Growth Hormone Reduces the Severity of Fibrosis Associated With Chronic Intestinal Inflammation

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Background & Aims: **Growth hormone (GH) is used to treat growth delay in children with Crohn's disease and in patients with short-bowel syndrome. GH can increase collagen accumulation in intestinal mesenchymal cells, raising concern that GH therapy could exacerbate fibrosis in patients with Crohn's disease. We tested if GH treatment altered inflammation or fibrosis during chronic, experimental granulomatous enterocolitis.** ***Methods:*** **Ileum and cecum of Lewis rats were subserosally injected with peptidoglycan-polysaccharide (PG-APS) or control human serum albumin. At the onset of chronic PG-APS–induced inflammation, rats were administered recombinant human GH or vehicle for 14 days. Fibrosis and inflammation were quantified by gross gut disease scoring, histologic scoring, type I collagen, and cytokine expression in cecum. Abundance and localization of suppressor of cytokine signaling-3 (SOCS-3) messenger RNA and/or protein were determined in cecum. Effect of GH, cytokines, or PG-APS on SOCS-3 synthesis was measured in intestinal myofibroblasts. Myofibroblasts overexpressing SOCS-3 were used to test whether SOCS-3 inhibits collagen accumulation.** ***Results:*** **In PG-APS–injected rats, GH modestly reduced gross adhesions and mesenteric contractions, cecal fibrosis score, and collagen expression, but had no effect on intestinal inflammation. GH increased SOCS-3 messenger RNA and protein abundance in PG-APS rats and SOCS-3 messenger RNA was localized to the periphery of granulomas. GH in combination with cytokines or PG-APS, but not alone, induced SOCS-3 synthesis in intestinal myofibroblasts. Myofibroblasts overexpressing SOCS-3 showed reduced cytokine-induced collagen accumulation.** ***Conclusions:*** **GH modestly reduces intestinal fibrosis associated with chronic experimental enterocolitis and stimulates expression of antifibrogenic SOCS-3, suggesting that GH therapy in inflammatory bowel disease should not exacerbate fibrosis.**

Intestinal fibrosis is a common complication of Crohn's disease (CD) and generally is considered an excessive, irreversible, wound-healing response to chronic transmural inflammation.[1] Fibrosis in this disorder involves overgrowth of the muscularis mucosa and muscularis propria,[2] excessive collagen deposition,[2,3] and mesenchymal cell hyperplasia.[4–6] Although fibrosis is variable in presentation in CD, it can lead to stricture and partial or complete bowel obstruction, which are serious complications for which endoscopic balloon dilation or surgical resection are the only current therapies.[7,8] Unfortunately, high recurrence of inflammation and fibrosis in CD patients contributes to repeat surgeries and complications and can result in short-bowel syndrome (SBS), a malabsorption syndrome resulting from insufficient functional bowel.[7,8]

Recombinant human growth hormone (rhGH), alone or in combination with glutamine and modified diet, has been tested as therapy in SBS patients in a number of clinical trials and was reported to improve weight gain and lean body mass and reduce the need for parenteral nutrition.[9–13] Clinical trials of rhGH in SBS patients reported varied outcomes on nutrient absorption and provided no consistent evidence about direct effects of GH on intestinal function or growth.[9,11,14–16] The use of rhGH in SBS patients remains controversial, despite recent Food and Drug Administration approval.[17] Some SBS patients treated with rhGH had CD, but no information was reported regarding rhGH effects on inflammation or fibrosis.[9,10] rhGH therapy improved linear growth in pediatric CD patients with growth delay.[18,19] A clinical trial of a small number of patients with active CD reported that GH therapy in concert with a high-protein diet improved disease symptoms measured by the Crohn's Disease Activity Index and decreased the need

Abbreviations used in this paper: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSA, human serum albumin; IGF-I, insulin-like growth factor; IL, interleukin; PG-APS, peptidoglycan-polysaccharide; rhGH, recombinant human growth hormone; SBS, short bowel syndrome; SOCS-3, suppressor of cytokine signaling-3; TNFα, tumor necrosis factor α.

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for other medications.[20] Thus, there is clinical evidence for benefits of GH in the setting of SBS and CD. However, potential effects of GH on fibrosis, a serious complication of CD, have not been analyzed in the clinical studies or in animal models of inflammatory bowel disease (IBD).

Beneficial effects of GH on intestinal inflammation have been documented in animal models of colitis. Administration of rhGH reduced inflammation in the trinitrobenzene sulfonic acid rat model of IBD.[21,22] Transgenic mice overexpressing GH exhibited improved mucosal repair after acute colitis induced by dextran sodium sulfate compared with wild-type mice.[23] Improved repair in transgenic mice overexpressing GH was associated with more rapid but transient increases in crypt cell proliferation. However, GH action on intestinal fibrosis was not analyzed in any of these studies. Overall, fibrosis during chronic inflammation has been less well studied than inflammation. This reflects the fact that many animal models of IBD do not exhibit fibrosis and analyses in clinical trials generally are limited to the mucosa and not the submucosal layers, which are predominant sites of fibrosis. Concerns that GH treatment could exacerbate fibrosis stem from preliminary observations that GH stimulates collagen accumulation in cultured intestinal myofibroblasts,[24] the primary mesenchymal cell type thought to mediate intestinal fibrosis.[6] GH also induces insulin-like growth factor-I (IGF-I) expression in some tissues and considerable evidence suggests that IGF-I may play a role in mediating fibrosis in CD.[1] This raises the possibility that GH therapy in IBD patients may increase directly or indirectly intestinal collagen deposition or fibrosis. This study tested the hypothesis that therapeutically administered GH exacerbates fibrosis in a rat model of chronic intestinal inflammation induced by peptidoglycan-polysaccharide polymers derived from group A streptococci (PG-APS), one of the few animal models characterized by fibrosis associated with chronic intestinal inflammation.[25] When injected subserosally into ileum and cecum of susceptible rat strains, PG-APS polymers induce acute inflammation for 24–48 hours followed by remission of inflammation and then spontaneous reactivation between 10–20 days after injection with progression to chronic granulomatous inflammation.[26,27] This model particularly is relevant to CD for a number of reasons. Reactivation and chronic inflammation is T-cell mediated and accompanied by systemic responses including arthritis, which is also a complication in a significant number of CD patients.[28] Importantly, chronic inflammation induced by PG-APS is known to involve severe transmural fibrosis and smooth muscle hyperplasia,[25] making this model particularly useful for studying the possible effects of therapeutic interventions on fibrosis. In the present study, we tested whether GH given therapeutically after reactivation of chronic inflammation altered the severity of fibrosis associated with chronic PG-APS–induced enterocolitis. As well as testing the effects of GH on fibrosis, we examined whether GH altered the severity of intestinal inflammation, key cytokine mediators of inflammation, or local expression of IGF-I, which is upregulated at sites of fibrosis in the PG-APS model[29] and in involved intestine of patients with CD.[6,30,31] Our study provides evidence that GH improves rather than exacerbates intestinal fibrosis in the PG-APS model and that this is correlated with local induction of suppressor of cytokine signaling-3 (SOCS-3). SOCS-3 is a cytokine-inducible signaling molecule that, to date, has been linked primarily to negative feedback regulation of cytokine action, particularly interleukin (IL)-6 action on macrophages and T cells.[32,33] Given the unexpected antifibrogenic actions of GH and its induction of SOCS-3, follow-up studies were performed in intestinal myofibroblasts to examine directly whether GH induces SOCS-3 in intestinal mesenchymal cells and to test whether SOCS-3 negatively modulates the effects of GH or IGF-I on collagen accumulation. Increasing evidence suggests a role for tumor necrosis factor α (TNFα) in inflammation-induced fibrosis in other organs including lung and kidney[34,35] and that TNFα is a key proinflammatory mediator in CD.[28] Therefore, we tested whether SOCS-3 influenced TNFα action on collagen accumulation in intestinal myofibroblasts.

Materials and Methods

Bacterial Cell Wall Preparation

PG-APS fragments from cell walls of group A, type 3, strain D58 streptococci (*Streptococcus pyogenes*) were isolated and prepared as previously described.[36] The preparation was sonicated immediately before use to disperse aggregates. The final PG-APS concentration based on rhamnose measurements was 12 mg/mL.

Experimental Protocol

Female inbred specific pathogen-free Lewis rats (140–170 g) were obtained from Charles River Laboratories (Raleigh, NC). All animals were housed in standard cages with 6 animals per cage and were allowed food and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and conformed to National Institutes of Health guidelines. Animals were anesthetized with 1.5 mL/kg body weight Innovar (Pitman-Moore, Washington Crossing, NJ) and 80 mg/kg body weight ketamine hydrochloride, and their intestines were exposed

by laparotomy using aseptic technique. A total of 24 rats were injected subserosally with PG-APS (12.5 μg rhamnose/g body weight) into 7 sites of the distal ileum and cecum. A control group of rats (n = 16) were injected with human serum albumin (HSA; 37.5 μg/g body weight, Baxter Healthcare Corporation, Glendale, CA). The rats were monitored for arthritis and were weighed daily. Of the 24 rats injected with PG-APS, 16 rats showed spontaneous reactivation of a chronic inflammatory response to PG-APS indicated by an increase in joint diameter of .6 mm from the previous measurement accompanied by edema and redness in 1 or both hind ankle joints. On the day of reactivation, rats were implanted with osmotic minipumps (Durect Corporation, Cupertino, CA) to administer rhGH (500 μg/kg body weight/day, Genentech Inc., San Francisco, CA) or vehicle (saline) for 14 days. The dose of GH was chosen to match the low doses reported in the literature for clinical trials in humans.[17] This dose is at the low end of a range of doses of GH tested for effects on the intestine in rodent models.[21,22,37,38] PG-APS–injected rats showing reactivation were paired based on comparable joint diameter at onset of reactivation and one of each pair was administered GH and the other was administered vehicle. The aim in pairing animals for comparable joint diameter was to minimize any potential effects of differences in disease severity at the onset of treatment. An experienced laboratory research analyst assigned the paired animals to vehicle or GH treatment groups and assigned a code number to each animal such that experimenters performing all subsequent analyses and evaluation or scoring of disease severity were blinded to the treatment groups. A total of 8 pairs of GH-treated and vehicle-treated PG-APS–injected rats were studied. HSA control rats were administered GH (n = 8) or vehicle (n = 8) in parallel with PG-APS–treated groups. Joint diameter and body weight were monitored daily throughout the experiment. After the 14-day treatment period, rats were killed by an intramuscular injection of sodium pentobarbital (100 μg/g body weight, Abbot Laboratories, Chicago, IL). The abdomen was opened by a midline incision and a gross gut disease score was derived as one measure of disease severity. Well-established criteria for gross gut disease score have been detailed in previous reports using the PG-APS model.[39] Briefly, 4 independent parameters consisting of extent of cecal wall thickening, severity of adhesions, severity of mesenteric contractions, and number of cecal nodules were each given a score ranging from 0 to 4, where 4 indicates the most severe disease. The scores for each parameter were totaled to derive an overall gross gut disease score, with a maximum possible score of 16.[39]

