Suppressor of cytokine signaling-2 modulates the fibrogenic actions of GH and IGF-I in intestinal mesenchymal cells

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Submitted 1 October 2004; accepted in final form 6 April 2005

Fruchtmant, Shira, James G. Simmonst, Carmen Z. Michaylirat, Megan E. Miller, Christopher J. Greenhalgh, Denise M. Ney, and P. Kay Lund. Suppressor of cytokine signaling-2 modulates the fibrogenic actions of GH and IGF-I in intestinal mesenchymal cells. Am J Physiol Gastrointest Liver Physiol 289: G342–G350, 2005. First published April 14, 2005; doi:10.1152/ajpgi.00413.2004.—Growth hormone (GH) and IGF-I play important roles in wound healing during intestinal injury and inflammation, but there is also indirect evidence that locally expressed IGF-I may act to induce excessive collagen deposition, which can lead to intestinal fibrosis. Factors that dictate the balance between normal wound healing and excessive healing responses are unknown. Using RNAse protection assay and in situ hybridization, we determined whether GH and/or IGF-I increase type I collagen deposition in the intestine of rats fed by total parenteral nutrition (TPN), a feeding modality used for many patients following intestinal surgery and resection. We also used an in vitro model system to confirm our in vivo effects and to directly evaluate the relative potency of GH and IGF-I on DNA synthesis and collagen deposition in intestinal myofibroblasts. Both GH and IGF-I stimulated collagen production in vivo and in vitro, and IGF-I, but not GH, stimulated DNA synthesis in vitro. In collagen production, GH was less potent than IGF-I. Suppressors of cytokine signaling (SOC) are cytokine-inducible proteins that negatively feedback to inhibit the actions of cytokines and we recently found that GH selectively upregulates SOC-2 in the intestine of TPN-fed rats. We examined whether SOC-2 may be responsible for the difference in magnitude of action of GH and IGF-I on collagen accumulation. GH, but not IGF-I, induced SOC-2 in isolated myofibroblasts, and overexpression of SOC-2 led to a suppression of GH- and IGF-I-induced collagen accumulation. SOC-2 null mice infused with IGF-I showed greater collagen gene expression compared with wild-type (WT) mice. Myofibroblasts isolated from SOC-2 null mice showed increased IGF-I-stimulated DNA synthesis compared with WT cells. Taken together, these findings suggest that SOC-2 induced by GH may play an important role in suppressing collagen accumulation and mesenchymal cell proliferation induced by GH or GH-induced IGF-I, providing a mechanism for the differing potencies of GH and IGF-I on intestinal mesenchyme and collagen synthesis.

collagen; myofibroblasts; cell proliferation; fibrosis

THE INTESTINAL MUCOSA HAS a remarkable capacity to adapt its mass in response to altered nutrient intake and to restore or heal the mucosa after damage. Much attention has focused on the adaptive responses of the epithelial cells, particularly the proliferative rates of crypt epithelial cells essential for continued renewal of the epithelial lining. Less is known about adaptive responses of intestinal mesenchyme. Intestinal mesenchymal cells include subepithelial myofibroblasts that lie closely opposed to intestinal epithelial cells. Subepithelial myofibroblasts are prominent sources of type I collagen. Submucosa lying between the mucosa and muscularis layers is composed primarily of fibroblasts and also represents a rich source of type I collagen providing scaffolding for normal tissue integrity.

During situations such as total parenteral nutrition (TPN), the restriction of oral nutrient leads to atrophy of the intestinal epithelium (5, 39). Because this atrophy can delay or prevent resumption of oral feeding, a number of trophic factors have been tested for their ability to limit or reverse the mucosal atrophy associated with TPN. Growth hormone (GH) and IGF-I represent two such factors (7, 18, 20). GH infusion in TPN-fed rats increases plasma levels of IGF-I and improves some aspects of intestinal function, including glucose transport, but does not prevent the decline in crypt proliferation and atrophy of the intestinal epithelium (2, 4, 20).

To date, the responses of intestinal mesenchyme to TPN have not been extensively addressed, nor have their responses to trophic factors. Appropriate production of collagen and ECM by subepithelial mesenchymal cells is important to tissue integrity (36, 37). After intestinal resection, appropriate production of collagen by intestinal mesenchymal cells is important to healing of anastomoses (19). GH has recently been approved as therapy for patients with short bowel syndrome (1, 28–30) and is under testing as a potential therapy in Crohn’s disease (32), two conditions that can lead to TPN. Patients with Crohn’s disease show increased collagen expression in mucosa and submucosal layers, representing wound healing responses to inflammation (22). However, these responses, when excessive, lead to fibrosis, stricture, and obstruction, frequent complications of Crohn’s disease (41). Defining the effects of GH and IGF-I on collagen synthesis in intestine is therefore relevant to possible benefits and risks of GH therapy in short bowel syndrome and Crohn’s disease. One goal of this study was to assess whether TPN or supplementation with GH or IGF-I during TPN changes collagen production by intestinal mesenchyme.

