SMA and desmin-positive cells within the submucosa (Figure 5*D*, arrows; 5*F*, arrowheads). These results indicated the presence of myofibroblasts within areas of collagen deposits in the submucosa of the week 8 TNBS samples.

Monolayers of colonic cells in culture displayed a different morphology depending on whether they were derived from water control or TNBS-treated mice (data not shown). Monolayers from control colons became confluent readily and showed typical morphology of slender juxtaposed nonoverlapping spindle cells. In contrast, monolayers from chronically inflamed colons failed to achieve confluency despite active replication, and individual cells showed remarkably thin and interlacing long bodies with prominent nuclei. Mesenchymal cells from both control and TNBS-treated colons expressed high levels of α-SMA and minimal levels of vimentin (data not shown).

Effect of TNF-α and IFN-γ on MMP-1, MMP-3, IGF-1, TGF-β1, and TIMP-1 mRNA Expression by Colonic Mesenchymal Cells

Mesenchymal cells produce much of the ECM proteins and enzymes that remodel them and play a pivotal role in fibrogenic tissue damage. Therefore, we examined selected functional characteristics of murine colonic fibroblasts cells derived from control and inflamed tissues. Cells isolated from the colon of control and TNBS-treated mice were exposed to TNF-α and IFN-γ as representative cytokines known to be involved in experimental colitis. Both TNF-α and IFN-γ increased MMP-1 and MMP-3 mRNA expression in these TNBS-treated and control colonic mesenchymal cells (Figure 6). IGF-1 and TGF-β1 expression, however, was not significantly altered by IFN-γ or TNF-α in either mesenchymal cell cultures. Interestingly, in TNBS mesenchymal cells only, TIMP-1 mRNA expression was increased significantly, in response to IFN-γ and TNF-α (both at $P = 0.039$).

NF-κB p65 Subunit Antisense Oligonucleotide Therapy

Previous studies have shown that the TNBS colitis model has a prominent CD4+ T-cell component that favors Th1-type cytokine production.[21] Recent studies further showed that local administration of antisense oligonucleotide to NF-κB p65 down-regulated the expression of the p65 subunit of NF-κB and abrogated colonic inflammation.[19] To determine whether this would be effective for minimizing fibrosis associated with chronic long-term inflammation, we tested the effects of p65 antisense oligonucleotide administered locally in 2 models. In the prophylactic model 2, the oligonucleotide treatment was given intrarectally 1 day before each TNBS treatment. The change in body weight was followed-up weekly for each experimental group (n = 7–12 per group) and representative data are presented as change in body weight on day 3 after treatment compared with initial body weight on day 0 of each week. These results are shown for weeks 1, 3, and 5 (Figure 7). The saline controls (Figure 7, solid bar) showed a consistent increase in body weight during weeks 1, 3, and 5. The TNBS-treated animals showed a consistent decrease in body weight each week. The TNBS-treated group that was given the NF-κB antisense prophylaxis was the only one, other than the control saline group, that gained weight, at weeks 1 and 5, with no change at week 3 (Figure 7*A*). As in the TNBS group, the TNBS plus control oligonucleotide–treated mice lost body weight at each time point. In the therapeutic model