Sample Collection

Blood was collected by cardiac puncture for hematologic assays (performed by the Animal Clinical Core Facility at the University of North Carolina). Plasma was separated by centrifugation and plasma IGF-I concentrations were measured by enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories Inc., Webster, TX). Samples were pretreated as described in the enzyme-linked immunosorbent assay protocol to remove IGF-binding proteins complexed to IGF-I. Briefly, enzyme-linked immunosorbent assay samples were incubated with biotin-labeled rat IGF-I and goat anti-rat IGF-I antiserum in wells coated with rabbit anti-goat γ globulin. Unlabeled IGF-I in plasma and biotin-labeled IGF-I compete for limited anti-rat IGF-I binding sites. Streptavidin–horseradish peroxidase binds the antibody-free biotinylated rat IGF-I, whose concentration in standards and each sample is estimated based on enzymatic turnover of the substrate tetramethylbenzidine and absorbance at 450 and 620 nm.

The entire cecum was dissected and the contents were flushed with ice-cold .9% saline. The cecal tip was embedded in frozen tissue–embedding medium (Fisher Scientific, Fair Lawn, NJ) for in situ hybridization histochemistry. Adjacent samples were formalin-fixed and paraffin-embedded; sections were used for histologic evaluation of inflammation and fibrosis. The remaining cecum was cut longitudinally into 2 portions and homogenized immediately in 4 mol/L guanidine thiocyanate for RNA extraction or in protein extraction buffer (50 mmol/L Hepes, 150 mmol/L NaCl, 20 mmol/L Na pyrophosphate, 100 mmol/L NaF, 1.5% Triton X-100, and 100 mmol/L ethylenediaminetetraacetic acid containing protease inhibitors: 1 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 mmol/L vanadate; pH 7.4).

Histologic Scoring of Inflammation and Fibrosis

Coded paraffin-embedded, formalin-fixed sections of cecum were stained with H&E, Masson's trichrome, or Sirius red. H&E-stained sections were used to obtain a histologic score for inflammation using criteria previously described.[27,39] A score ranging from 0 to 4 (4 being the most severe) was assigned for both acute and chronic inflammation of each layer of the cecal wall. The acute and chronic scores were summed to derive an overall inflammatory score for each rat, and the maximum possible score was 32. The acute inflammatory score was based on hemorrhage, edema, polymorphonuclear leukocytic infiltration, and necrosis. The chronic inflammatory score was based on the number of mononuclear cells present.

Masson's trichrome– and Sirius red–stained sections of cecum were used to obtain a histologic score for fibrosis. Masson's trichrome stains collagen, whereas Sirius red stains only fibrillar collagen. Experimental sections were compared with sections from normal untreated control rats to derive a score ranging from 0 to 5 for increases in collagen deposition where 0 represents no increase and 1–5 represent a progressive increase in collagen in different layers of the cecal wall (Table 1). A score of 5 is the most severe fibrosis representing increased collagen deposition throughout all layers from mucosa to serosa (Table 1). The fibrosis score was multiplied by 1–4 to reflect 0%–100% extent of the section exhibiting fibrosis. At least 2 sections of cecum per animal were scored.

Total RNA Extraction and Northern Blot Hybridization Assays

Total RNA was prepared by the guanidine thiocyanate, cesium chloride procedure as previously described.[29] Northern blot hybridization was performed as previously de-

Table 1. Criteria for Histologic Fibrosis Score of Intestine

	Score	Description
Fibrosis	0	No increased collagen deposition
	1	Increased collagen deposition in submucosa
	2	Increased collagen deposition in submucosa and mucosa
	3	Increased collagen deposition in muscularis mucosa, submucosa, and mucosa; thickening, disorganization of the muscularis mucosa
	4	Increased collagen deposition in muscularis propria, muscularis mucosa, submucosa, and mucosa
	5	Increased collagen deposition throughout all layers including serosa
Percent involvement	1	0–25% of section
	2	25–50% of section
	3	50–75% of section
	4	75–100% of section

scribed[40] using ^{32}P-deoxycytidine triphosphate–labeled complementary DNA probes (Random Primed DNA Labeling Kit; Roche Diagnostics Corporation, Indianapolis, IN) specific for rat procollagen α1(I)[25], rat TNFα (a generous gift of Dr Karl Decker, University of Freiburg, Freiburg, Germany), and mouse SOCS-3 (a generous gift of Dr Douglas Hilton, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). ^{32}P-uridine triphosphate–labeled mouse IL-1β and rat IGF-I antisense RNAs also were used as probes.[41] To account for minor variations in RNA loading across samples, blots were reprobed for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) by using an antisense RNA probe prepared from a commercially available DNA template (Ambion Inc., Austin, TX). Blots were scanned on a Molecular Dynamics Storm 840 Phosphorimager and mRNA abundance was quantified using Image Quant software (version 1.2) for Macintosh (Amersham Biosciences, Piscataway, NJ). The abundance of each test mRNA was normalized to the abundance of GAPDH mRNA in each sample.

Western Immunoblot and Immunoprecipitation

A total of 100 μg cecal protein extracts were separated in 7.5% polyacrylamide reducing gels followed by semidry transfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) and blocked in .1% casein and .2× phosphate-buffered saline. Blots then were immunoblotted with a rabbit antibody specific for procollagen α1(I) (Rockland, Gilbertsville, PA) and incubated with a goat anti-rabbit secondary antibody conjugated to an infrared dye (IR Dye 800 Conjugated Goat Anti-Rabbit IgG; Rockland) at a 1:5000 dilution and signal visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The infrared-conjugated secondary antibody allows detection of signal without using the chemiluminescence detection method. Instead, the Odyssey System uses excitatory lasers, enabling fluorescence detection of the infrared-labeled secondary antibody. The benefits of using the Odyssey system vs chemiluminescence include detection of proteins across a wide linear range without the limitation of enzyme/substrate kinetics and greater sensitivity. The signal was quantified using the Odyssey Infrared Imaging System Application software version 1.2 (Li-Cor Biosciences). Subsequent blotting with actin antibody (Sigma, St. Louis, MO) was performed to control for differences in protein loading.

SOCS-3 was immunoprecipitated from 500 μg cecal protein extracts with 1 μg goat anti–SOCS-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μL 50% protein G agarose beads (Sigma). Nonspecific proteins were removed by washing 2 times in cold protein extraction buffer. The samples were heated in Promega (Madison, WI) 2× loading buffer at 100°C for 5 minutes and separated in 12.5% polyacrylamide reducing gels followed by semidry transfer onto polyvinylidene difluoride membranes. Membranes were blocked in .1% casein and .2× phosphate-buffered saline and immunoblotted with a rabbit anti–SOCS-3 antibody (a generous gift of Dr Douglas Hilton). Blots then were incubated with the goat anti-rabbit secondary antibody conjugated to an infrared dye and signal visualized using the Odyssey Infrared Imaging System. Immunoprecipitation followed by Western immunoblotting for SOCS-3 was performed because Western immunoblotting alone lacked the sensitivity needed to detect SOCS-3 in cecal protein extracts. Complete SOCS-3 recovery was ensured by follow-up immunoprecipitation of the cecal extracts with additional primary antibody and Western analysis.

In Situ Hybridization Histochemistry

Antisense and sense SOCS-3 RNA probes were generated by polymerase chain reaction using primers described by Rosell et al,[42] with the addition of a T7 RNA polymerase site 5′ to the antisense primer and a SP6 RNA polymerase site 5′ to the sense primer. Polymerase chain reaction products were used as a complementary DNA template to generate ^{35}S–uridine triphosphate–labeled sense and antisense RNA probes using either SP6 or T7 RNA polymerases, respectively. In situ hybridization was performed on 10-μm cryostat sections of cecum that were processed as previously described[6] with some modifications. Briefly, frozen sections were fixed in 4% paraformaldehyde, treated with proteinase K (.5 μg/mL), and acetylated with triethanolamine (.1 mol/L) and .25% (vol/vol) acetic anhydride. Sections were prehybridized for 1 hour at 62°C in hybridization buffer containing 75% formamide. Sections were hybridized with ^{35}S–uridine triphosphate–labeled probes in hybridization buffer for 18 hours at 62°C in a humidified chamber, treated with RNase, and washed in low-salt buffers at 62°C. Slides were exposed to NTB-2 emulsion (Kodak, Rochester, NY) for 14 days at 4°C, developed with D-19 developer (Kodak) and counterstained with hematoxylin. Sections were photographed under dark- and bright-field illu-

mination. Adjacent sections were stained with Sirius red to localize collagen and H&E for histology.