Although some effects of GH are direct, GH mediates many of its actions indirectly via IGF-I (12). The ability of IGF-I, but not GH, to prevent the mucosal atrophy and normalize crypt proliferation during TPN (20) indicates that this traditional view of GH and IGF-I interaction may not be operative in the...
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intestine. Recent studies (17) indicate that GH may, in fact, induce reduced sensitivity of intestinal epithelial cells to the proliferative actions of GH and IGF-I by inducing a suppressor of cytokine signaling-2 (SOC-2), which limits the proliferative effects of GH and IGF-I in intestinal epithelial cells. SOC-2 is an intracellular signaling molecule known to limit the ability of GH to activate the downstream JAK and STATs which mediate some of the actions of GH (8, 9, 13, 24, 23). The possibility that SOC-2 may limit the actions of IGF-I is novel because IGF-I action is mediated by the type 1 IGF receptor tyrosine kinase rather than the cytokine receptors whose actions are typically modulated by SOC (10). The possibility is supported, however, by findings that SOC-2 interacts with the type 1 IGF receptor in yeast two-hybrid assays (3). Furthermore, IGF-I can, in some circumstances, induce STATs, particularly STAT-3, indicating that the IGF-I receptor is linked to pathways known to be modulated by SOC (42).

A role of SOC-2 as an inhibitor of collagen production is suggested by findings that mice with targeted disruption of both SOC-2 alleles show increased collagen accumulation in skin and vasculature (15). However, the direct role of SOC-2 in collagen synthesis by mesenchymal cells from any organ, including the intestine, has not, to our knowledge, been tested. In addition, although IGF-I has been shown to increase proliferation and collagen synthesis in fibroblasts from a variety of organs, GH has been less well studied, and the relative potencies of GH vs. IGF-I in stimulating collagen production or stimulating proliferation of collagen-producing mesenchymal cells has not been defined. Given the induction of SOC-2 by GH and not by IGF-I in the rat TPN model, we predicted that GH would have less potent actions than IGF-I on collagen expression in the intestine in vivo and that SOC-2 acts to inhibit or limit the actions of GH and/or IGF-I on collagen synthesis. This hypothesis was tested by examining collagen expression in the intestine of TPN-fed rats given vehicle, GH, or IGF-I. Isolated intestinal myofibroblasts from wild-type (WT) mice and mice with targeted deletion of both SOC-2 genes (SOC-2 null) were used to directly compare the effects of GH and IGF-I on proliferation and collagen production by intestinal mesenchymal cells and to establish whether SOC-2 modulates these actions. SOC-2 null and WT mice infused with IGF-I were also used to establish definitively whether in vivo SOC-2 limits collagen production within intestine in response to IGF-I. Our studies indicate that GH and IGF-I both stimulate increased collagen synthesis in intestinal mesenchyme in vivo and in vitro but that IGF-I is more potent. SOC-2 is induced by GH, but not by IGF-I, and limits the collagen accumulation stimulated by GH and IGF-I. Together, these findings indicate that SOC-2 could play an important role in limiting the fibrogenic actions of enterotrophic hormones and thereby protect against excessive wound healing responses and fibrosis.

MATERIALS AND METHODS

Animal Experiments

TPN and hormone treatments. All animal experiment protocols were approved by the Institutional Animal Care Committees of the University of North Carolina-Chapel Hill and the University of Wisconsin-Madison. TPN experiments were performed at the University of Wisconsin-Madison. These experiments have been described in detail elsewhere (2). Briefly, male Sprague-Dawley rats weighing 250 g (Harlan, Indianapolis, IN) were acclimated and fed ad libitum for 7 days. Animals were then fasted for 18 h and anesthetized by intramuscular injection of 80 mg ketamine plus 8 mg xylazine per kg body wt before installation of a catheter into the superior vena cava as previously described (20). Rats received a hypocaloric TPN solution for the first 3 days (10 ml/day on day 1, gradually increasing to 40 ml/day by day 3) and then isocaloric TPN for the final 5 days on TPN (55 ml/day). Animals were given recombinant human GH (rhGH) or rhIGF-I at a dose of 800 μg/day or an equal volume of saline vehicle during the final 5 of 8 days on TPN. The rhIGF-I and rhGH was added to the TPN solution daily for continuous infusion.

Tissue collection. Corresponding segments of jejunum were collected from each treatment group and flushed with cold saline. For RNA extraction, 15-cm long segments were flash frozen in liquid nitrogen and kept at −80°C until use. Adjacent segments were embedded in optimal cutting temperature compound (Miles, Elkhart, IN), frozen in cold isopentane on dry ice (−40 to −50°C), and stored at −80°C for in situ hybridization histochemistry.