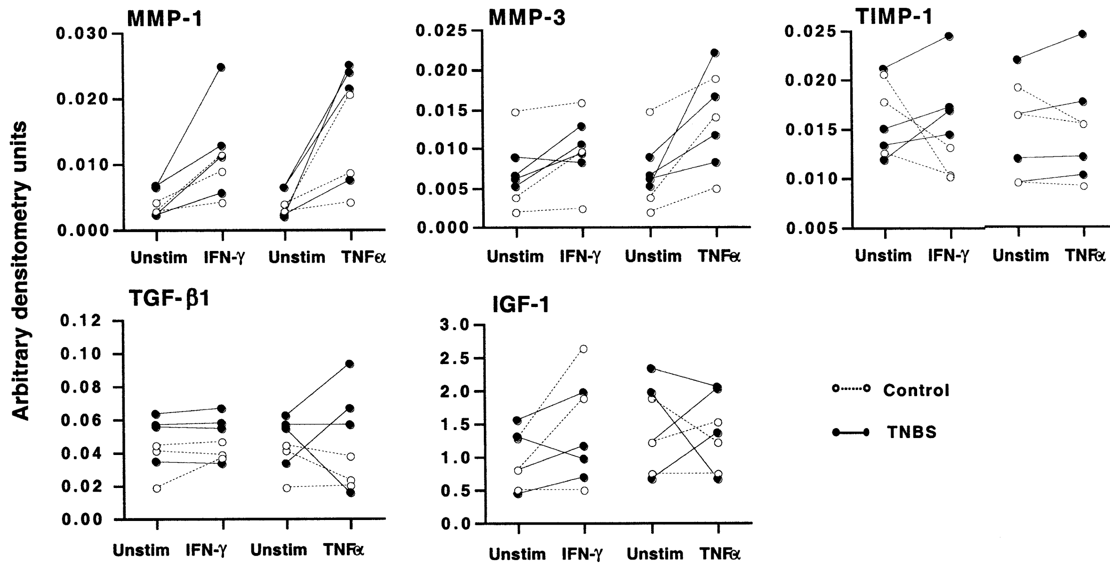

Figure 6. Effects of inflammatory cytokines on MMP-1, MMP-3, IGF-1, TGF-β1, and TIMP-1 mRNA on mesenchymal cells from inflamed and control colons. All experiments were performed using cells isolated from colons of 8th week water control and TNBS-treated CD-1 mice (model 1). Bands of individual polymerase chain reaction products were measured by densitometry normalized to β-actin. Lines link values derived from the same culture under nonstimulated and stimulated conditions. IFN-γ and TNF-α stimulation greatly increased MMP-1 and MMP-3 mRNA in cells from both TNBS-treated and control colons. IFN-γ and TNF-α increased TIMP-1 mRNA in mesenchymal cells from TNBS-treated colons, but inhibited TIMP-1 mRNA in cells from control colons. TIMP-1 mRNA expression was significantly different between cells from TNBS-treated and control colons on stimulation by IFN-γ or TNF-α ($P = 0.039$ and $P = 0.039$). No statistically significant differences were observed in the expression of IGF-1 and TGF-β1 when exposed to either IFN-γ or TNF-α.

3, the antisense therapeutically treated group did not gain weight as the prophylactically treated group in model 2 (Figure 7*B*). The p65 antisense given prophylactically alleviated piloerection and lethargy (data not shown).

Extent of inflammation and fibrosis was assessed in these 2 models in animals harvested on week 8, 2 weeks after the last TNBS treatment. Percent of animals harboring mild, moderate, and severe inflammation and fibrosis are shown as bar graphs below representative histology sections of each group (Figure 8). In model 2 (Figure 8*C* and *D*), the antisense given prophylactically was highly effective in preventing moderate and severe inflammation, compared with the TNBS-treated group (which showed moderate and severe inflammation in 33% and 22% of the mice, respectively) only 17% of the antisense-treated group still showed mild inflammation. Moderate and severe fibrosis seen in 45% and 22% of the TNBS group (Figure 8*B*) was absent in the antisense prophylaxis–treated group (Figure 8*C*), with only 33% of this group showing mild fibrosis. In model 3 (Figure 8*E* and *F*), the therapeutically treated group receiving the antisense treatment at weeks 5 and 6 of the TNBS regimen had mild inflammation in 43% of the animals, with 14% still showing signs of moderate fibrosis. Thus, in our long-term TNBS model, the abundant trichrome-positive connective tissue build-up seen in the submucosa of TNBS-treated mice (Figure 8*B*) and those receiving TNBS plus control oligonucleotide (Figure 8*D*) was minimal in mice treated concomitantly with the NF-κB antisense oligonucleotide (model 2, Figure 8*C*). The therapeutic model 3 was effective in resolving moderate and severe inflammation, but less effective in clearing moderate fibrosis (Figure 8*E*).

Discussion

There is a strong yet poorly understood connection between chronic inflammation and fibrogenic changes in the intestine. Existing models of TNBS colitis based on 2 or 3 enemas only result in severe, but transient, inflammation that resolves over a short period of time.[18,21,28,29] To induce long-term colonic inflammation and associated fibrosis, we modified the TNBS administration regimen to allow the development of chronic colitis and associated colonic fibrosis.

Early in the regimen, clinical signs of severe inflammation such as rectal bleeding and prolapse were rare and diarrhea was only transitory. In model 1, with weekly low doses of TNBS, the mice developed inflammation reflected by the increase in colon to body weight ratio. Acute and chronic inflammation, as well as increased connective tissue deposition, was evident by histologic examination. Increased trichrome staining, indicative of

