Glutamine Prevents Fibrosis Development in Rats with Colitis Induced by 2,4,6-Trinitrobenzene Sulfonic Acid\textsuperscript{1,2}

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Abstract

We investigated the effects of glutamine on the development of colonic fibrosis and on the expression of the major fibrogenic factors in a rat model of experimental colitis. Colitis was induced in one-half of the male Wistar rats by intracolonic administration of 30 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS). L-Glutamine (25 mg/kg) was administered rectally to one-half of the controls and one-half of the colitic rats. The control, control+glutamine, TNBS, and TNBS+glutamine groups were studied at d 2 and 7 after colitis induction. Glutamine induced a significant decrease in the area of colon fibrosis and in the staining of α-smooth muscle actin positive cells within areas of extracellular matrix deposits in the submucosa. Collagen synthesis was stimulated following TNBS administration, with a significant increase in procollagen IV, collagen III, and collagen Iα2 mRNA levels in the colon by d 2 after TNBS instillation. Tissue inhibitor of metalloproteinase, connective tissue growth factor, transforming growth factor-β, platelet-derived growth factor, and phosphorylated Smad3 were overexpressed in the colon of TNBS-treated rats. These effects were significantly abrogated in the colitic rats treated with glutamine. Our findings suggest that glutamine treatment not only attenuates the outcome of TNBS-induced colitis by reducing the inflammatory response but also by downregulating the increased expression of several gene pathways that contribute to the accumulation of matrix proteins. This molecule may be an interesting candidate for reducing the risk of fibrosis and stricture formation in inflammatory bowel disease. J. Nutr. 140: 1065–1071, 2010.

Introduction

Crohn’s disease is a recurrent chronic inflammatory enteropathy that may discontinuously affect the gastrointestinal tract as a consequence of the imbalance in the production of proinflammatory and immunoregulatory cytokines (1). Diseases characterized by chronic inflammation often result in irreversible organ dysfunction due to extensive tissue fibrosis, with luminal narrowing and stricture formation due to excessive extracellular matrix (ECM)\textsuperscript{5} deposition (2). This progression can be seen in Crohn’s disease, in which the transmural granulomatous inflammation frequently leads to extensive fibrosis (3).

The main effector cells that mediate gastrointestinal fibrosis are the intestinal myofibroblasts, which are activated and multiply in response to inflammatory signals, forming an expanded poll of mesenchymal cells and leading to increased deposition of ECM (4,5). A hallmark of cell activation is altered collagen synthesis, associated to changes in matrix-degrading metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) (6). The most potent profibrogenic cytokine is transforming growth factor-β (TGFβ), and blocking of TGFβ signaling prevents progression of fibrosis in experimental animals (7). The TGFβ intracellular signal transduction pathway is mediated primarily by Smad3 (8), resulting in stimulation of α-smooth muscle actin (α-SMA) and collagens (9). Another key cytokine is platelet-derived growth factor (PDGF), which plays an important role as an inducer of fibrosis. PDGF is a potent mitogen for intestinal myofibroblasts (10) and is upregulated in patients with Crohn’s disease (11). Connective tissue deposition is also stimulated by connective tissue growth factor (CTGF), which also favors TGFβ autoinduction and increases the expression of proinflammatory cytokines (12).

Existing therapies, predominantly glucocorticoids, 5-amino-salicylic acid, and immunosuppressive agents, can relieve the inflammatory symptoms of Crohn’s disease, but patients may

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\textsuperscript{1} Supported by the Programa de Cooperación Interuniversitaria España-Brasil (ref. PHB2006-0031-PC). CIBERehd is funded by Instituto de Salud Carlos III (Spain).

\textsuperscript{2} Author disclosures: B. San-Miguel, I. Crespo, N. A. Kretzmann, J. L. Mauriz, N. Marroni, M. J. Tuñón, and J. González-Gallego, no conflicts of interest.

\textsuperscript{3} Abbreviations used: α-SMA, α-smooth muscle actin; CTGF, connective tissue growth factor; ECM, extracellular matrix; IL, interleukin; MMP, matrix-degrading metalloproteinase; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinase; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNFa, tumor necrosis factor-α.