RNA extraction and RNase protection assay. Total RNA was isolated by the guanidine thiocyanate-cesium chloride method previously described (27). Abundance of procollagen α1(I) mRNA was assayed in 20-μg aliquots of total RNA by RNase protection assay (RPA) using a [32P]-labeled procollagen α1(I) cRNA probe (25) and an RPA II kit according to manufacturer’s instructions (Ambion, Austin, TX). A rat glyceraldehyde-3-phosphate dehydrogenase cRNA (GAPDH; Ambion) was used as control. Protected RNA fragments were electrophoresed through a 4% urea-acrylamide denaturing gel. The gel was fixed in acetic acid, transferred to filter paper, dried, and exposed to a phosphorimaging screen. Quantification of type I collagen mRNA was performed using ImageQuant software (Version 1.2; Molecular Dynamics, Sunnyvale, CA). Abundance of type I collagen mRNA in each sample was normalized to that of GAPDH to control for differences in RNA loading.

In situ hybridization histochemistry. In situ hybridization histochemistry was performed on frozen sections of jejunum using antisense RNA probes for type I collagen and control sense probes labeled with [35S]UTP, as described previously (27, 33). After hybridization and washing, slides were dehydrated and exposed to Ilford Nuclear Autoradiographic Emulsion (Polysciences, Warrington, PA) at 4°C for 10–21 days. Slides were developed and counterstained with Mayer’s hematoxylin and then examined and photographed under both dark- and brightfield illumination. Positive hybridization with antisense probes was defined as clusters of silver grains observed over cells at densities higher than in sections hybridized with sense probe.

SOC-2 null and WT mice. Derivation of mice with targeted disruption of both SOC-2 alleles has been described previously (15). SOC-2 null mice on the C57Bl/6 background were bred with WT C57Bl/6 mice to generate male and female mice heterozygous for SOC-2 gene deletion. Male and female heterozygous SOC-2 mice were then bred to provide WT and SOC-2 null homozygous for analyses. Animal genotypes were established by PCR on tail DNA using oligomers for the WT (5’-CAGAGCTCAGTCACAAACAGGTAAG-3’, 5’-GCTTTCAAGTGTAAGGGTCTTCC-3’) or targeted allele (5’-GCACAGAGTGTGCAAGATTCC-3’, 5’-GATGCGACAGATTGCAACGC-3’).

Assays of IGF-I action on collagen expression in SOC-2 null and WT mice. To test whether SOC-2 affects basal or IGF-I-stimulated collagen synthesis in vivo, SOC-2 null and WT mice were treated with vehicle (saline, 12 μl/day) or IGF-I (2.5 μg·kg−1·day−1) for 5 days. Mice were anesthetized and ALZET microosmotic pumps (Durect, Cupertino, CA) were implanted subcutaneously to deliver vehicle, GH, or IGF-I. Colon was dissected, and samples were collected for extraction of total RNA. Collagen expression was assayed by Northern blot hybridization using [32P]-labeled cDNA probes for mouse type I collagen and with GAPDH probe (Ambion) as control as previously described (27). Collagen histology was assessed...
on dissected distal segments of colon (0.5 cm) fixed in 4% formalin, paraffin-embedded, and stained with Sirius red.

**Mouse intestinal myofibroblasts.** Cultured intestinal myofibroblasts were used as the primary in vitro model, because preliminary experiments established that they were more easily transfected than primary cultures of rat intestinal myofibroblasts and use of mouse cells permitted comparisons of myofibroblasts from small intestine of WT and SOC-2 null mice. Initial studies were performed in existing intestinal myofibroblast cell lines prepared from WT mice on the C57Bl/6 background. To assess the effects of SOC-2 deletion on cell responses, intestinal myofibroblast cell lines were also prepared from age- and sex-matched SOC-2 null and WT littermates. Mouse intestinal myofibroblasts were prepared based on a modification of a method used previously (6). Briefly, ~8 cm of mid-small intestine were removed, flushed with DMEM (GIBCO-BRL, Grand Island, NY) plus antibiotics, cut longitudinally and transversely into 2-mm segments, and washed repeatedly with medium. Following washing, fragments were digested in DMEM plus 0.1 mg/ml dispase (Worthington Biochemicals, Freehold, NJ) plus 300 U/ml collagenase (Worthington Biochemicals). Cells were subsequently pipetted, centrifuged, and plated. Cells were propagated in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Experiments were performed on cells between passages 3 and 8.