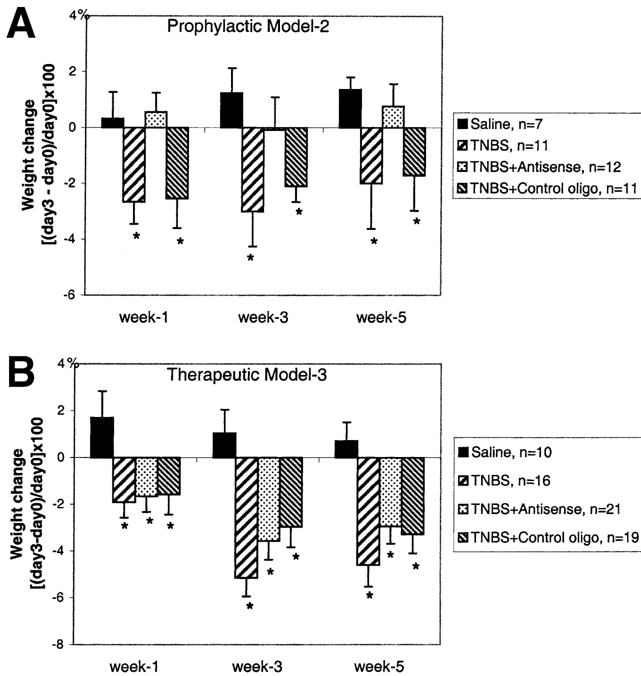

Figure 7. Change in body weight in response to the NF-κB p65 subunit antisense oligonucleotide given (*A*) prophylactically (model 2) or (*B*) therapeutically (model 3). (*A*) Model 2: mice pretreated with NF-κB antisense or control oligonucleotide at day 0 were given TNBS enemas or saline control at day 1 of each week as described in the Materials and Methods section. (*B*) Model 3: mice were given TNBS weekly for 4 weeks to establish chronic inflammation. At weeks 5 and 6, mice were pretreated with NF-κB antisense or control oligonucleotide 1 day before TNBS. Each model contains 4 groups of mice (numbers as indicated). Saline: those receiving saline instead of TNBS enemas; TNBS: mice receiving TNBS enemas; TNBS + antisense: mice receiving TNBS and the antisense treatment; TNBS + control oligo: TNBS-treated mice receiving the control oligonucleotide. The mice were weighed each week on days 0 and 3 and the change in weight was calculated ([day 3 − day 0]/day 0) × 100 for each animal. Mean for each treatment group ± standard error of the mean are shown here for weeks 1, 3, and 5. Note that compared with the TNBS and control oligo group, which lost weight, the antisense-treated group is the only one, other than the saline group, that gained weight in model 2. In the therapeutic model 3, the saline group is the only one that gained weight each week.

increased connective tissue, clearly was evident at week 6, but not at week 2. Most importantly, in mice that were not given any additional TNBS after 6 weeks, increased connective tissue staining persisted for 4 additional weeks. Model 1 applied to the BALB/c strain of mice also maintained colonic fibrosis for 2 additional weeks (based on our observation period) after the sixth TNBS treatment. This confirmed that the low-dose, long-term TNBS treatment that we have developed is capable of causing fibrosis that is maintained for 2–4 weeks without further TNBS induction. Also, our new model is not limited to the outbred strain of mice only, but is effective in the BALB/c inbred strain of mice as well. In fact, compared with the CD-1 strain, the BALB/c displayed higher levels of inflammation and fibrosis at the week 6 time point (mice harvested 3 days after the sixth TNBS treatment). At week 8, the percentage of mice with moderate/severe fibrosis was comparable in the 2 strains. The sequence of clinical and histologic events noted in this model better resembles active remodeling and fibrosis typically observed in human CD,[30,31] and differs from the short-term inflammatory response seen in traditional TNBS-induced colitis used to study associated immune abnormalities.[18,21]

An increase in Col1a2 mRNA levels and an increase in hydroxyproline, a collagen-specific amino acid, indicate that total collagen had increased significantly in the TNBS-treated colons. Increased expression of the profibrogenic growth factors TGF-β1 and IGF-1 in the TNBS-treated colons may be stimulating proliferation of mesenchymal cells that contribute to increased ECM synthesis as suggested by studies on IBD and on fibrosis in other systems.[26,32–34]

By immunohistology we showed that the submucosa, a primary site of increased collagen deposition in the TNBS-treated colon, contained α-SMA-positive, desmin-positive, and weakly vimentin-positive cells. Although the definition of myofibroblast is controversial,[35] this staining pattern strongly suggests the presence of mesenchymal myofibroblasts in the submucosa that are likely the source of the fibrotic tissue. Intestinal myofibroblasts also were implicated in fibrosis, in an earlier Lewis rat model, injected with bacterial cell wall lipopolysaccharides.[36] Mouse intestinal mesenchymal cells established from control and the TNBS groups were also α-SMA positive, but unlike the colonic sections were weakly positive for vimentin and desmin. Culture conditions often do not reflect tissue conditions in their entirety and may contribute to these apparent differences with the vimentin and desmin staining. However, high levels of α-SMA and low vimentin levels are consistent with a typical myofibroblast phenotype.

It is widely accepted that immune cell activation leads to nonimmune cell activation and profibrogenic ECM changes.[37,38] Although mRNA levels may not reflect secretion or activity of cytokines, growth factors, and ECM proteins, we explored the cytokine/growth factor–ECM relationship by investigating their gene expression in colon tissues and colonic fibroblast cultures by RT-PCR. T-cell activation, a key event in intestinal inflammation, leads to increased IFN-γ and TNF-α.[39–41] These are particularly prominent in the early stages of inflammation in IBD and in the TNBS models. These cytokines possibly are required for initiation of inflammation, but are not necessarily crucial for its maintenance.[18,42] Thus,

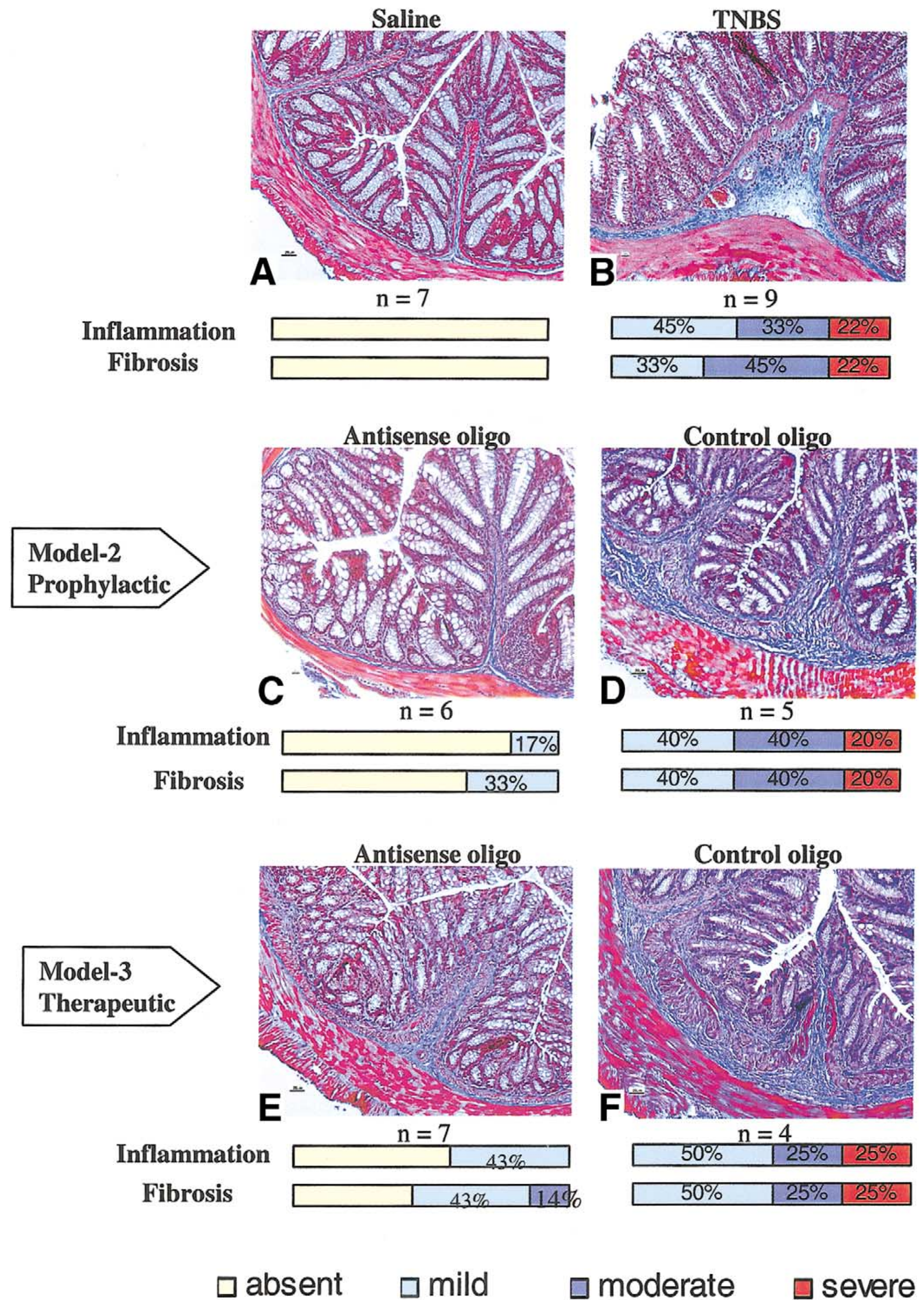

Figure 8. Histology of colonic cross-sections at week 8 from models 2 and 3. Percent of animals harboring mild, moderate, and severe inflammation and fibrosis are shown as bar graphs. Representative histology sections of each group are (*A*) saline-treated, (*B*) TNBS-treated, (*C*) TNBS plus NF-κB p65 antisense–treated (model 2) mice in which the antisense or the (*D*) control oligonucleotide treatment was given 1 day before every TNBS treatment for 6 weeks, and TNBS + the (*E*) antisense or (*F*) control oligonucleotide given on weeks 5 and 6. Note increased trichrome-positive connective tissue in the submucosa of (*B*) TNBS mice and those given (*D* and *F*) TNBS and control oligonucleotide. Model 2 with the NF-κB antisense oligonucleotide given as a prophylaxis (*C*) shows complete lack of connective tissue build-up in contrast to (*E*) a mild increase in submucosal connective tissue in the therapeutic model 3.

in our long-term model 1, at the week 8 stage, there was only a modest increase in TNF-α message and no significant change in IFN-γ.

In a prior study, by microarray gene expression profiling, we found increased expression of genes associated with ECM deposition without a balanced increase in MMP production in CD but not UC, an observation that may explain why fibrosis and stenosis are seen commonly in CD.[43] In our TNBS models here we also noted increased mRNA levels for Col1a2 and only one of the MMPs, namely MMP-1. Additionally, TIMP-1, an inhibitor of proteolytic activities of MMPs, also was up-regulated, suggesting conditions that discourage remodeling and favor ECM deposition. Intestinal myofibroblasts from CD patients also were reported to express higher levels of TIMP-1.[44]

We investigated the effectiveness of antisense NF-κB oligonucleotide given intrarectally to not only alleviate

inflammation, which has been shown in the past,[19] but also to reduce fibrogenic connective tissue changes associated with chronic inflammation in a prophylactic (model 2) and a therapeutic model (model 3). In model 2, TNBS-treated mice that were given the antisense oligonucleotide, macroscopic signs of the disease, namely piloerection, diarrhea, and significant weight loss, were minimal. More importantly, trichrome staining of colonic cross-sections showed lack of fibrogenic tissue architecture and abnormal collagen build-up. The therapeutic model 3, in which the antisense NF-κB treatment was given twice (at weeks 5 and 6) after establishing inflammation and fibrosis with 4 prior TNBS treatments, was somewhat less effective than model 2 in resolving inflammation and fibrosis. Overall, the therapeutic model 3 was more effective in resolving inflammation than fibrosis. After the NF-κB antisense treatment no animals showed moderate or severe inflammation, whereas 14% of the animals still showed moderate fibrosis. Thus, NF-κB antisense treatment given prophylactically during development of chronic inflammation may provide an early block in the signaling cascade that leads to both inflammatory and downstream fibrogenic changes. Additionally, the promoters of several ECM genes have NF-κB–responsive elements that may be regulated by NF-κB in developing fibrosis. Blocking NF-κB thus may down-regulate these ECM components and contribute toward lowering fibrogenic response. The antisense treatment given after inflammation and fibrosis was established is able to down-regulate inflammation well, and reduce severe fibrosis. Thus, our study shows that NF-κB blockade by antisense oligonucleotides decreases intestinal inflammation and reduces fibrosis. On the other hand, the idea that defective NF-κB signaling may lead to intestinal inflammation, as in CD associated with NOD2 variants,[45–47] may appear paradoxic.[48] At present, the mechanism by which NOD2 mutations increase susceptibility to CD is unknown. A possibility is that sensing of enteric bacterial components in the absence of physiologic NF-κB activation may trigger gut inflammation through a NOD2-independent, inappropriate T-cell response.[49]

In conclusion, we developed a novel murine model of chronic immune-mediated inflammation and associated fibrosis of the colon displaying certain features of CD. Histologically, CD and our long-term TNBS models show a full-thickness chronic inflammation associated with increased ECM deposition and distortion of colonic tissue architecture. The antisense NF-κB treatment given prophylactically prevented weight loss and dramatically reduced TNBS-induced inflammation and fibrosis. Given twice therapeutically after establishment of inflammation and fibrosis, the antisense treatment resolved inflammation well and reduced the number of animals with moderate/severe fibrosis, but was ineffective at preventing weight loss.

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