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develop side effects or resistance and they do not significantly improve the stricture lesions of the bowel (21,13). Therefore, investigating new therapeutic strategies, including the use of drugs with antifibrotic properties, is highly needed in fibrotic enteropathy, especially in Crohn’s disease. Experimental studies have demonstrated an inhibition of colonic fibrosis by captopril (7) or by Boswellia and Scutellaria extracts (9) in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-treated rats, and resistance to the development of TNBS-induced fibrosis in Smad3 null mice (8).

Because enteral glutamine is the main source of amino acids for the enteral mucosa and is metabolized at high rates by enterocytes and immunocytes (14), it has been used in many clinical conditions such as extended trauma or burning, prolonged fasting, and chemotherapy to prevent enteritis and colitis (15). Administration of glutamine by gastric gavage (16,17) or enema (18) before TNBS instillation reduces colonic damage in rats with experimental colitis. Moreover, it has been shown that dietary glutamine improves mucosal function in a dextran sodium sulfate-induced model of experimental colitis (19) and we previously reported that glutamine prevents nuclear factor-xB (NF-xB) activation in rats with acetic acid-induced colitis (20) and inhibits proinflammatory gene expression in TNBS-induced treated rats (21). Some clinical studies of oral glutamine in patients with Crohn’s disease have been disappointing (22), but new formulations and local administration of glutamine on the mucosal lesions could enhance its efficacy.

In this study, we assessed the antifibrogenic effect of glutamine using the TNBS model in rats. Treatment with 25 mg/kg of glutamine markedly decreased the severity of macroscopic damage to the colon and prevented extensive fibrotic connective tissue deposition at 2 and 7 d after TNBS instillation.

Materials and Methods

Materials. TaqMan primers and probes for interleukin (IL)-6 (GenBank accession no. M26744.1 and Rn09999011_m1), TNFx (GenBank accession no. A011227.1 and Rn09999017_m1), procollagen type IV (GenBank accession no. BC089096.1 and Rn01482927_m1), collagen Iα2 (GenBank accession no. AF121217.1 and Rn01526721_m1), collagen III (GenBank accession no. M21354.1 and Rn01437681_m1), MMP-2 (GenBank accession no. U65656.1 and Rn01538170_m1), TIMP-1 (GenBank accession no. L31883.1 and Rn00557855_m1), TGFβ (GenBank accession no. X52498.1 and Rn00572010_m1), Smad7 (GenBank accession no. AF042999.1 and Rn01528319_m1), Smad3 (GenBank accession no. U64797.1 and Rn01528319_m1), PDGFβ (GenBank accession no. Z14117.1 and Rn01502593_m1), CTGF (GenBank accession no. AB023068.1 and Rn01537279_g1), and glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. X00223.1 and Rn09999916_s1) genes were derived from the commercially available TaqMan Gene Expression Assays (Applied Biosystems). Antibodies against collagen Iα2, TIMP-1, TGFβ, Smad7, phospho Smad3, Smad3, and PDGF were from Santa Cruz Biotechnology and antibodies against MMP-2, CTGF, and collagen III were from Abcam.

Induction of colitis. Experimental colitis was induced by TNBS (23). Briefly, rats that had been deprived of feed for 24 h were lightly anesthetized with isoflurane and a polyethylene catheter was inserted rectally until the splenic flexure. TNBS (30 mg) dissolved in a volume of 0.25 mL of ethanol 50% (v/v) was administered through the catheter. TNBS was retained in the colon for 30 s, after which the fluid was withdrawn. Control rats received the vehicle (0.9% saline).

Rats and treatments. Male Wistar rats weighing 300–350 g were caged at 24°C with a 12-h-light:dark cycle and free access to food and water. The rats were randomly divided into 4 groups (n = 8 rats each): a control group that received only vehicle, a colitic group that received TNBS, and a control and colitic group that received L-glutamine (25 mg·kg⁻¹·day⁻¹) in a volume of 3 mL rectally 4 h after the induction of colitis and once daily through the end of the study (21). The control, control+glutamine, TNBS, and TNBS+glutamine groups were studied at d 2 and 7 after colitis induction.