In Vitro Analyses

Mouse intestinal myofibroblasts were used to evaluate directly the effect of GH, IGF-I, and TNFα on collagen accumulation and the possible modulation of their actions by overexpression of SOCS-3. Mouse, rather than rat, intestinal myofibroblasts were used because mouse-derived cells have proved easier to transfect. Methods for preparation of early passage cultures of mouse intestinal myofibroblasts were modified from previously described methods used to isolate rat intestinal myofibroblasts.[25] Briefly, adult (40- to 50-day old) mice were killed and the ileum was dissected and washed in Dulbecco's modified essential medium plus antibiotics (100 U/mL penicillin and 50 μg/mL streptomycin). Whole-tissue pieces were washed repeatedly by pipetting into suspension and collection by centrifugation. After the final wash, the tissue pieces were resuspended in 5 mL Dulbecco's modified essential medium plus collagenase (300 U/mL CLS-1; Worthington, Lakewood, NJ) and dispase (.1 mg/mL; Roche Diagnostics Corporation) and placed on an orbital shaker (80 cycles/min) for 25 minutes at room temperature. Two volumes of Dulbecco's modified essential medium plus 10% fetal bovine serum then were added and the cell slurry was dispersed by vigorous pipetting. The cell slurry was allowed to settle and the supernatant was collected and centrifuged. The supernatant was discarded and the cell pellet was washed in Dulbecco's modified essential medium plus 10% fetal bovine serum and plated into a T-75 tissue culture flask in the same medium. Medium was changed after 48 hours to remove nonadherent/dead cells. Adherent cells were maintained in medium plus 10% fetal bovine serum with media changes biweekly. Myofibroblast phenotype was confirmed based on positive expression for vimentin and α-smooth muscle actin as evaluated by Western immunoblot.[25,43] Subconfluent cells were studied at passages 3–8.

Intestinal myofibroblasts were serum deprived and treated with GH (10^{-9} mol/L), TNFα (1 ng/mL), IL-6 (10 ng/mL), IL-10 (10 ng/mL), or PG-APS (20 μg/mL) alone or in combination with GH for 60 minutes. Doses of GH and PG-APS were based on maximal responses found by a dose response (data not shown). Doses of TNFα, IL-6, and IL-10 were based on maximal doses reported in other studies and the duration was chosen based on the time course of SOCS-3 induction by GH or IL-6 in other cell types.[44–46] Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). SOCS-3 mRNA abundance was determined by Northern blot hybridization as described earlier.

We previously have reported direct effects of GH and IGF-I to stimulate collagen accumulation in cultured intestinal myofibroblasts.[24] In the present study, intestinal myofibroblasts were transfected with a human pBIG2i-FLAGSOCS-3 expression vector or empty vector provided by Dr Richard Furlanetto (University of Rochester School of Medicine and Dentistry, New York, NY). pBIG2i-FLAGSOCS-3 contains the complete coding sequence of human SOCS-3 with a FLAG-tag epitope at the amino terminus. The empty vector is identical except that it lacks the inserted SOCS-3 coding sequence. Both vectors were purified by using an endotoxin-free plasmid kit (Qiagen, Valencia, CA). Cells were transfected with empty vector or pBIG2i-FLAGSOCS-3 using Effectene transfection reagent (Qiagen) and selected continuously with media containing 100 μg/mL Hygromycin B (Roche). Stable transfectants maintained their myofibroblast phenotype, as indicated by positive expression for vimentin and α-smooth muscle actin. Transfected cells were serum deprived and treated with rhGH (10^{-10} mol/L), human IGF-I (20 ng/mL), or mouse TNFα (1 ng/mL) and compared with no-treatment control cells. TNFα was tested as well as IGF-I because of increasing evidence for profibrogenic actions of TNFα in animal models of inflammation-induced fibrosis of other organs[34,35] and because of the established role of TNFα as a proinflammatory mediator in CD[28] and the PG-APS model. Reverse-transcription polymerase chain reaction verified overexpression of SOCS-3 in myofibroblasts transfected with the SOCS-3 expression vector (data not shown). Collagen accumulation in total cell lysates was determined by Western immunoblot. Cells were solubilized in sodium dodecyl sulfate sample buffer and size fractionated on 8.5% sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked in phosphate-buffered saline containing 3% nonfat dry milk and incubated for 16 hours at 4°C with a rabbit polyclonal antibody specific for procollagen α1(I) (Rockland). Blots were washed and then exposed to peroxidase-conjugated secondary antibody for 1 hour at room temperature. Blots were washed and immunoreactive proteins were detected by using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Blots were reprobed with rabbit polyclonal anti-actin as a loading control (Sigma). Radiograph films were analyzed by densitometry and signal intensity for procollagen α1(I), or loading control was quantified using National Institutes of Health image software (version 1.61; available at: http://rsb.info.nih.gov/nih-image/).

Statistical Analyses

Values are expressed as mean ± SEM. Data from in vivo experiments were analyzed by 2-way analysis of variance to test for significant effects of PG-APS or GH, and a significant interaction between PG-APS and GH, which provides statistical evidence for an effect of GH on the response to PG-APS. Subsequent pair-wise comparisons used Tukey's post hoc test to test for significant differences between 2 particular groups. Linear regression analyses were performed to test for possible correlations between particular parameters measured. Data from in vitro experiments were compared by 1-way analysis of variance for significant effects of treatments vs control followed by planned pairwise comparisons. A P value $<.05$ was considered statistically significant in all analyses.

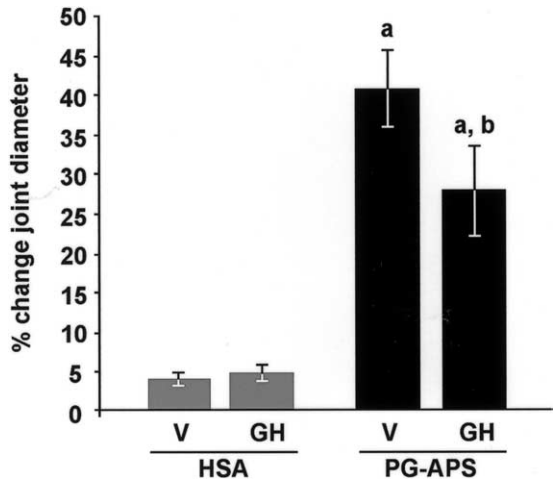

Figure 1. Mean percent change in joint diameter during GH or vehicle infusion in HSA- and PG-APS–injected rats. Values are mean ± SEM of percent joint diameter change in each animal after 14 days of infusion with GH or vehicle relative to diameter at the start of GH or vehicle (v) infusion. $^{a}P < .005$ vs HSA controls; $^{b}P < .05$ vs PG-APS + V.

Results

GH Reduces Gross Intestinal Injury and Joint Inflammation During PG-APS–Induced Colitis

We previously established that increased joint diameter provides a reliable and readily measurable sign of reactivation and progression of chronic inflammation and enterocolitis in the PG-APS model.[26] In this study, the diameter of both hind ankle joints was measured daily to monitor reactivation and as an overt measure of disease severity over the course of GH or vehicle treatment. Joint diameter at the onset of reactivation in PG-APS–injected rats was comparable in animals assigned to GH (15.26 ± .38 mm, pooled left and right joints) or vehicle (15.45 ± .35 mm) treatment groups and was significantly higher than in control animals injected with HSA and assigned to vehicle (13.32 ± .16 mm) or GH (13.08 ± .15 mm; $P < .05$) groups. Figure 1 shows the mean percentage change in joint diameter from start of vehicle or GH infusion to the end of the 14-day infusion period. HSA control animals showed no signs of inflammation or redness and only small increases in joint diameter, which are typical of normal growth, and did not differ significantly between vehicle- and GH-treated rats over the course of the 14-day treatment (Figure 1). PG-APS–injected rats showed significant increases in joint diameter typical of the worsening joint inflammation as chronic inflammation progressed. PG-APS–injected animals given GH showed significantly smaller increases in joint diameter compared with vehicle-treated PG-APS–injected animals (Figure 1).

Body weight, white blood cell count, and hemoglobin concentration were used as clinical measures of disease (Table 2). As a group, HSA-injected rats gained more weight than PG-APS–injected rats during the 14-day infusion period (7.6% ± 1.5% HSA combined vehicle and GH vs 2.1% ± 1.5% PG-APS; $P < .05$). When separated into vehicle or GH treatment groups, GH did not significantly increase body weight gain in either HSA- or PG-APS–injected rats compared with vehicle (Table 2). PG-APS–injected rats showed increased white blood cell counts and reduced hemoglobin concentrations, which are typical signs of disease in this model,[39] and GH did not alter these measures of inflammation.

HSA control rats given vehicle or GH had little or no gross evidence of intestinal injury, as indicated by low gross gut disease scores (Table 3). PG-APS rats showed extensive adhesions, mesentery contractions, cecal wall thickening, cecal nodules, and hepatic nodules (Table 3). PG-APS rats administered GH had small but significant reductions in gross disease score compared with PG-APS–injected rats given vehicle (Table 3). Significantly lower mean scores for adhesions and mesentery contractions, which are 2 complications of intestinal fibrosis, primarily contributed to the lower gross injury scores in GH-treated PG-APS–injected rats (Table 3).

Modest Reductions in Histologic and Biochemical Measures of Fibrosis in GH-Treated Rats

Bright-field photomicrographs of representative cecum sections stained with Sirius red for collagen are shown in Figure 2. Mean, blinded histologic scores for severity of fibrosis in cecum sections stained with Sirius red or Masson's trichrome are shown in Table 4. HSA control rats treated with vehicle or GH had low fibrosis scores (<1.0) with the majority of collagen deposition located within the submucosa, as is found in the normal cecum (Figures 2*A* and *B*). PG-APS rats showed obvious

Table 2. Percent Body Weight Change and Hematologic Profile in Rats Injected With PG-APS or HSA and Given GH or Vehicle

	% Increase body weight	WBC ($\times 10^3$/mL)	Hemoglobin (g/dL)
HSA + V	6.7 ± 1.4	5.2 ± .4	16.5 ± .2
HSA + GH	8.3 ± 1.3	7.4 ± 1.1	15.5 ± .8
PG-APS + V	1.4 ± 1.8[b]	19.3 ± 1.9[a]	13.4 ± .3[b]
PG-APS + GH	2.8 ± 2.4	17.9 ± 1.9[a]	12.6 ± .6[a]

NOTE. Values are expressed as mean ± SEM. n = 8 animals in each treatment group.
WBC, white blood cells; V, vehicle.
$^{a}P < .001$ vs HSA controls.
$^{b}P < .05$ vs HSA controls.

Table 3. Gross Findings of Rats Injected With HSA or PG-APS and Administered Vehicle or GH

	Gross injury score	Adhesions	Mesentery contractions	Cecal-wall thickening	Cecal nodules	Liver nodules
HSA + V	.2 ± .2	.1 ± .1	.1 ± .1	.0 ± .0	.0 ± .0	.0 ± .0
HSA + GH	.1 ± .1	.1 ± .1	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0
PG-APS + V	10.0 ± .9[a]	2.9 ± .4[a]	3.0 ± .3[a]	2.1 ± .2[a]	2.0 ± .3[a]	2.5 ± .4[a]
PG-APS + GH	8.0 ± .4[a,b]	1.7 ± .3[a,c]	2.1 ± .4[a,b]	1.9 ± .1[a]	2.2 ± .3[a]	1.7 ± .3[a]

NOTE. Values are expressed as mean ± SEM. n = 8 animals in each treatment group.
V, vehicle.
[a]$P < .001$ vs HSA controls.
[b]$P < .05$ vs PG-APS + V.
[c]$P < .01$ vs PG-APS + V.

increases in transmural collagen deposition (Figures 2*C*–*F*), validated by significant increases in mean histologic scores for fibrosis (Table 4). PG-APS rats treated with GH had a modest but significant reduction in histologic scores for fibrosis (Table 4) compared with PG-APS–injected rats given vehicle (Table 4), as shown also by the representative sections in Figures 2*C* and *E* vs Figure 2*D* and *F*.

Expression of procollagen α1(I) mRNA and protein in cecal extracts were assayed as biochemical markers of fibrosis. GH had no effect on collagen mRNA or protein abundance in HSA control rats. However, during PG-

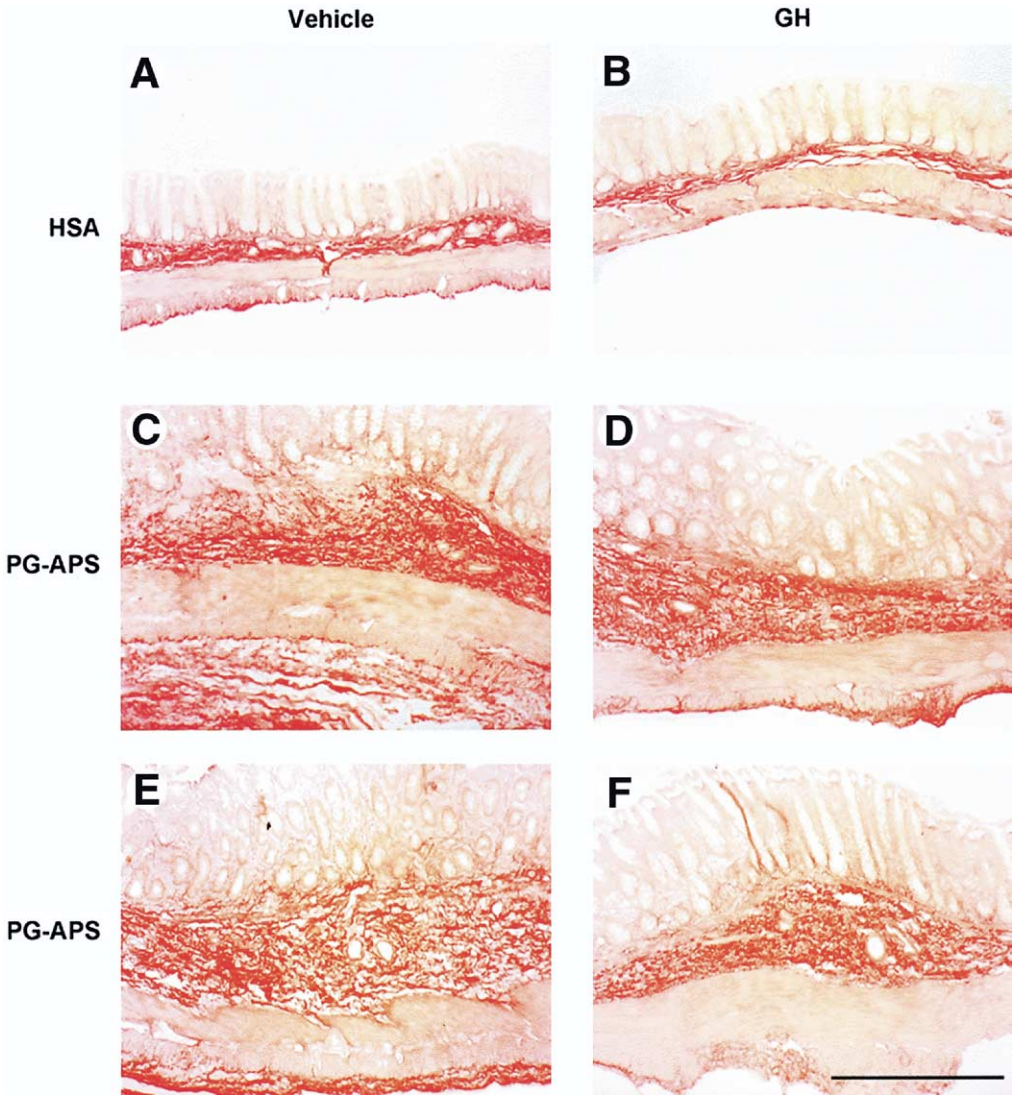

Figure 2. Representative bright-field photomicrographs of sections of cecum stained with Sirius red for collagen. (*A*) HSA + vehicle; (*B*) HSA + GH; (*C*, *E*) PG-APS + vehicle; (*D*, *F*) PG-APS + GH. (*F*) *Bar* = 500 μm.

Table 4. Fibrosis Scoring of Rat Cecum Injected With HSA or PG-APS and Administered Vehicle or GH

	Fibrosis score
HSA + V	.3 ± .1
HSA + GH	.7 ± .2
PG-APS + V	9.7 ± .5[a]
PG-APS + GH	7.9 ± .5[a,b]

NOTE. Values are expressed as mean ± SEM. n = 8 animals in each treatment group. Scores reflect evaluation of 4 sections of cecum for each animal.
V, vehicle.
[a]$P < .001$ vs HSA controls.
[b]$P < .05$ vs PG-APS + V.

APS–induced colitis, GH modestly reduced collagen mRNA (Figure 3*A*) and protein (Figure 3*B*) abundance compared with vehicle control rats. Regression analysis revealed highly significant correlations in individual animals across the independent measures of fibrosis, indicating consistent results: cecal collagen mRNA vs histologic fibrosis scores ($r = .66$; $P < .01$), cecal collagen mRNA vs cecal collagen protein ($r = .70$; $P < .005$), cecal collagen protein vs histologic fibrosis scores ($r = .51$; $P < .05$).

GH Treatment Does Not Affect Severity of Inflammation in the Cecum

To determine if effects of GH on fibrosis were associated with effects on intestinal inflammation, severity of colitis in the cecum was measured by an established blinded scoring system for acute and chronic inflammation.[27,39] PG-APS–injected rats showed dramatic increases in scores for acute and chronic intestinal inflammation compared with HSA controls (Table 5). GH treatment had no significant effect on acute or chronic inflammation scores in either HSA- or PG-APS–injected rats (Table 5).

Expression of IL-1β and TNFα mRNA, two proinflammatory cytokines known to be up-regulated at the mRNA level in the PG-APS model, also were quantified as independent biochemical markers of inflammation. Figure 4 shows representative Northern blots of IL-1β and TNFα mRNA expression in the cecum of HSA and PG-APS rats treated with vehicle or GH. PG-APS–injected rats had significantly increased levels of cecal IL-1β and TNFα mRNAs. GH treatment had no significant effect on IL-1β or TNFα mRNA abundance in cecum of PG-APS–injected rats. Histo-

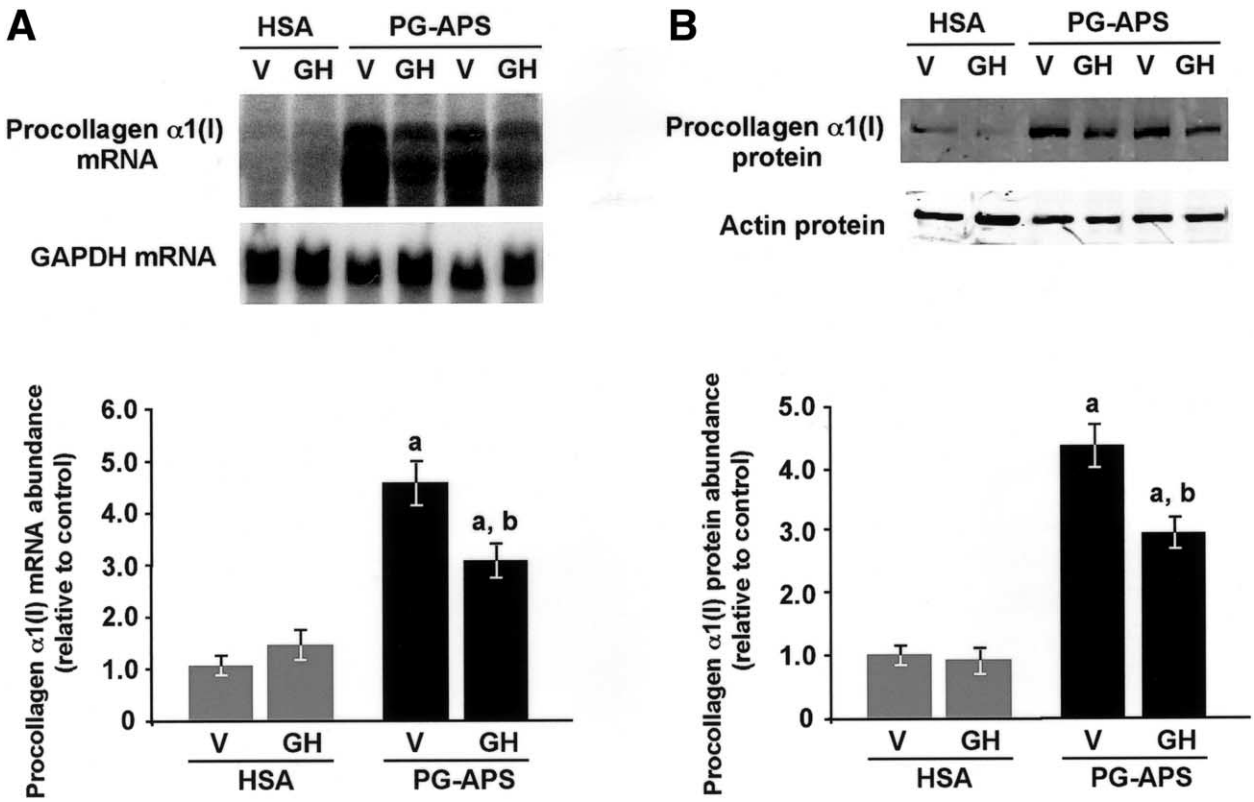

Figure 3. (*A*) Representative Northern blots showing procollagen α1(I) and control (GAPDH) mRNA abundance in total RNA isolated from cecum and (*B*) representative Western immunoblots for procollagen α1(I) protein and actin protein as a loading control in cecal protein extracts. Treatments are indicated. Histograms showing mean ± SEM of the fold-difference relative to the mean value of HSA + vehicle controls analyzed on the same blot. [a]$P < .005$ vs HSA controls, [b]$P < .05$ vs PG-APS + V.

Table 5. Histologic Scoring of Inflammation in Cecum Sections Stained With H&E From Rats Injected With HSA or PG-APS and Administered Vehicle or GH

	Total inflammation score	Acute inflammation score	Chronic inflammation score
HSA + V	.8 ± .2	.0 ± .0	.8 ± .2
HSA + GH	.9 ± .4	.0 ± .0	.9 ± .4
PG-APS + V	9.1 ± .9[a]	2.2 ± .5[a]	6.7 ± .3[a]
PG-APS + GH	9.7 ± .8[a]	3.1 ± .6[a]	6.9 ± .3[a]

NOTE. Values are expressed as mean ± SEM. n = 8 animals in each treatment group. Scores reflect evaluation of 2 sections of cecum for each animal.
V, vehicle.
[a]$P < .001$ vs HSA controls.

logic inflammatory scores were correlated directly with IL-1β expression ($r = .47$; $P < .05$) and TNFα expression ($r = .51$; $P < .05$) in individual animals, indicating consistent results from independent histologic and biochemical measures.

Table 6. Concentration of Plasma IGF-I in Rats Injected With HSA or PG-APS Followed by Treatment With Vehicle or GH

	Plasma IGF-I (ng/mL)
HSA + V	1102.9 ± 49.2
HSA + GH	1063.6 ± 50.3
PG-APS + V	1550.3 ± 97.0[a]
PG-APS + GH	1405.5 ± 50.8[a]

NOTE. Values are expressed as mean ± SEM. n = 8 animals in each treatment group, performed in duplicate. V, vehicle.
[a]$P < .05$ vs HSA controls.

Plasma Levels of IGF-I and Cecal Expression of IGF-I

Because many actions of GH are mediated by IGF-I, plasma IGF-I and local IGF-I expression in the cecum were measured to determine if the effects of GH on fibrosis or joint diameter were related to altered IGF-I. Plasma IGF-I concentration was increased significantly by approximately 50% during PG-APS–induced inflammation compared with controls (Table 6). GH treatment caused no further increase in plasma IGF-I

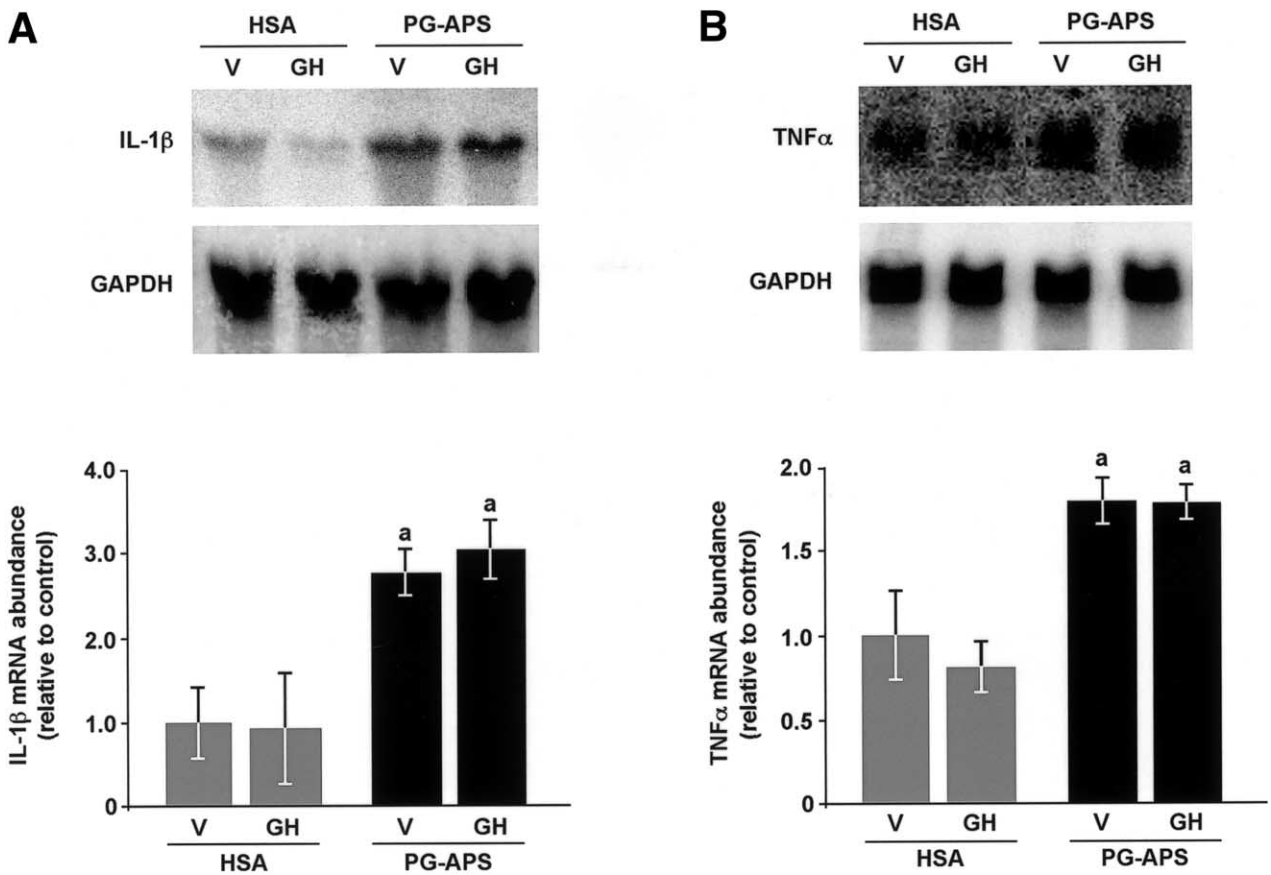

Figure 4. Representative Northern blots showing (*A*) IL-1β and (*B*) TNFα and control (GAPDH) mRNA expression in cecums of HSA- or PG-APS–injected rats treated with vehicle or GH. Histograms showing mean ± SEM of the fold-difference relative to the mean value of HSA + vehicle controls analyzed on the same blot. [a]$P < .05$ vs HSA controls.

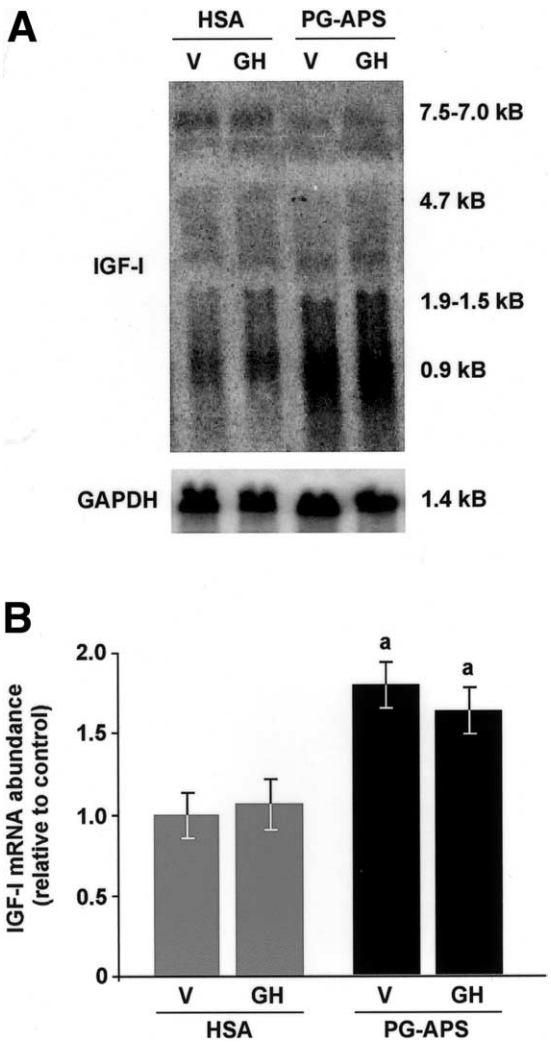

Figure 5. (*A*) Representative Northern blot showing IGF-I and control (GAPDH) mRNA expression in total RNA isolated from cecum. Treatments are indicated. (*B*) Histograms showing mean ± SEM of the fold-difference relative to the mean value of HSA + vehicle controls analyzed on the same blot. $^{a}P < .05$ vs HSA controls.

levels in PG-APS–injected rats and did not significantly alter plasma IGF-I concentrations in HSA-injected rats. Abundance of cecal IGF-I mRNA was increased during PG-APS–induced colitis as shown previously in this animal model,[29] but did not differ in GH vs vehicle-treated rats (Figure 5).

GH Increases SOCS-3 mRNA and Protein Abundance During PG-APS–Induced Colitis

Expression of SOCS-3, which has been implicated as a negative regulator of intestinal inflammation,[47] was assessed by Northern blot hybridization in total cecal RNA. SOCS-3 mRNA abundance was increased by 3-fold in PG-APS–injected rats that were administered GH ($P < .005$) compared with PG-APS–injected rats given vehicle, but was not increased significantly in GH-treated HSA-injected rats relative to vehicle control HSA rats (Figure 6*A*). SOCS-3 protein also was increased in GH-treated PG-APS–injected rats compared with vehicle-treated rats (Figure 6*B*). Linear regression analysis revealed that SOCS-3 mRNA abundance showed a significant inverse correlation with histologic fibrosis score ($r = -.74$; $P < .05$) and cecal collagen mRNA abundance ($r = -.69$; $P < .05$) in individual PG-APS–treated rats, and SOCS-3 protein abundance showed a significant inverse correlation with cecal collagen protein ($r = -.62$; $P < .05$), providing indirect evidence that increased SOCS-3 expression induced by GH during PG-APS–induced colitis could contribute to GH-dependent reduction in intestinal fibrosis. In HSA-injected control rats, there was no significant correlation between SOCS-3 mRNA levels and cecal fibrosis score ($r = .02$; $P = .51$) or collagen mRNA expression ($r = .04$; $P = .89$), indicating that the inverse association between SOCS-3 and fibrosis occurs only during inflammation. Analyses of SOCS-1 and SOCS-2 mRNAs, other members of the family of suppressor of cytokine signaling known to be induced in intestine by GH in some settings,[48] revealed very low constitutive expression and no induction by GH in HSA- or PG-APS–injected rats in total RNA isolated from cecum (data not shown).

SOCS-3 mRNA Localizes to the Periphery of Granulomas

In situ hybridization on cecal sections from PG-APS– and GH-treated rats localized SOCS-3 mRNA to the periphery of serosal granulomas (Figure 7*E* and *F*), correlating with sites of collagen accumulation visualized by Sirius red staining (compare Figure 7*C* and *E*). In adjacent sections, SOCS-3 sense control was negative, validating the specificity of hybridization signals obtained with the antisense probe. Cells at the periphery of granulomas in the PG-APS model were previously characterized as myofibroblasts[25] that deposit collagen protein, resulting in collagen surrounding the granuloma. SOCS-3 mRNA was barely detected in cecum of PG-APS given vehicle and HSA control animals, and expression could not be attributed conclusively to specific cell populations (data not shown).

GH in Combination With TNFα, IL-6, IL-10, or PG-APS Rapidly Up-Regulates SOCS-3 mRNA Abundance in Cultured Intestinal Myofibroblasts

Cultured intestinal myofibroblasts were treated with GH, TNFα, IL-6, IL-10, and PG-APS, alone or in combination with GH, to determine which factors induce SOCS-3 expression in cultured intestinal myofibroblasts. No cytokine or PG-APS alone up-regulated

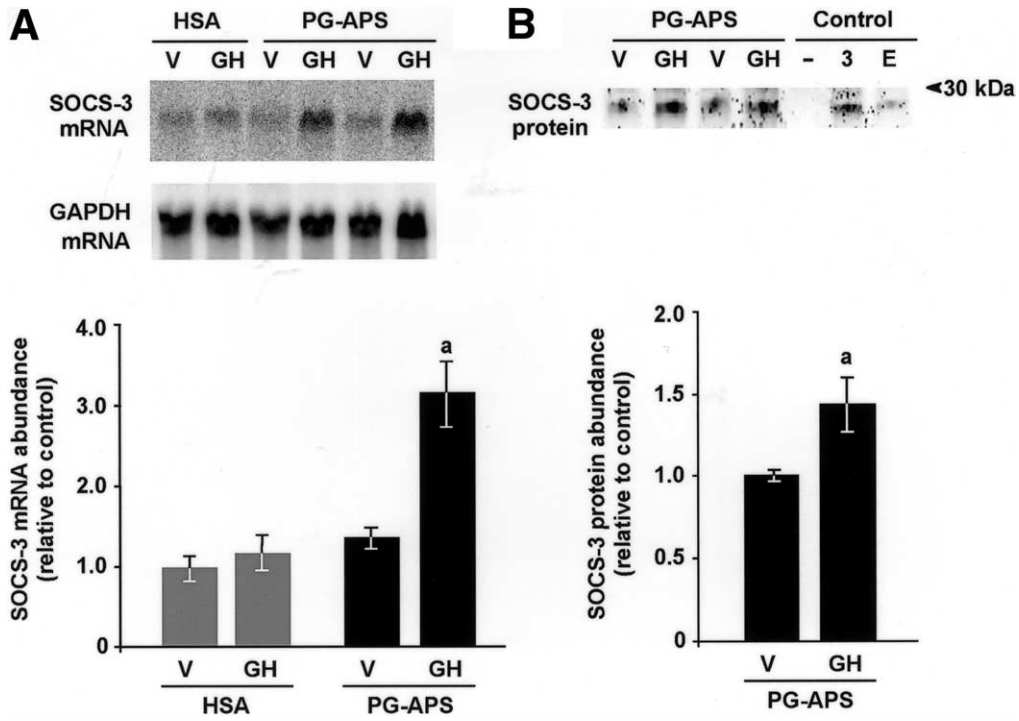

Figure 6. (*A*) Representative Northern blot showing SOCS-3 and control (GAPDH) mRNA expression in cecums of HSA- or PG-APS–injected rats treated with vehicle or GH. Values are mean ± SEM of SOCS-3 mRNA abundance normalized to GAPDH expression relative to the mean value of HSA + vehicle controls analyzed on the same blot. $^{a}P < .005$. (*B*) Representative Western immunoblot for SOCS-3 protein from cecal protein extracts immunoprecipitated for SOCS-3. Omission of primary antibody during the immunoprecipitation was performed as a negative control (−). Protein isolated from Caco-2 cells transfected with a SOCS-3 expression vector was used as a positive control (3) compared with cells transfected with empty vector (E). Histograms showing mean ± SEM of the fold-difference relative to the mean value of PG-APS + vehicle controls analyzed on the same blot. $^{a}P < .05$ vs PG-APS + V.

SOCS-3 mRNA abundance, but TNFα, IL-6, IL-10, and PG-APS, when given in combination with GH, all induced SOCS-3 expression (Figure 8).

GH, IGF-I, and TNFα Induce Collagen Accumulation in Cultured Intestinal Myofibroblasts and SOCS-3 Inhibits This Effect

To test whether SOCS-3 can modulate GH action on collagen accumulation, intestinal myofibroblasts stably transfected with a SOCS-3 expression vector or empty vector control were treated with GH. GH robustly stimulated collagen accumulation in empty vector–transfected cells and this effect was attenuated in SOCS-3–overexpressing cells (Figure 9). The effect of SOCS-3 was not limited to GH because IGF-I and TNFα each induced collagen accumulation in empty vector–transfected cells, but SOCS-3 overexpression attenuated the effects of GH and IGF-I and eliminated the effects of TNFα (Figure 9).

Discussion

Prior studies in animal models have indicated that GH promotes mucosal repair after chemically induced mucosal damage and acute intestinal inflammation.[21,23] Our study showed that GH does not exacerbate fibrosis associated with chronic PG-APS–induced transmural granulomatous enterocolitis. Rather, GH moderately reduced gross complications of inflammation associated with fibrosis such as adhesions and mesentery contractions and modestly reduced histologic and biochemical measures of fibrosis. These results of GH infusion in an animal model of chronic enterocolitis and fibrosis are reassuring with respect to potential complications of GH therapy in patients with IBD because they provide no evidence that GH worsens fibrosis, a serious complication that is common in patients with CD.

A second experiment in a small number of animals using an entirely different preparation of PG-APS revealed similar effects of GH to reduce gross gut scores for adhesions (vehicle, 3.5 ± .5; vs GH, 1.5 ± .5) and mesentery contractions (vehicle, 3.0 ± .0; vs GH, 1.5 ± .5), as well as histologic fibrosis score (vehicle, 11.7 ± .4; vs GH, 8.9 ± .5), validating the reproducibility of the antifibrogenic effects of GH. The reduced fibrosis in GH-treated rats could represent decreased synthesis and deposition of collagen, decreased myofibroblast proliferation, or a combination of both. The decrease in collagen

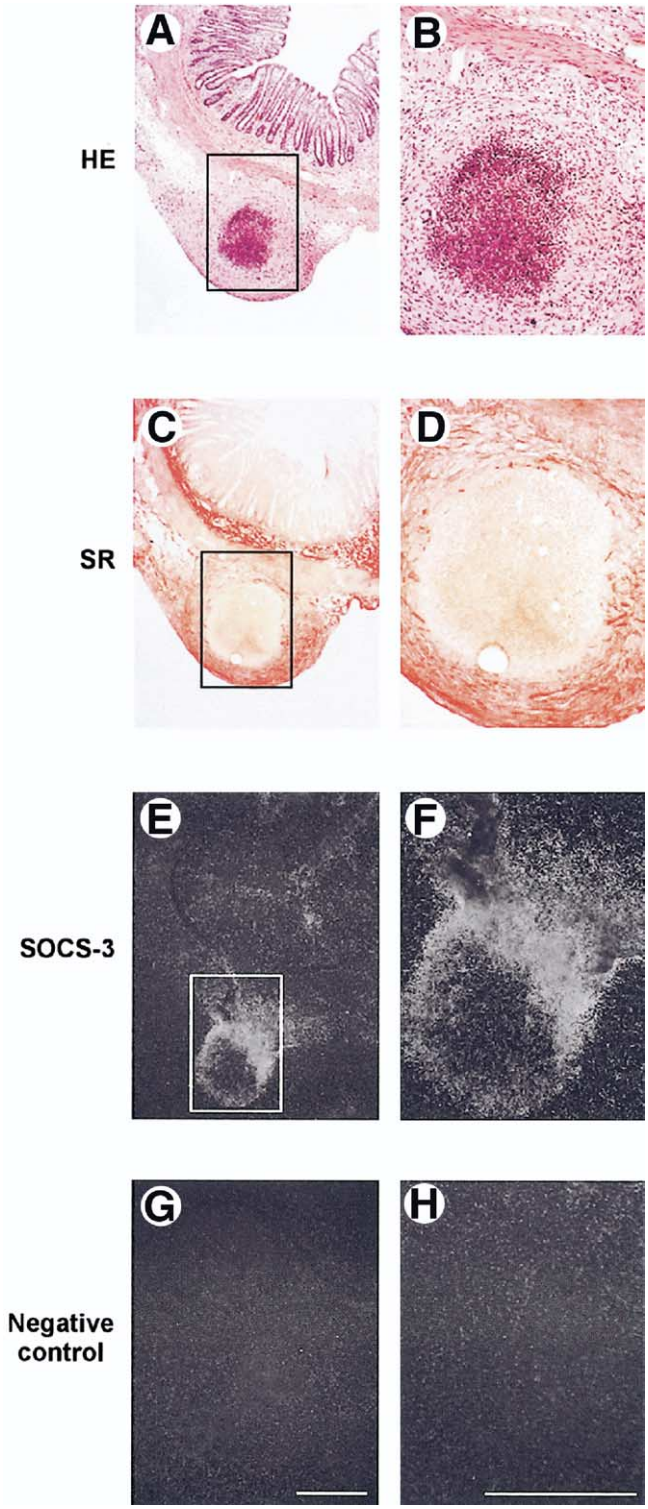

Figure 7. (*A–D*) Bright-field and (*E*, *F*) dark-field photomicrographs show in situ hybridization of SOCS-3 mRNA (*E*, *F*) at the periphery of a granuloma (inside box) in the serosa of a PG-APS + GH rat. (*C*, *D*) SOCS-3 mRNA localizes to sites of collagen deposition as revealed in adjacent sections by Sirius red staining. (*G*, *H*) A SOCS-3 sense RNA probe was used as a negative control and gave a negative signal. HE, H&E-stained adjacent section to show histology. SR, Sirius red–stained adjacent section to indicate localization of collagen protein (*red stain*). Box indicates area shown in higher magnification in the adjacent column. (*G*, *H*) Bar = 500 μm.

mRNA, protein, and histologic fibrosis scores in GH-treated rats supports an effect on collagen synthesis and accumulation. We did not evaluate directly the effects of GH on proliferation of mesenchymal cells at sites of collagen deposition. Attempts to identify proliferating mesenchymal cells by bromodeoxyuridine incorporation or immunostaining have proved problematic (Lund, unpublished data, November 2000). This is because it is difficult to distinguish mesenchymal cells from numerous immune cells labeled with bromodeoxyuridine. Thus, we cannot formally exclude an effect of GH on mesenchymal cell proliferation, although it should be noted that in cultured myofibroblasts GH has little or no effect on proliferation.[49] Although the reduction in fibrosis by GH was modest, it was verified by 4 different measures that include gross gut disease scores for adhesions and mesentery contractions, histologic fibrosis

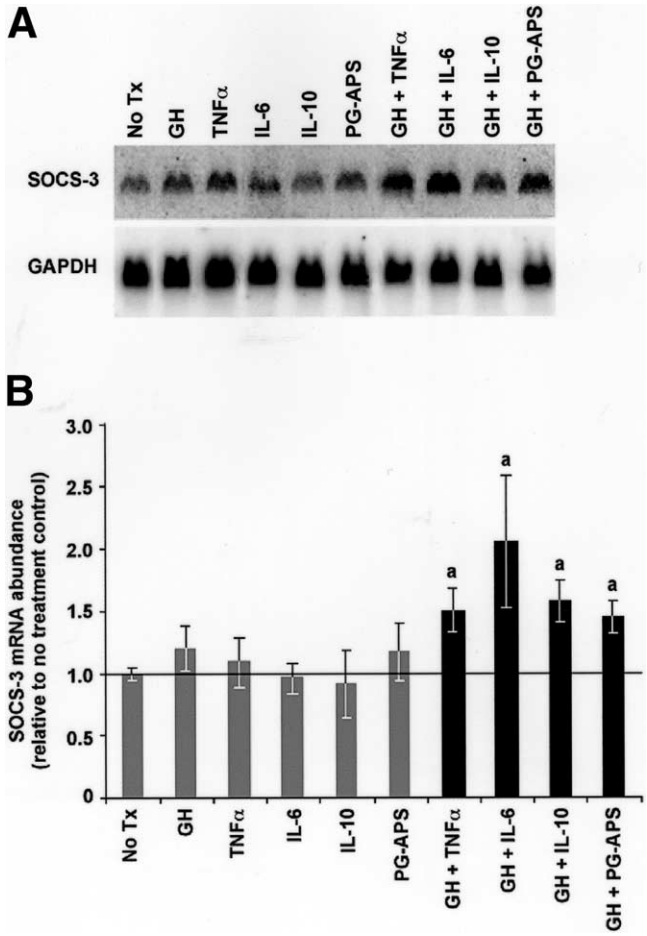

Figure 8. Representative Northern blot showing SOCS-3 and control (GAPDH) mRNA expression in cultured intestinal myofibroblasts treated with GH alone or in combination with various cytokines or PG-APS. Values are mean ± SEM of SOCS-3 mRNA abundance normalized to GAPDH expression relative to the mean value of no treatment (No Tx) controls analyzed on the same blot. $^{a}P < .05$ vs no treatment; n ≥ 3 per treatment.

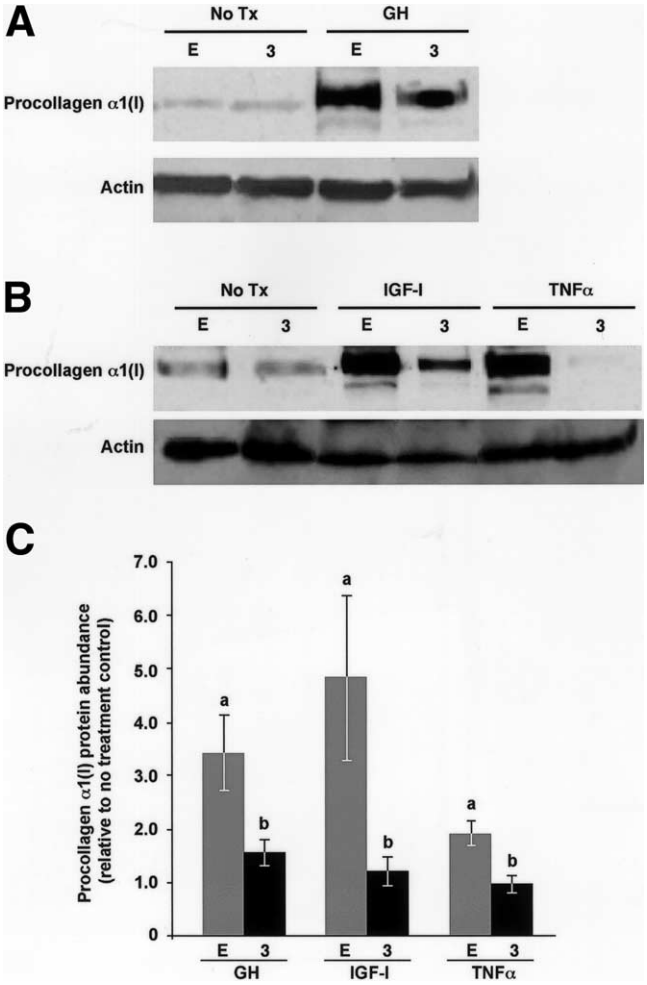

Figure 9. (*A*) Western blots of procollagen α1(I) protein accumulation and actin (control) in intestinal myofibroblasts transfected with SOCS-3 (3) or empty vector (E) expression constructs and treated with GH. (*B*) Western blots showing procollagen α1(I) and control protein abundance in intestinal myofibroblasts transfected with SOCS-3 or empty vector expression constructs and treated with IGF-I or TNFα. (*C*) Procollagen α1(I) protein abundance normalized to control protein abundance, relative to no treatment control value (set at 1) analyzed on the same blot. n ≥ 5 per treatment group. [a]$P < .05$ vs no treatment control, [b]$P < .05$ vs empty vector–transfected cells of the same treatment.

score, and type I collagen mRNA and protein abundance. Importantly, these results provide no evidence that GH will exacerbate fibrosis in patients with IBD. It is important to emphasize that GH was given therapeutically in a situation of ongoing chronic inflammation. Given that few, if any, current treatments are known to reduce complications of fibrosis, this effect in an experimental model indicates that evaluation of GH action on fibrosis in humans with SBS or IBD is warranted.

Despite modest beneficial effects of GH on fibrosis, therapeutic GH had no effect on intestinal inflammation assessed by histologic scoring of acute and chronic colitis, grossly evident cecal wall thickening and granulomas, or the level of expression of IL-1β and TNFα. The lack of effect of GH on inflammation is consistent with our prior findings in GH-transgenic mice in which GH overexpression did not alter the severity of inflammation or mucosal damage induced by dextran sodium sulfate administration, although GH-transgenic mice did show more rapid epithelial repair and crypt regeneration after dextran sodium sulfate treatment.[23] We did not examine effects of GH on epithelial responses to PG-APS–induced colitis in the present study, largely because the PG-APS model is one of progressive worsening of chronic submucosal-predominant inflammation that does not lend itself readily to studies of epithelial repair. However, the lack of effect of GH on inflammation indicates that even if GH did affect the epithelium in this model, this does not improve the course of chronic inflammation. Prior studies reporting anti-inflammatory actions of GH[22] or effects of GH on mucosal repair[50] involved GH administration before and during the induction of mucosal damage and inflammation. We deliberately administered GH therapeutically after the onset of chronic inflammation to best mimic the clinical actions of GH treatment during active, chronic IBD. The absence of an anti-inflammatory effect of GH on the intestine when given during active, chronic disease in the PG-APS model, but anti-inflammatory actions when given before disease onset, indicates that timing of GH therapy relative to time of disease onset or remission may be critical to maximizing the benefits of GH therapy in CD patients.

In the PG-APS model, GH treatment did result in moderate but significant reductions in the severity of joint inflammation, indicated by the smaller increase in joint diameter in GH-treated rats. Thus, GH did improve one of the extraintestinal inflammatory complications secondary to PG-APS–induced colitis. We did not perform joint histology or biochemical analysis of joint inflammation in this study, but the induction of SOCS-3 in intestine by GH is intriguing given findings that adenovirus therapy with SOCS-3 expression constructs improved arthritis in mouse models of antigen-induced and collagen-induced arthritis.[51] Therefore, GH may improve joint inflammation by inducing SOCS-3 and the study of the effects of GH on SOCS-3 or of other mechanisms of action in joint inflammation in animal models of IBD or in patients with IBD is warranted in the future.

Because many actions of GH are mediated through IGF-I, and IGF-I is implicated as a mediator of fibrosis in CD,[1] we had anticipated that GH may increase plasma or local levels of IGF-I and thus indirectly increase collagen synthesis or fibrosis during PG-APS–induced colitis. PG-APS inflammation increased plasma IGF-I concentrations

compared with control rats, but GH treatment showed no effect compared with vehicle infusion. This lack of response could be caused by the relatively low dose of GH used during the infusion, which was chosen to more closely replicate dosing in humans. In addition, it is well established that inflammation leads to a situation of GH resistance in the liver such that GH is unable to induce IGF-I synthesis.[52] Thus, the absence of an effect of GH to increase plasma IGF-I or body weight gain likely reflects this phenomenon. Cecal expression of IGF-I was increased during PG-APS inflammation, as shown previously,[29] but did not change with GH treatment. This indicates that systemic administration of low doses of GH does not increase local IGF-I expression further in the intestine above the already increased levels associated with chronic intestinal inflammation. This finding is especially important because considerable evidence suggests locally expressed, mesenchymal cell–derived IGF-I, acting in a paracrine and/or autocrine manner, may be a mediator of inflammation-induced intestinal fibrosis.[1]

In the liver, SOCS proteins have been shown to be induced by GH, and in settings of hepatic or systemic inflammation to prevent GH induction of IGF-I.[52] Recent findings in a rat model of total parenteral nutrition indicate that GH induces SOCS-2 in the small intestine and colon and that SOCS-2 can limit the proliferative actions of GH and IGF-I on the intestinal epithelium.[48] We therefore analyzed SOCS expression in cecum of HSA- or PG-APS–injected rats given vehicle or GH with the rationale that GH induction of SOCS could limit the fibrogenic actions of GH or endogenously up-regulated IGF-I that appears to mediate or contribute to fibrosis during intestinal inflammation. In contrast to the total parenteral nutrition model, GH administered to HSA control rats induced neither SOCS-2 nor SOCS-3 mRNA. At present, we have no experimental evidence to establish why cecal levels of SOCS-2 were not up-regulated in HSA rats by GH treatment as observed in total parenteral nutrition controls, except that the dose of GH was lower and the duration of treatment was longer. In addition, oral feeding in the current study vs total parenteral nutrition feeding, when GH was found to induce SOCS-2, could impact on GH induction of SOCS.

However, in PG-APS–injected rats, GH robustly induced SOCS-3 mRNA and protein relative to vehicle-injected controls. This is intriguing because SOCS-3 is implicated increasingly as a negative modulator of the actions of proinflammatory cytokines on macrophages and T cells.[32,33] However, in the PG-APS model, GH induction of SOCS-3 was not associated with reduced inflammation, nor did SOCS-3 levels show a significant negative correlation with measures of inflammation, as might be anticipated if, in this setting, SOCS-3 had anti-inflammatory actions. Instead, regression analysis revealed SOCS-3 mRNA abundance showed a significant inverse correlation with cecum fibrosis score and collagen mRNA abundance in individual PG-APS–treated rats and SOCS-3 protein abundance showed a significant inverse correlation with collagen protein abundance. However, HSA control rats showed no correlation between SOCS-3 mRNA expression and cecum fibrosis score or collagen mRNA expression. Furthermore, in situ hybridization revealed SOCS-3 mRNA localized to the periphery of granulomas in PG-APS–injected rats treated with GH, indicating SOCS-3 is induced in a relevant cell population. Previous studies have localized GH receptor expression in the intestinal mucosa layer and to mesenchymal cells in submucosa, muscularis, and serosa layers,[53] consistent with responsiveness of these cells to GH in vivo. Because it is difficult in in vivo studies to show directly that GH induction of SOCS-3 mediates the reduction in fibrosis, we used simple cell culture models to test this possibility.

Treatment of intestinal myofibroblasts with GH, TNFα, IL-6, IL-10, or PG-APS alone failed to induce SOCS-3 expression. However, GH in combination with these factors rapidly induced SOCS-3. These results indicate GH, in combination with other cytokines expressed during inflammation, can up-regulate SOCS-3 expression in intestinal myofibroblasts, but SOCS-3 is not up-regulated when GH is administered alone. This is important mechanistically given our in vivo evidence that SOCS-3 induction correlated with reduced fibrosis and more direct evidence in cultured myofibroblasts that SOCS-3 overexpression directly inhibited GH-, IGF-I– or TNFα-induced collagen accumulation. These results support a model (Figure 10) that induction of SOCS-3 expression in the inflamed intestine by GH, in combination with other cytokines, decreases the fibrogenic actions of IGF-I and TNFα, which are favored as mediators of fibrosis, and therefore SOCS-3 mediates antifibrogenic rather than profibrogenic actions of GH. One surprising finding in the present study was that SOCS-3 mRNA expression was not up-regulated in the cecum of rats with chronic PG-APS–induced enterocolitis vs HSA controls in the absence of GH therapy. This contrasts with increases in SOCS-3 expression reported in a number of IBD models, including the dextran sodium sulfate model and the IL-10 knockout mouse.[47] It seems likely that the difference in up-regulation of endogenous SOCS-3 across IBD models reflects variations in chronicity of inflammation and the particular cytokine milieu because it is known that SOCS-3 mRNA is induced cyclically, has a short half-life, and has been linked most

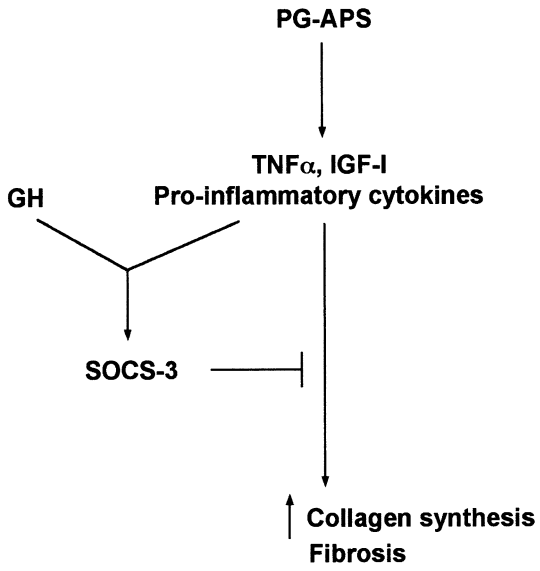

Figure 10. Hypothetical model showing PG-APS induction of proinflammatory cytokines that, together with therapeutic GH, induce SOCS-3. SOCS-3 decreases the fibrogenic actions of proinflammatory cytokines including TNFα, IGF-I, and GH itself, resulting in decreased intestinal fibrosis.

closely to IL-6 up-regulation in other models.[54] Nonetheless, our findings that GH induces SOCS-3 in a chronic granulomatous enterocolitis model that shares features with CD and the new evidence that this induction inversely correlates with severity of fibrosis, rather than inflammation, suggest a role of SOCS-3 as an antifibrogenic signaling intermediate in mesenchymal cells that warrants further investigation.

In conclusion, our findings in the PG-APS rat model of chronic intestinal inflammation and fibrosis indicate that GH treatment does not exacerbate intestinal fibrosis and has a modest therapeutic benefit to decrease intestinal fibrosis during chronic disease. These data extend previous findings suggesting beneficial effects of rhGH used as therapy in patients with CD and SBS.[10–13,16,18–20] Our study of therapeutic GH in an animal model of chronic intestinal inflammation and fibrosis provides no evidence that GH will exacerbate fibrosis in IBD, but instead provides new evidence that GH may have antifibrogenic actions mediated by the induction of SOCS-3 expression.

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