Using this procedure, we have verified that all cells express α-smooth muscle actin and vimentin, typical of myofibroblast phenotype. However, because whole thickness of intestine is used, we recognize that cells may originate from any mesenchymal layer within the intestinal wall including muscularis layers. During serum-starvation or treatment with GH or IGF-I, cells were cultured in medium minus serum, but supplemented with 5 mg/ml transferrin, 50 U/ml penicillin, 50 mg/ml streptomycin, and 0.05% BSA (Sigma, St. Louis MO). rhGH (Protropin) and rhIGF-I used for these experiments were from Genentech (South San Francisco, CA) and kindly provided by Dr. Louis Underground (University of North Carolina, Chapel Hill, NC).

**[3H]thymidine incorporation assay.** Cells were seeded in 24-well plates at a density of 1 × 10^4^ cells per well and allowed to attach for 24 h. Cells were then serum starved for 24 h to synchronize cell cycling and then treated with GH (10^{-10} M) or IGF-I (10^{-10} M) for 24 h in the presence of 2 µCi/ml [3H]thymidine. [3H]thymidine incorporation was measured as an indicator of cell proliferation (DNA synthesis) using methods previously described (31).

**Protein extraction and immunoblotting.** Cells (2 × 10^5^/well) were grown in 12-well plates treated with either GH (10^{-10} to 10^{-4} M) or IGF-I (10^{-13} to 10^{-10} M) for 48 h and collected directly into 80 µl of 2× sample buffer for loading onto SDS polyacrylamide gels. Whole cell extracts were run on 7.5% polyacrylamide reducing gels followed by semi-dry transfer onto Immobilon-P membrane (Millipore, Billerica, MA). Membranes were then blocked with 5% nonfat dry milk in TBS buffer and 2% azide overnight then immunoblotted with type I collagen antibody (Rockland, Gilbertsville, PA). Blots were then incubated with a secondary antibody and signal visualized by enhanced chemiluminescence (New England Nuclear Life Science Products, Boston, MA). Blots were then incubated with a secondary antibody and signal visualized by enhanced chemiluminescence (New England Nuclear Life Science Products, Boston, MA). Subsequent immunoblotting with antibody to either actin or β-tubulin (Sigma) was performed to control for differences in protein loading.

**RNA isolation and quantification (RT-PCR).** Myofibroblasts were seeded in 100-mm dishes, grown in DMEM-H plus 10% FBS, serum-deprived to synchronize cells, and then treated with 10 nM GH or 1 nM (10 ng/ml) IGF-I for 0–18 h. Total RNA was isolated using TRIzol reagent according to manufacturer’s instructions (GIBCO-BRL). Measurements of SOC-2 and GAPDH mRNAs were performed by reverse-transcribing total RNA into cDNAs and amplifying specific transcripts by PCR using specific oligonucleotide primers (mouse SOC-2, sense: 5’-GTGAGTGACAGTGTTCTGGAGAGAC-3’, anti-sense: 5’-GCATGAGTCCCCACACCTGTGG-3’; mouse GAPDH, sense: 5’-CTACTGGCGTCTGAAAAAGCTT-3’, antisense: 5’-GCCATGAGTCCCCACACCTGTGG-3’). PCR products were electrophoresed through a 1% agarose gel and visualized under UV light. The specificity of the SOC-2 amplicon was verified by DNA sequence analysis.

**Transfection.** To study the functional effects of SOC-2 on type I collagen induction by GH or IGF-I, mouse intestinal myofibroblasts were transfected with either an empty vector or human SOC-2 expression vector, pBIG2i-FLAGSOC-2, provided by Dr. Richard Furlanetto (Juvenile Diabetes Research Foundation). This expression vector contains the complete coding sequence of human SOC-2 with a FLAG tag epitope (3, 34). Both vectors were purified using an endotoxin-free plasmid kit (Qiagen, Valencia, CA) and cells were transfected with empty vector or pBig2i-FLAGSOC-2 using Effectene Transfection Reagent (Qiagen). Cells were then selected continuously with media containing 100 µg/ml hygromycin B (Roche, Indianapolis, IN). Stable transfectants maintained their myofibroblast phenotype (actin and vimentin positive). Expression of transfected vector was ascertained by RT-PCR of human SOC-2. To assess changes in type I collagen protein in SOC-overexpressing cells, cells were seeded in growth medium and collagen measured by immunoblot as described above.

**Statistical Analyses**

Values are expressed as means ± SE. One-way ANOVA followed by Fisher’s protected least significant differences multiple Student’s t-test was used to test for differences among groups. Two-way ANOVA followed by Tukey’s post hoc test was used in experiments using SOC-2 null and WT mice. Statistical significance was set at P < 0.05.

**RESULTS**

**Type I Collagen Expression in Jejunum of Rats on TPN, TPN plus GH, or TPN plus IGF-I**

RNome protection assays revealed that the amount of type I collagen mRNA within total jejunal RNA did not differ significantly in intestine of orally-fed and TPN-fed rats, but that both GH and IGF-I significantly increased type I collagen RNA (Fig. 1). In situ hybridization was used to assess type I collagen mRNA at the cellular level. This was particularly important because the atrophy of mucosal epithelium in TPN compared with orally fed rats and an increase in mass of epithelial cells in IGF-I- but not GH-treated rats (20) indicates possible differences across groups in the relative mass of collagen-producing mesenchymal cells compared with epithelial cells, which are not primary sources of collagen. Analyses of collagen expression on total jejunal RNA therefore could mask differences in type I collagen expression at the cellular level. Consistent with this possibility, in situ hybridization revealed that type I collagen expression was lower in subepithelial mesenchymal cells and, to a lesser extent, submucosa of TPN-fed vs. orally-fed controls. In situ hybridization also revealed that IGF-I potently induced type I collagen in subepithelial mesenchyme and submucosa (Fig. 2). GH did not have discernible effects on type I collagen mRNA in subepithelial mesenchyme and had less potent effects than IGF-I in submucosa (Fig. 2). Because these in vivo studies suggested differing potencies of GH and IGF-I on collagen synthesis, we used cultured intestinal myofibroblasts to directly compare GH and IGF-I action.
Type I Collagen Production and DNA Synthesis in Response to GH/IGF-I in Mouse Intestinal Myofibroblasts

Immunoblot assays revealed that GH or IGF-I treatment induced type I collagen production in intestinal myofibroblasts. At a similar concentration (10^{-10}M), IGF-I was more potent than GH (Fig. 3A). Although the focus of our analyses is on collagen production, we examined incorporation of [3H]thymidine into DNA as a measure of mitogenic actions of GH and IGF-I. This is because GH or IGF-I could influence net collagen accumulation or fibrosis by effects on mesenchymal cell.

Fig. 1. Jejunal type I collagen mRNA abundance in rats fed orally, total parenteral nutrition (TPN) alone, TPN + growth hormone (GH), or TPN + IGF-I. Procollagen α1(I) and control GAPDH mRNAs were quantified by RNase protection assay on 20 μg of total RNA extracted from jejunum of rats fed orally (Oral) or on TPN alone, TPN + GH (+GH) and TPN + IGF-I (+IGF). A: representative autoradiogram. B: histograms representing mean ± SE values for type I collagen normalized to GAPDH for each group. *P < 0.05 vs. TPN alone; n = 3.

Fig. 2. Localization of type I collagen mRNA in jejunum of rats fed orally (Oral) or TPN alone, TPN +GH or TPN + IGF-I, and sense control. Brightfield (BF) and darkfield (DF) photomicrographs illustrate in situ hybridization data. Sections of rat jejunum were hybridized with antisense mRNA probe complementary to rat procollagen α1(I). Signal is visualized under DF as white grains. BF represents a hematoxylin-stained section after hybridization with type I collagen probe to show histology of jejunum. Additional adjacent sections (from IGF-I group) were hybridized with a sense probe as a negative control. Lack of detectable hybridization signal with sense control probe indicates specificity of signals obtained with antisense probe. Note higher expression of procollagen α1(I) mRNA in IGF-I-treated rats fed by TPN compared with those treated with GH.

Fig. 3. Effect of GH or IGF-I on type I collagen deposition (A) and DNA synthesis in mouse intestinal myofibroblasts (B). A: Western immunoblots of type I collagen accumulation in GH (10^{-8} to 10^{-10}M)- or IGF-I (10^{-10}M)-treated intestinal myofibroblasts. Whole cell lysates were prepared from mouse intestinal myofibroblasts and treated for 48 h with serum-free medium without supplements (No Tx) or supplemented with GH or IGF-I. Blots were incubated with antibodies specific for type I collagen and actin (as loading control). A gel representative of three separate experiments is shown. Note that IGF-I is more potent than GH in stimulating DNA synthesis and inducing collagen deposition. B: histogram shows [3H]thymidine incorporation in mouse intestinal myofibroblasts treated with GH (10^{-10}M) or IGF-I (10^{-10}M) for 24 h. Results are expressed as degree of stimulation (means ± SE) compared with cells incubated in serum-free medium (No Tx). *P < 0.05 vs. no treatment (No Tx) control; n = 4.
proliferation as well as collagen synthesis (14), and differential effects of GH and IGF-I on proliferation of other cell types have been noted (17). IGF-I significantly stimulated DNA synthesis (2.3 ± 0.2-fold) in intestinal myofibroblasts compared with cells incubated in serum-free medium, whereas GH had little or no effect (Fig. 3B).

**SOC-2 Expression in Response to GH and IGF-I in Myofibroblasts**

We have previously established that GH induces SOC-2 mRNA in intestine of TPN-fed rats. We tested whether GH induces SOC-2 in isolated myofibroblasts. GH induced SOC-2 mRNA expression in mouse intestinal myofibroblasts within 1 h of treatment, and the increased expression persisted up to 18 h, the longest time point tested. GAPDH was used as an invariant control (Fig. 4A). SOC-2 mRNA was undetectable in cells treated with IGF-I (Fig. 4B).

**Overexpression of SOC-2 in Myofibroblasts Inhibits Collagen Induction by GH and IGF-I**

Intestinal myofibroblasts were transfected with empty vector (E) or SOC-2 expression vector (2) and treated with GH or IGF-I. GH and IGF-I stimulated type I collagen production in cells transfected with empty vector (Fig. 5), confirming our previous results in Fig. 3A. Compared with empty vector-transfected myofibroblasts, overexpression of SOC-2 inhibited the induction of type I collagen by GH and IGF-I (Fig. 5). Actin and β-tubulin (not shown) were used as loading controls.

**DNA Synthesis in GH- and IGF-I-Treated Myofibroblasts Isolated from SOC-2 Null and WT Mice**

To determine whether SOC-2 deficiency leads to increased IGF-I- or GH-induced DNA synthesis, we isolated myofibroblasts from SOC-2 null and WT mice. The myofibroblasts were serum deprived for 24 h and treated separately with GH or IGF-I. SOC-2 null myofibroblasts showed a dose-dependent increase in [3H]thymidine incorporation with IGF-I (0–10 ng/ml), whereas WT myofibroblasts were only responsive to IGF-I at the highest dose tested (10 ng/ml). Neither WT or SOC-2 null myofibroblasts treated with GH showed an increase in [3H]thymidine incorporation (Fig. 6).

**IGF-I More Potently Induces Collagen mRNA in SOC-2 Null Mice Relative to WT mice**

Because SOC-2 null myofibroblasts showed increased responsiveness to IGF-I, we examined whether they showed increased sensitivity to IGF-I in vivo. Microosmotic pumps containing IGF-I were inserted into SOC-2 null and WT mice for 5 days. IGF-I infusion stimulated type I collagen mRNA in colon of both genotypes, but SOC-2 null mice showed a greater induction (2.00 ± 0.18-fold) compared with WT mice (1.50 ± 0.11-fold) vs. vehicle controls of the same genotype (Fig. 7). Sirius red-stained sections of colon showed more collagen present in SOC-2 null mice infused with IGF-I compared with WT (Fig. 7C).

**DISCUSSION**

Both RPA and in situ hybridization histochemistry were used to evaluate type I collagen expression in jejunum of orally-fed and TPN-fed rats and in TPN-fed rats given GH and IGF-I. Both methods demonstrated that GH and IGF-I increase...
type I collagen mRNA abundance, indicating that, as well as documented effects on intestinal epithelial cells (2, 20), these peptides modulate mesenchymal function in vivo. Despite a similar abundance of type I collagen mRNA in orally and TPN-fed rats and similar increases in type I collagen mRNA by GH and IGF-I when assayed on total RNA extracted from jejunum, in situ hybridization provided evidence for differences in jejunal type I collagen mRNA expression at the cellular level. TPN-fed rats exhibited lower levels of collagen expression than orally fed rats in both subepithelial mesenchyme and submucosa. GH-treated rats exhibited induction of collagen only in submucosal and not subepithelial mesenchyme and IGF-I more strongly induced type I collagen in both regions. The apparently incongruous results from the two assays likely reflect the fact that epithelial cells, which do not express collagen, represent a greater proportion of cells contributing to total RNA extracted from jejunum in orally-fed and TPN-fed rats given IGF-I than TPN alone and TPN plus GH groups. This is because of the documented profound atrophy and hypoplasia of epithelium in TPN-fed rats and the ability of IGF-I, but not GH, to normalize epithelial mass during TPN (2, 20). Thus, in these circumstances of differences in relative abundance of collagen expressing mesenchymal cells across treatment groups, the in situ hybridization analyses provide important evidence for changes in type I collagen mRNA expression at the cellular level, which was not evident from biochemical assays on total RNA. Our observations of reduced type I collagen expression in subepithelial and submucosal mesenchyme during TPN provides, to our knowledge, the first evidence that TPN alters function of intestinal mesenchymal cells by reducing the expression of a major extracellular matrix component. The induction of type I collagen by IGF-I and, to a lesser extent by GH, indicates that, as well as documented effects of IGF-I to preserve epithelial mass or GH to maintain some aspects of epithelial cell function, these peptides also preserve collagen production by intestinal mesenchyme, which may be relevant to preservation of tissue integrity and tensile strength during TPN.

The less potent effects of GH on type I collagen expression than IGF-I are, at first sight, somewhat surprising given prior observation that GH increases body weight gain and plasma IGF-I levels as effectively as IGF-I in these same TPN-fed animals (2). Recent analyses of c-Fos and c-Jun mRNAs in...
TPN-fed animals given short-term GH and IGF-I treatments provided evidence that IGF-I more potently induced these early markers of activation of GH and IGF-I signaling pathways (2). c-Fos induction in particular was prominent in the mesenchyme layers of IGF-I- but not GH-treated rats (2). Given this observation and other studies indicating more potent effects of IGF-I than GH in intestine of TPN-fed rats, we have proposed that a state of postreceptor resistance to GH exists in the intestine of TPN-fed rats (2). The present observations of type I collagen suggest that this leads to less potent effects of GH compared with IGF-I on intestinal mesenchyme.

Our prior studies demonstrated that TPN-fed animals show reduced circulating levels of IGF-I compared with orally fed rats (2, 20), providing direct evidence that reduced plasma IGF-I may contribute to the reduction in collagen expression during TPN. However, both GH and IGF-I normalize circulating levels of IGF-I in TPN-fed rats (2, 20). Also, the levels of locally expressed IGF-I mRNA, which is expressed primarily in intestinal mesenchymal layers, does not differ in TPN-fed rats and those given GH or IGF-I (21). Thus the differential effects of GH and IGF-I on collagen synthesis during TPN cannot be readily explained by differences in circulating IGF-I or locally expressed intestinal IGF-I.

Because in vivo studies cannot reveal direct effects of GH and IGF-I on intestinal mesenchymal cells, we compared their ability to stimulate collagen accumulation and proliferation in isolated intestinal myofibroblasts. Consistent with in vivo data, IGF-I proved more potent than GH in isolated mesenchymal cells, even when GH was used at higher doses than IGF-I. Although less potent than IGF-I, our findings provide, to our knowledge, the first evidence for direct effects of GH on collagen synthesis in isolated intestinal myofibroblasts.

Our prior studies (17) indicated that GH differed from IGF-I in the induction of SOC-2 in intestine of TPN-fed rats. Because the phenotype of SOC-2 null mice indicated that SOC-2 may limit the growth-promoting and collagen-inducing actions of GH and possibly GH-induced IGF-I (15), we tested whether SOC-2 may underlie or contribute to the differing potencies of GH and IGF-I in intestinal mesenchyme. In vitro studies demonstrated that SOC-2 is induced in intestinal myofibroblasts by GH, but not by IGF-I, and that overexpression of SOC-2 limits the ability of both GH and IGF-I to induce collagen. In vivo studies demonstrated enhanced IGF-I-induced collagen expression in intestine of SOC-2 null mice compared with WT mice, providing compelling evidence that SOC-2 limits the in vivo actions of IGF-I on intestinal mesenchyme.

SOC-2 is a member of a family of intracellular signaling molecules known to act by negative feedback to limit or terminate the actions of cytokines such as GH, which act via the JAK-STAT pathway (10). A wealth of evidence suggests that SOC act by binding to cytokine receptors to prevent STAT activation or by reducing JAK activation (10). The concept that SOC limit the actions of growth factors such as IGF-I, which typically signal via receptor-tyrosine kinases distinct from cytokine receptors, is more novel. Our evidence that SOC-2 deficiency leads to increased IGF-I-induced mesenchymal cell proliferation and type I collagen gene expression while SOC-2 overexpression results in a reduction in IGF-I-induced collagen accumulation is the first, to our knowledge, to demonstrate a direct effect of SOC-2 on a functional response to IGF-I.

Although our studies do not define the GH- or IGF-I-activated signaling pathways modulated by SOC-2, studies on other systems indicate that SOC-2 associates with the type 1 IGF receptor (3) and may inhibit GH activation of STAT5b (8) or IGF-I activation of STAT3 (42). In addition, recent studies indicate a role for the ERK/MAP kinase pathway in mediating IGF-I action on collagen synthesis in smooth muscle cells (38) and in regulating GH induction of SOC-2 and IGF-I in skeletal muscle (26). Future experiments examining the role of these and STAT pathways in GH induction of SOC-2 or in SOC-2 regulation of IGF-I action in intestinal myofibroblasts will clearly be of interest.

The type I collagen gene contains response elements for the AP-1 transcription factor, comprised of fos and jun dimers, which are important determinants of collagen gene transcription (35) and are transcriptionally regulated by STATs (16, 11). Therefore, induction of type I collagen expression by GH or IGF-I may occur via AP-1 induction, as is the case for other cytokines and growth factor actions on collagen and suppression of GH, or IGF-I-induced collagen by SOC may occur via diminished STAT induction of AP-1. Our prior findings that GH but not IGF-I induces SOC-2 in intestine of TPN-fed rats and that IGF-I but not GH induces c-Fos in intestinal mesenchyme (17, 2) support the possibility that SOC-2 may limit AP-1 activation by GH or IGF-I. Additionally, it is important to consider the possibility that GH may induce collagen production in intestinal myofibroblasts via IGF-I and that the ability of SOC to limit fibrogenic response to GH could reflect reduced induction of IGF-I within myofibroblasts themselves.

Support for this possibility stems from findings that SOC-2 null mice have elevated local IGF-I expression in some tissues. We have attempted to explore whether GH induces IGF-I in cultured myofibroblasts and whether this is altered by SOC-2. Preliminary data did not reveal GH induction of IGF-I mRNA in cultured intestinal myofibroblasts and did not reveal differences in basal levels of IGF-I in the intestine of SOC-2 null mice (S. Fruchtman, P. K. Lund, and C. Michaylira, unpublished observations). We feel that these negative data should be viewed with caution, because we have observed that basal levels of IGF-I and IGF-II mRNA can vary across different myofibroblast cultures and we have only examined basal IGF-I expression in intestine of adult WT and null mice and not at other ages. Thus we feel that more extensive experiments will be required to definitively establish whether SOC-2 impacts on the ability of GH to induce IGF-I. However, the key point of the present study is that SOC-2 limits the collagen induction in intestinal myofibroblasts by exogenous IGF-I in vivo and directly inhibits IGF-I action on intestinal myofibroblasts in vitro. This latter effect has been observed in independent myofibroblast cultures derived from different animals and therefore seems unlikely to reflect different levels of endogenous IGF-I. We have also reported previously that IGF-I, but not GH, induces IGFBP5 in intestinal mesenchyme in vivo (21). Available evidence suggests that IGFBP5 is induced by IGF-I in cultured intestinal smooth muscle cells (40) and may impact on IGF-I regulation of collagen expression in intestinal mesenchymal cells (38, 40). It will therefore be of considerable interest in the future to examine the role of SOC-2 in IGFBP5 induction by IGF-I or in its modulatory effects on IGF-I action.

In vivo observations that SOC-2 null mice show enhanced type I collagen mRNA induction in response to infused IGF-I
could be complicated by effects of SOC-2 deficiency on relative mass of nonmesenchymal cells or cells that do not express type I collagen as was the case in TPN experiments. Indeed, our prior studies (17) suggest that SOC-2 null mice may show enhanced epithelial cell mass. This would tend to mask or reduce our ability to detect increases in collagen expression in SOC-2 null mice in the basal state or in response to IGF-I. Histological evidence, however, supports our observations that SOC-2 null mice infused with IGF-I show increased collagen deposition visualized by Sirius red staining, indicating that the enhanced collagen response to IGF-I is evident at the cellular level.

The differential induction of SOC-2 by GH and not by IGF-I in intestinal myofibroblasts has in vivo implications for the role of the GH-IGF-I axis in mesenchymal cell responses that may promote normal tissue healing and architecture or excessive responses that may lead to fibrosis. In multiple animal models of intestinal injury and inflammation or inflammatory bowel disease, there is induction of locally expressed IGF-I, which appears to be independent of GH and accompanied by inflammation-induced GH resistance (34a). Inflammation may therefore disturb normal balance of the GH-SOC-2-IGF-I axis in favor of excess IGF-I. This would permit excessive collagen production in response to IGF-I that is unrestrained or not normally limited by SOC-2. Normalizing such a balance could therefore promote restoration of tissue architecture and limit fibrosis. This model is speculative at present, and testing of such a model will require evaluation of the actions of GH on SOC-2 and collagen responses to IGF-I during mucosal injury or inflammation in vivo. Such studies will be of interest in the future.

In conclusion, these studies provide the first direct evidence that IGF-I and GH induce intestinal type I collagen expression in vivo and that induction of SOC-2 by GH treatment in intestinal myofibroblasts, a cell type implicated in intestinal wound healing and fibrosis, limits the fibrogenic response of these cells to GH or IGF-I. These results extend our understanding of the mechanisms by which GH and IGF-I promote wound healing in the intestine and provide new information to demonstrate less potent effects of GH compared with IGF-I on collagen expression. These outcomes are encouraging with respect to potential fibrogenic complications of GH therapy in patients with short bowel syndrome or intestinal injury and inflammation, such as Crohn’s disease, because GH induces a signaling molecule that limits the collagen stimulating actions of GH as well as IGF-I.

ACKNOWLEDGMENTS

We thank Randy Fuller, Brooks Scull, and Kirk McNaughton for their expert technical assistance. We also appreciate Dr. Douglas Hilton for providing the breeder stocks of SOC-2 null mice.

GRANTS

This work was supported by a National Institute of Diabetes and Digestive and Kidney Diseases Research Service Award DK-063748 (to S. Fruchtman) and Grants DK-47769 and DK-40247 (to F. K. Lund) and DK-42835 (to D. M. Ney). Work was also supported by the United States Department of Agriculture Cooperative State Research, Education and Extension Services project WISO4672 (to D. M. Ney).

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Soc-2 limits fibrogenic actions of gh and IGF-I