The rats were killed by cervical dislocation and the distal 8 cm of the colon was excised, opened by longitudinal incision, rinsed with saline, immediately snap-frozen in liquid nitrogen, and stored at −80°C. The Ethical Committee of the University of León approved all experimental procedures.

Macroscopic and microscopic analysis. Macroscopic damage of the colonic mucosa was assessed by 2 observers who were unaware of the treatment applied. The scale for macroscopic damage ranged from 0 to 10 and was based on thickening of the wall, sites of ulceration, and sites of inflammation (21). Samples for histology were rinsed with saline, fixed in 10% buffered formalin, and embedded in paraffin blocks. Slices (5-μm sections) were stained with picrosirisus red stain. Intestinal fibrosis was scored as mild, moderate, or severe, depending on the density and extent of picrosirisus red-positive connective tissue staining and disruption of tissue architecture (6).

Immunohistochemistry. Immunoreactivity of α-SMA was studied in sections deparaffinized by xylene and rehydrated with graded alcohols (24), which were incubated with antibody α-SMA at its working dilution of 1:200. Pathological findings were assessed by one of the authors who did not know the group assignments.

Real-time quantitative RT-PCR. Total RNA was extracted from colon and reverse transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems). cDNA was amplified using a TaqMan Universal PCR Master mix (Applied Biosystems) on a Step One Plus (Applied Biosystems). Relative differences in expression levels were determined using the 2−ΔΔCT method (25). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of GAPDH gene detection, referred to as ΔCT.

Western blot analysis. Lysate proteins were fractionated by SDS-PAGE and Western blotting was performed using the corresponding primary antibodies. Bound antibody was detected by enhanced chemiluminescence. Membrane rehybridization with β-actin antibody was conducted to check loading accuracy. The density of the specific bands was quantified with an imaging densitometer (Scion Image).

Statistical analysis. Results were expressed as means ± SEM. Data were analyzed using multifactorial ANOVA with a 2 (glutamine) × 2 (TNBS) × 2 (time) design. A Welch ANOVA was performed when Levene’s test indicated that variances were unequal. When a significant effect was found, post hoc comparisons were carried out using the Tukey’s honestly significant difference test. Differences were considered significant at P < 0.05.

Results

Glutamine reduces the severity of TNBS-induced fibrosis in rats. In rats receiving TNBS, severe macroscopic damage of the colon was observed at d 2 and 7 after rectal administration. The severity of macroscopic damage was significantly lower in those treated with glutamine at both times (Table 1). The histological features of the colons from untreated control rats and control rats receiving glutamine were normal. The colon of the TNBS-treated rats showed evidence of fibrosis in the submucosa and serosa, and a severe disarrangement of colonic architecture was clearly present at d 7. The extent and severity of colon injury was less in colitic rats treated with glutamine, with a significantly lower fibrosis score than in TNBS-treated controls on d 7 (Fig. 1; Table 1).
Glutamine influences activation of intestinal myofibroblasts. We identified the activated myofibroblast phenotype in the colon by immunostaining with an antibody for α-SMA. Anti-α-SMA stained myofibroblasts in the muscle layers and muscularis mucosa of colons from both control and TNBS-treated rats. However, only sections from TNBS-treated rats showed α-SMA positive cells within areas of ECM deposits in the submucosa. There was less α-SMA staining in the colon of TNBS+glutamine-treated rats compared with the colon of TNBS-treated rats (Fig. 1).

Glutamine modulates the expression of genes related to inflammation and fibrosis. We previously reported that glutamine abolishes neutrophil infiltration and reduces the expression of cytokines and proinflammatory genes in rats with TNBS-induced colitis (21). This was confirmed in the present study by significantly greater colon IL-6 and TNFα mRNA levels at d 2 and 7 after TNBS instillation compared with controls. These effects were partially abrogated by glutamine treatment (Table 2).

To define mechanisms underlying the fibrogenic process, we elucidated by RT-PCR differences in gene expression for collagens, enzymes responsible for tissue destruction and remodeling, and profibrogenic cytokines. Collagen synthesis was stimulated following TNBS administration as indicated by the fibrosis scores (Table 1), with significantly greater levels of procollagen IV, collagen III, and collagen Iα2 mRNA levels at d 2 and 7 after TNBS instillation compared with controls. These effects were partially or completely abrogated by glutamine treatment (Table 2).

In addition to the synthesis and extracellular deposition of ECM components, the fibrogenic process also involves inhibition of ECM degradation due to an imbalance between MMP and their inhibitors, TIMP (26). MMP-2 and TIMP-1 mRNA levels were significantly higher at d 2 and 7 after TNBS administration. Treatment with glutamine partly prevented MMP-2 and TIMP-1 overexpression (Table 2).

Because TGFβ is the most potent procytokine, acting through changes in the activation of members of the Smad family (8), we investigated expression of TGFβ, Smad3, and Smad7 in the different experimental groups (Table 2). TGFβ mRNA levels were significantly higher than in controls at d 2 and 7 after TNBS administration. On d 7 after TNBS instillation, the Smad3 mRNA level was slightly but significantly lower than in the control, whereas that of Smad 7 did not differ at either time. Glutamine completely or partially abolished the effects on Smad3 and TGFβ. Another potent mitogen for intestinal myofibroblasts, PDGF, was also overexpressed in TNBS-treated rats on d 7 and again that effect was partly prevented in rats receiving glutamine. Finally, in rats treated with TNBS, there was by d 2 of treatment a significant overexpression of CTGF, a potent stimulator of ECM deposition, which is linked to both TGFβ autoinduction and also to the synthesis of proinflammatory factors (12). On d 7, CTGF mRNA levels were partially normalized by glutamine in colitic rats (Table 2).

The described transcriptional effects on the expression of genes related to ECM deposition and tissue destruction and remodeling were maintained at a post-translational level, as confirmed by the assessment of gene expression for collagens, enzymes responsible for tissue destruction and remodeling, and profibrogenic cytokines by Western blot. Changes in protein concentration induced by TNBS administration and by glutamine treatment were generally parallel to those in mRNA levels except for reduced MMP-2 protein concentration in TNBS-treated rats, which was partially abolished by glutamine. Moreover, TNBS-treated rats had a marked expression of phosphorylated Smad3 that was absent in rats receiving glutamine (Fig. 2).

Discussion

The TNBS model of colitis resembles human Crohn’s disease in terms of its various histological features, including infiltration of colonic mucosa by neutrophils and macrophages, fibrosis, and increased production of inflammatory and profibrogenic mediators (27,28). We have previously reported that in this animal model, treatment with glutamine attenuates the inflammatory damage to the colon, as verified by macroscopic and histological findings, the abolishment of myeloperoxidase accumulation, and the downregulation of various proinflammatory mediators and related signaling pathways (21). Despite recent advances in the therapy of Crohn’s disease, the development of intestinal fibrosis and strictures remains a challenging complication and the use of drugs with antibacterial properties is highly needed in fibrotic enteropathy. We therefore investigated in the present study whether glutamine administration was also able to inhibit or mitigate the fibrogenic process in TNBS-treated rats.

Increased connective tissue disposition was evident in TNBS-treated rats by histological examination, which demonstrated that within 48 h of TNBS instillation, there was an increased picrosirius red staining that had further progressed by 7 d. Using immunohistochemistry, we found that the submucosa, a primary site of increased ECM deposition in the TNBS-treated colon, contained α-SMA positive cells, which suggests the presence of mesenchimal myofibroblasts that are likely the source of the fibrotic tissue (6). Glutamine administration significantly decreased the number of α-SMA positive cells. Those results were further supported by the fact that expression of components of the ECM, procollagen IV, collagen III, and collagen Iα2 was markedly reduced in rats treated with glutamine.
The role of profibrogenic cytokines is central for the development of fibrosis, with progression greatly dependent on TGFβ production. TGFβ plays a role in the immunoregulation of inflammatory responses and tissue remodeling in inflammatory bowel disease (26). TGFβ is upregulated in Crohn’s disease (29,30), but its antiinflammatory function is ineffective due to impairment of intracellular TGFβ signaling, with a loss of the inhibitory effect on NF-κB activation (31,32). Overexpression of TGFβ contributes, however, to mesenchymal cell activation and ECM production and accumulation. One of the intracellular mediators of TGFβ, Smad3, plays a major role in its fibrogenic effects, because Smad3 loss confers resistance to the development of TNBS-induced fibrosis (8). Our data demonstrate that upregulation of TGFβ in TNBS-treated rats was associated with an increased phosphorylation of Smad3 and that these effects were partly abrogated in rats receiving glutamine. The results obtained are similar to those indicating that the combined oral administration of *Boswellia* extracts with antiinflammatory activity and *Scutellaria* extracts with antifibrotic effects significantly improves the course and macroscopic finding of TNBS-induced chronic colitis as well as the histological severity of the fibrosis of the colonic wall through inhibition of the TGFβ/Smad3 pathway (9). However, the authors of the study did not assess at which level (transcriptional or post-translational) the TGFβ/Smad3 pathway was affected by the *Boswellia/Scutellaria* treatment. The present research goes a step further...
by demonstrating the transcriptional upregulation of TGFβ by glutamine. This signaling pathway is downregulated by Smad7, an antagonist of TGFβ that inhibits phosphorylation of Smad3 (31). In fact, targeting Smad7 has been suggested as a plausible method for treating inflammatory bowel disease patients (33,34). However, blocking Smad7 may result in an artificial enhancement of the TGFβ signaling pathways and the potential induction of stricture formation (35). In any case, Smad7 expression was not affected in our study, although it was reported previously that Smad7 expression is reduced 21 d after

**FIGURE 2** Glutamine modulates the expression of fibrosis-related genes in rats with TNBS-induced colitis. Protein from colonic extracts from samples taken at d 2 and 7 after instillation of TNBS was separated by SDS-PAGE followed by immunoblotting for collagen Iα2, collagen III, MMP-2, TIMP-1, TGFβ, Smad7, phosphorylated Smad2, Smad3, PDGF, and CTGF. Results are representative of 4 independent experiments. Equal loading of proteins is illustrated by β-actin bands.

**TABLE 2** Effect of treatment with glutamine on colon mRNA levels of genes related to inflammation and fibrosis in rats with TNBS-induced colitis1,2

<table>
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<tr>
<th>mRNA</th>
<th>Time</th>
<th>Control</th>
<th>Gln</th>
<th>TNBS</th>
<th>TNBS+Gln</th>
<th>Gln</th>
<th>TNBS</th>
<th>T</th>
<th>Gln × TNS</th>
<th>ANOVA P-values</th>
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<tr>
<td>d Fold of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-6</td>
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<td>1.0 ± 0.1</td>
<td>97</td>
<td>0.1</td>
<td>7.9 ± 0.3</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.007</td>
<td>0.014</td>
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<tr>
<td>TNFα</td>
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<td>42</td>
<td>0.1</td>
<td>5.2 ± 0.3</td>
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<td>0.001</td>
<td>0.001</td>
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<td>Collagen Iα2</td>
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<td>29</td>
<td>0.1</td>
<td>4.1 ± 0.2</td>
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<td>0.001</td>
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<td>1.9 ± 0.3</td>
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<td>0.1</td>
<td>2.1 ± 0.3</td>
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<td>0.001</td>
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<td>0.001</td>
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<td>TGFβ</td>
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<td>0.1</td>
<td>4.9 ± 0.3</td>
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<td>0.001</td>
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<td>1.0 ± 0.1</td>
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<td>0.1</td>
<td>0.8 ± 0.1</td>
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| 1 Values are means ± SEM, n = 8. Means for a variable with superscripts without a common letter differ, P < 0.05.  
2 Levels of mRNA were analyzed by real-time PCR assays and were normalized to GADPH.  
3 T, Time. |
TNBS administration (9). Therefore, the role of Smad7 still remains to be fully established.

While Smad proteins may be critical for TGFβ signaling, interaction with other signaling cascades is necessary for regulation of target gene expression. Of particular interest is the interaction between TGFβ and reactive oxygen species formation. TGFβ is a redox-sensitive gene whose expression is upregulated by reactive oxygen species and blocked by catalase (36). We have previously reported that glutamine prevents the increase in markers of oxidative stress in rats with TNBS-induced colitis (21). Moreover, glutamine induces a down-regulation of TNFα, which has been shown to increase in response to oxidative stress and to lead to enhanced TGFβ expression (37). Therefore, antioxidant properties of glutamine could contribute to the antifibrotic effects of this molecule.

TGFβ provides a strong stimulus for the synthesis of CTGF, a potent stimulator of fibroblast proliferation and of the production of collagen and other components of the ECM (38). Our data indicate that CTGF expression is inhibited by glutamine treatment, supporting previous research that indicates that in Smad3 null mice, there is a decrease in the immunostaining of both TGFβ and CTGF (8) or that oral administration of Boswellia and Scutellaria extracts inhibits the colonic expression of TGFβ and CTGF in TNBS-treated rats (9). Inhibition of CTGF expression by glutamine would also play a role in the maintenance of the fibrotic process, because CTGF is a necessary contributor to the autoinduction of TGFβ (12). In addition to its role as a fibrotic factor, CTGF has been reported to activate the NF-κB pathway and the cascade of mitogen-activated protein kinases (39), demonstrating cross-talk between both signaling pathways and a release of proinflammatory cytokines that may accelerate the process of chronic inflammation (40). Therefore, effects on CTGF allow the establishment of a link among the antifibrotic properties of glutamine.

So far, the major interest in PDGF has focused on cancer and little is known of its involvement in inflammatory diseases of the gastrointestinal tract. More recently, however, PDGF has been found to be a promising target for antifibrotic therapies (41) and it was reported that expression of PDGF mRNA and protein increases in inflammatory bowel disease patients, both in areas of active inflammation and also in areas of active fibrosis, where intensive repair processes are occurring (11). It has been also shown that inflammatory bowel disease is associated with increased circulating PDGF levels, which reflect the inflammatory and clinical disease activity (42). Results from the present research suggest that downregulation of PDGF could also be a contributor to the antifibrotic properties of glutamine. Another link among glutamine’s antifibrotic and antifibrotic effects comes from the fact that glutamine inhibits NF-κB activation in TNBS-treated rats (21). The promoters of several ECM genes have NF-κB responsive elements that may be regulated by NF-κB in developing fibrosis (6). Blocking NF-κB may thus downregulate these ECM components and contribute toward lowering the fibrogenic response.

An additional finding from our study was the reversal by glutamine of changes in the expression of TIMP-1 and MMP-2 induced by experimental colitis. MMP are a family of metalloendopeptidases that act to degrade many important extracellular proteins and their activity is negatively regulated by TIMP (24). Cronh’s stricture myofibroblasts overexpress TIMP-1 (43) and, in TNBS-treated mice, fibrosis is associated with an increase in TIMP-1 (6). Upregulation of the MMP-2 mRNA level has also been reported in mice with experimental colitis (6,44). However, that effect is not accompanied by an increase in MMP-2 protein activity (45) and, in our study, the MMP-2 protein concentration was reduced in TNBS-treated rats. These data suggest a sequestering in MMP/TIMP complexes and indicate that intestinal fibrosis is associated with conditions that discourage matrix degradation and remodeling and favor ECM deposition. TGFβ has been reported to stimulate TIMP-1 production (46) and its inhibition by glutamine could contribute to its effects on the mediators of ECM turnover. Moreover, because TIMP-1 has antiapoptotic effects (47), decreased expression can be beneficial for fibrosis resolution.

In conclusion, our results revealed that administration of glutamine was associated in TNBS-treated rats with an early prevention of fibrosis development and the effect was related to the inhibition of the colonic expression of collagen Iα2, collagen III, TGFβ, phosphorylated Smad3, PDGF, and CTGF. The results of our experiments demonstrate that glutamine treatment not only attenuates the outcome of TNBS-induced colitis by impairing the inflammatory response but also by downregulating increased expression of several gene pathways that contribute to the accumulation of matrix proteins. Although the TNBS model is not the same as the disease in humans and results from human trials have been underwhelming, this molecule may be an interesting candidate for reducing the risk of fibrosis and stricture formation in inflammatory bowel disease and deserves further investigation.

Acknowledgments
M.J.T., N.M., and J.G.G. designed research; M.J.T. and J.L.M. conducted research; B.S.M., I.C., and N.K. analyzed data; M.J.T., N.M., and J.G.G. wrote the paper. J.G.G. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited