IGF-I induces collagen and IGFBP-5 mRNA in rat intestinal smooth muscle

E. M. Zimmermann, L. Li, Y. T. Hou, M. Cannon, G. M. Christman, and K. N. Bitar. Insulin-like growth factor (IGF) binding protein 5 (IGFBP-5) mRNA was studied in intestines of rats with peptidoglycan-polysaccharide enterocolitis by Northern analysis and in situ hybridization. IGFBP-5 mRNA was increased 2.4 ± 0.5-fold in inflamed rat colon compared with controls and was highly expressed in smooth muscle. Cultured rat intestinal smooth muscle cells were used to study the regulation of IGFBP-5 and type I collagen synthesis. IGF-I (100 ng/ml) increased IGFBP-5 mRNA (1.9 ± 0.1-fold) and collagen type I mRNA (1.6 ± 0.2-fold) in cultured smooth muscle cells. IGF-I induced a dose- and time-dependent increase in IGFBP-5 in conditioned medium by Western ligand blot and by immunoblot. IGF-I did not affect the IGFBP-5 mRNA decay rate after transcriptional blockade. Cycloheximide abolished IGFBP-5 mRNA. In conclusion, IGFBP-5 mRNA is expressed by intestinal smooth muscle and is increased during chronic inflammation. IGF-I increases IGFBP-5 and collagen mRNAs in intestinal smooth muscle cells.

Inflammatory bowel disease; Crohn's disease; intestinal fibrosis; growth factors; insulin-like growth factor I; insulin-like growth factor binding protein 5

Methods

Animals. PG-PS enterocolitis was induced by the method of Sartor et al. (22) and Zimmermann et al. (27). Briefly, Lewis strain rats (135–150 g body wt, female, specific pathogen free; Charles River Laboratories, Wilmington, MA) were anesthetized (50 mg/kg ketamine and 5 mg/kg body wt xylazine by im injection) and underwent laparotomy using an aseptic technique. Rats received a total dose of either 37.5 μg/g body wt PG-PS (10S preparation; Lee Laboratories, Grayson, GA) or a control solution of human serum albumin (HSA; Baxter Health Care, Glendale, CA) by subserosal injection in seven standardized sites in the distal ileum and cecum (27). After laparotomy, the rats were given free access to food and water and were cared for according to standards established by the University Committee for the Use and Care of Animals at the University of Michigan. Rear ankle joint diameter was measured three times per week. Rear ankle arthritis has been studied three times per week. Rear ankle arthritis has been studied three times per week.
shown to correlate with the development of chronic intestinal inflammation (22, 27) and developed in 90% of PG-PS injected rats ~15 days after laparotomy. Rats were killed by inhalation of 100% CO₂ 28 days after laparotomy. The cecum was removed, rinsed in ice-cold phosphate-buffered saline (PBS), and frozen in liquid N₂ for Northern analysis or placed in plastic capsules of optimum cutting temperature compound (Miles, Elkhart, IN), and frozen in isopentane at ~50°C for in situ hybridization. Tissues were stored at ~80°C until the time of study.

Smooth muscle cell culture. RISM were prepared by a modification of an established method (15). Briefly, two Lewis strain rats were killed by inhalation of 100% CO₂, and the cecums were dissected from the peritoneal reflection to the cecum. The colon was slit longitudinally with tunotomony scissors and rinsed with ice-cold PBS with 3% penicillin-streptomycin (P/S; GIBCO-BRL, Gaithersburg, MD) and then in cold 70% ethanol, followed by PBS with 3% P/S. Mucosa was gently scraped from the deep intestinal layers with a scalpel blade and discarded. Fat and connective tissue were removed from the serosal surface. Tissue was minced by hand into ~5-mm² pieces and was placed in a 25-cm² culture flask (Corning, Corning, NY) containing 10–12 ml of sterile Hanks’ balanced salt solution (GIBCO-BRL) with 1 mg/ml collagenase (type 2; Worthington Biochemical, Freehold, NJ) and 3% P/S. Tissue was incubated in a 5% CO₂ incubator for 2 h at 37°C. Tissue fragments were rinsed in Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum (FBS; GIBCO-BRL) and 3% P/S and were triturated using a sterile plastic 10-ml pipette. Isolated cells were collected by filtration. Approximately 5 ml of the cell suspension were placed in a 25-cm² culture flask and incubated at 37°C in 5% CO₂. Culture medium was changed every 3 days, and cells were passed when they were 80% confluent. Experiments were performed on cell passages 6–12.

For culture experiments, cells were passed into 100-mm diameter dishes and were grown in DMEM with 15% FBS. When they were 80% confluent, cells were washed three times with serum-free DMEM to remove serum IGFs and then were incubated in serum-free DMEM for 2 h. Medium was removed, fresh serum-free DMEM was added, and the cells were incubated for 24 h. Cells were exposed to triplicate 10–200 ng/ml IGF-I (UBI, Lake Placid, NY) for 4 to 24 h. Conditioned medium was collected for Western ligand blot analysis, and RNA was extracted from cells for Northern analysis. In some experiments, 5 µg/ml actinomycin D, 5–10 µg/ml cycloheximide, or 75 µM 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) were added at the same time or 24 h after addition of IGF-I. Cells were collected for RNA analysis from 0 to 24 h after addition of actinomycin D, cycloheximide, or DRB.

In situ hybridization. The plasmid containing the rat IGFBP-5 cDNA (kindly provided by Drs. N. Ling and S. Shimisaki, Whittier Institute, San Diego, CA) was linearized using appropriate restriction enzymes. Sense and antisense 35S-labeled probes were generated using the T3 and T7 DNA-dependent RNA polymerases (GIBCO-BRL), respectively, in a standard in vitro transcription protocol (27).

In situ hybridization was performed on 10-µm-thick sections of rat cecum using methods previously described (27). Briefly, sections were fixed in 4% neutral buffered formaldehyde for 30 min, washed in 0.1 M PBS, and treated with 1 µg/ml proteinase K for 10 min. Slides were exposed to triethanolamine and acetic anhydride (Sigma Chemical, St. Louis, MO) for 10 min, washed, and dehydrated through graded alcohols. Sections were exposed to standard hybridization buffer (27) containing 75% formamide (GIBCO-BRL) and 1–2 x 10⁶ counts/min (cpm) radiolabeled probe for 18 h at 55°C. After hybridization, coverslips were removed, and the slides were treated with 200 µg/ml ribonuclease (RNase) A (Sigma Chemical) for 30 min, then washed in increasingly stringent standard sodium citrate (SSC) buffers with the most stringent being 0.5× SSC for 1 h at 55°C. Slides were dehydrated, exposed to X-ray film for 24 h, dipped in radiographic emulsion, and maintained at 4°C for 2 wk. Slides were developed and then viewed and photographed under light- and dark-field illumination using a Zeiss axiophot microscope (Carl Zeiss, Thornwood, NJ). Control experiments included slides exposed to RNase A for 60 min before hybridization with the antisense probe and slides hybridized with a sense probe.

RNA analysis. RNA was extracted from ~0.5 g of whole rat cecum using the method of Chirgwin et al. (3) with minor modifications (27). The RNA from cecal tissue was enriched for poly(A)+ using oligo(dT) cellulose chromatography (27). For RNA extraction from RISM, cells were grown to 80% confluence in a 100-mm dish. Cells were washed and lysed with 500 µl of quinidine/isothiocyanate. A rubber spatula was used to collect the lysed cells. RNA was extracted with phenol and chloroform-isomyelalcohol (49:1) and was precipitated with isopropanol. The pellet was dissolved in guanidine isothiocyanate and precipitated in ethanol. The pellet was washed with 70% ethanol, air-dried, and dissolved in diethyl pyrocarbonate-treated H₂O. The optical density at 260 nm was used to determine the RNA concentration of each sample.

The IGFBP-5 cDNA was isolated from the IGFBP-5/pBluescript SK(+) plasmid (Stratagene, La Jolla, CA) using appropriate restriction enzymes. The cDNA was purified from 1% agarose gel by electrodereution (IBI, New Haven, CT) and was radiolabeled with 32P (Amersham, Arlington Heights, IL) using a random priming kit (Boehringer Mannheim, Indianapolis, IN). Antisense oligonucleotide probes for rat procollagen aI(1) (25) and human a-smooth muscle actin (a-sm actin; see Ref. 26) were synthesized by the University of Michigan Biomedical Research DNA Synthesis Core Facility using an automated synthesizer (Applied Biosystems, Foster City, CA). Oligonucleotides were purified by high-performance liquid chromatography and were 32P 5’-end labeled by the kinase reaction (Boehringer Mannheim).

For Northern analysis, RNA was electrophoresed on 1% agarose gel (GIBCO-BRL) with 6% formaldehyde (Sigma Chemical). The gel was soaked in H₂O₂ to decrease the formaldehyde concentration, stained with ethidium bromide for 30 min, and then destained for 3 h. The presence of sharp bands corresponding to the 18S and 28S ribosomal RNAs were confirmed by ultraviolet illumination. RNA was transferred overnight to Nytran (Schleicher & Schuell, Keene, NH) using capillary action, and the blots were baked at 80°C. For slot blots, RNA was loaded onto the slot-blot apparatus (Schleicher & Schuell) and transferred to the membrane. Blots were prehybridized for 3 h and then hybridized over night in buffer containing 50% formamide, 5× SSC, 150 µg/ml salmon sperm DNA, 32P-labeled DNA probe, and a buffer containing 250 mM tris(hydroxymethyl)aminomethane (pH 7.5), 0.5% sodium pyrophosphate, 5% sodium dodecyl sulfate, 1% polyvinylpyrrolidone, 1% Ficoll, 25 mM EDTA, and 1% bovine serum albumin. Membranes were washed in increasingly stringent SSC washes, with the most stringent being 0.5× SSC and 0.1% SDS at 55°C for 30 min. Membranes were exposed to radiographic film overnight at ~80°C with intensi-
IGFBP-5 mRNA in normal and inflamed rat intestine. The abundance of IGFBP-5 mRNA was studied in intestinal tissue from PG-PS-injected and control rats. Northern analysis of RNA extracted from the ceca of rats showed a single 6.0-kb IGFBP-5 mRNA transcript consistent with prior reports of IGFBP-5 mRNA size (2). There was a 2.4 ± 0.5-fold increase in IGFBP-5 mRNA in RNA extracted from PG-PS-injected rats compared with control rats (Fig. 1; n = 6 pairs of rats; P = 0.02).

In situ hybridization was used to determine the cellular sites of IGFBP-5 synthesis in inflamed and control bowel. In situ hybridization using the antisense probe for IGFBP-5 demonstrated IGFBP-5 mRNA in smooth muscle cells of the muscularis externa of PG-PS- and HSA-injected rat colons (Fig. 2). IGFBP-5 mRNA was expressed in inflamed submucosa, including in macrophage-like cells within PG-PS-induced granulomas (Fig. 2; see Ref. 27). There was also expression of IGFBP-5 mRNA in individual cells within the expanded submucosa and serosa (Fig. 2). Many of these cells had large pale nuclei similar to the cells at the center of granulomas and likely represent macrophages. Inflamed submucosa and serosa contain a variety of tightly packed cell types, making it impossible to determine with certainty which cell types expressed IGFBP-5 mRNA by this technique. IGFBP-5 mRNA was expressed in inflamed foci in the lamina propria of the PG-PS injected rat colonic mucosa but was undetectable in the lamina propria of control rat intestines (data not shown). There was no detectable expression of IGFBP-5 mRNA in the epithelial layer of the inflamed or normal gut. Sections hybridized with the sense probe or pretreated with RNase A before hybridization with the antisense probe showed only low-level background labeling over the tissue (Fig. 2E).

Northern analysis and in situ hybridization demonstrated increased IGFBP-5 mRNA in inflamed regions of the intestinal mucosa, submucosa, and serosa with the highest expression in smooth muscle cells of the muscularis externa.

IGFBP-5 and procollagen synthesis in cultured intestinal smooth muscle cells. Given the expression of IGFBP-5 mRNA in intestinal smooth muscle and the data from Crohn’s disease suggesting that smooth muscle cells are important sites of intestinal collagen synthesis (16), we cultured smooth muscle cells from the rat muscularis externa for use as an in vitro system to study the expression of IGFBP-5 and collagen. RISM maintained a smooth muscle phenotype in culture as determined by continued expression of α-sm actin. There was no evidence of decreasing expression of α-sm actin mRNA at least through passage 15 (data not shown; n = 3, r² = 0.1, P = 0.32). Confluent cells exhibited typical growth characteristics, including the development of ridges and valleys (15).

RISM expressed IGFBP-5 mRNA consistent with in situ hybridization data that demonstrate IGFBP-5...
mRNA in normal smooth muscle of the muscularis externa (Fig. 2, D and E). There was a $1.9 \pm 0.1$-fold increase in IGFBP-5 mRNA after 24 h of exposure to 100 ng/ml IGF-I ($n = 3$ experiments in triplicate, $P < 0.01$; Fig. 3A). IGF-I also induced procollagen $\alpha_1(I)$ mRNA in RISM $1.6 \pm 0.2$-fold ($n = 2$ experiments in triplicate, $P = 0.04$; Fig. 3B). There was a dose-dependent increase in IGFBP-5 mRNA with increasing doses of IGF-I ($r^2 = 0.55$, $P = 0.006$; Fig. 4). Consistent with mRNA data from in situ hybridization and Northern analysis, IGFBP-5 was barely detectable in medium from control cells, as determined by Western ligand blot and immunoblot (Figs. 5 and 6). There was a dose-dependent increase in IGFBP-5 with increasing doses of IGF-I (Fig. 5). IGFBP-5 accumulated in conditioned medium of RISM exposed to IGF-I for increasing lengths of time from 4 to 24 h (Fig. 6). There was a $1.6 \pm 0.2$-fold increase in IGFBP-5 mRNA 8 h after IGF-I exposure compared with control cells at the same time point ($n = 3$ experiments in triplicate, $P = 0.04$).
IGFBP-5 mRNA remained increased at least 24 h after IGF-1 exposure (Fig. 3A).

Western ligand blot demonstrated a 32-kDa protein consistent with the size for IGFBP-5 (1) and a second faint 24-kDa band that is consistent with the published size for IGFBP-4 (Figs. 5A and 6A; see Ref. 2). The 32-kDa band was confirmed by immunoblot to be IGFBP-5; the 24-kDa band was not seen on immunoblot and was not characterized further.

Effect of transcriptional and translational blockade on IGFBP-5 mRNA abundance. The transcriptional blocker actinomycin D was used to determine the effect of blocking new gene transcription on the abundance of IGFBP-5 mRNA. When mRNA abundance was studied 24 h after addition of IGF-I and actinomycin D, actinomycin D had little effect on IGFBP-5 mRNA abundance. When studied at earlier time points (2 and 4 h), however, actinomycin D caused an increase in IGFBP-5 mRNA (data not shown). This paradoxical increase in mRNA with the transcriptional blocker has been previously described (21) and made actinomycin D noninformative in our system. DRB, another blocker of transcription, caused a decrease in IGFBP-5 mRNA when mRNA abundance was studied 0, 4, 8, and 24 h after addition of DRB (Fig. 7A). The slopes of the curves for cells with and without exposure to IGF-I were no different, suggesting that IGF-I did not increase IGFBP-5 mRNA abundance by increasing mRNA stability and thereby supporting the hypothesis that IGF-I increases transcription of IGFBP-5 mRNA. Cycloheximide, an inhibitor of protein synthesis, abolished IGFBP-5 mRNA (Fig. 7B), suggesting that new protein synthesis is important for integrity of IGFBP-5 mRNA.
DISCUSSION

Morphological studies of strictured intestine from patients with IBD suggest that smooth muscle cells are major sites of collagen synthesis (10, 16). Recent studies demonstrate that IGF-I stimulates proliferation of smooth muscle cells in vivo, suggesting that smooth muscle cells are important targets for IGF-I actions (24). We have previously shown that IGF-I mRNA is increased in inflamed and fibrotic intestine from patients with IBD and rats with PG-PS enterocolitis (5, 27). Here, we demonstrate that IGF-I increases expression of type I collagen mRNA in cultured intestinal smooth muscle cells. The data are consistent with a mechanism whereby IGF-I is increased during the development of chronic inflammation and acts on smooth muscle cells to increase collagen synthesis and promote fibrogenesis.

IGF binding proteins are emerging as key factors in the determination of the sites of IGF-I actions and the magnitude or extent of the biological effects of IGF-I (4, 20). Classically, the IGFBind-proteins inhibit the actions of IGF-I in vitro probably by competing for IGF-I with the target cell IGF type I receptor. Like other IGFBind-proteins, IGFBind-5 has been shown to inhibit the mitogenic actions of IGF-I in vitro; however, IGFBind-5 is unique in that, under certain experimental conditions, it enhances the mitogenic actions of IGF-I. The potentiating effects of IGFBind-5 are seen when IGFBind-5 is associated with ECM underlying the target cell monolayer (19). ECM-associated IGFBind-5 may enhance IGF-I actions by accumulating IGF-I near its receptor and/or protecting it from proteolysis (19). In addition, ECM-associated IGFBind-5 has a lower affinity for IGF-I than soluble IGFBind-5 and may act as a less effective competitor for IGF-I, thereby facilitating the interaction of IGF-I with its receptor (19). Here we demonstrate expression of IGFBind-5 mRNA in rat intestinal smooth muscle and increased IGFBind-5 mRNA in chronically inflamed intestine. Cultured intestinal smooth muscle cells were shown to express IGFBind-5 and type I collagen mRNAs, and these mRNAs increased after exposure of the cells to IGF-I. IGF-I increased IGFBind-5 mRNA and secretion of IGFBind-5 into conditioned medium. Our hypothesis is that IGF-I is increased in inflamed intestine and, in turn, induces synthesis of IGFBind-5 and collagen by smooth muscle cells. Secreted IGFBind-5 associates with the ECM adjacent to smooth muscle cells.

Fig. 5. Dose-dependent increase in IGFBind-5 in RISM exposed to IGF-I. Cells were exposed to 10–200 ng/ml IGF-I for 24 h. Conditioned medium was collected and Western ligand blot (A; exposed to film for 2 days) and immunoblot (B; exposed to film for 20 min) were performed. Each lane represents concentrated conditioned medium from a different cell culture dish (26.5 µg protein/lane). IGFBind-5 (32 kDa) in conditioned medium of control cells was barely detectable. IGFBind-5 in conditioned medium increased with increasing concentration of IGF-I. A faint 24-kDa band was detectable in cells exposed to 200 ng/ml IGF-I, consistent with the size for IGFBind-4 (18). A representative of 2 experiments is shown.

Fig. 6. IGFBind-5 in RISM conditioned medium increased with increasing duration of IGF-I exposure. RISM were exposed to 100 ng/ml IGF-I for 4–24 h. Control cells were cultured for 24 h in serum-free DMEM. Conditioned medium was collected, and Western ligand blot and immunoblot were performed. Each lane in Western ligand blot and immunoblot represents concentrated conditioned medium from a different cell culture dish (26.5 µg protein/lane). IGFBind-5 was barely detectable in control cells and increased with increasing length of exposure to IGF-I as shown by Western ligand blot (A; exposed to film for 3 days) and immunoblot (B; exposed to film for 5 min). Shown are representative blots of 2 experiments.
muscle cells and, as demonstrated in vitro, enhances the action of IGF-I to increase collagen synthesis, thereby promoting fibrogenesis. In vivo modulation of IGF-I or IGFBP-5 synthesis in the inflamed intestinal environment will be required to definitively determine the roles of IGF-I and IGFBP-5 in intestinal fibrogenesis.

The effect of IGF-I on IGFBP-5 mRNA and protein levels in conditioned medium appears to depend on the cell system studied. In human U-2 osteosarcoma cells (7) and human fibroblasts (1), IGF-I increased IGFBP-5 in conditioned medium but had little effect on IGFBP-5 mRNA. In other cell systems, including vascular smooth muscle, IGF-I increased IGFBP-5 mRNA and protein accumulation, as was observed in our study (6, 8, 9). Depending on the cell system, the effect of IGF-I on IGFBP-5 has been determined to be the result of increased transcription (6, 8, 9) or posttranslational mechanisms (1, 7). Our results differ slightly from prior studies in that the IGFBP-5 protein accumulation in response to IGF-I was much more pronounced than the mRNA induction. This may be related to lower activity of IGFBP-5 proteolysis in our system than in others. In vascular smooth muscle, a 22-kDa IGFBP-5 immunoreactive proteolytic fragment, but not intact IGFBP-5, was detectable in control cells (cells not exposed to IGF-I or heparin), and proteolytic fragments were as abundant as intact IGFBP-5 in conditioned medium from cells exposed to IGF-I (9). In RISM, IGFBP-5 fragments are not detectable, even with long exposures of blots, in medium from control cells or cells exposed to IGF-I.

Factors that increase mRNA abundance and ultimately IGFBP-5 concentrations may be key to the determination of the actions of IGF-I in vivo. In RISM, IGF-I does not increase the half-life of IGFBP-5 mRNA as determined by comparison of mRNA abundance in IGF-I-treated and control cells after addition of the transcriptional blocker DRB. The finding that IGF-I did not affect mRNA stability indirectly supports the hypothesis that IGF-I increases transcription of IGFBP-5. Direct methods of analysis would include nuclear run-on assay. However, this proved technically difficult in our system because IGF-I induced only a twofold increase in IGFBP-5 mRNA. Data from nuclear run-on assays in vascular smooth muscle (9) suggest that IGF-I mediates the increase in IGFBP-5 through increased IGFBP-5 gene transcription. The finding that cycloheximide completely abolished IGFBP-5 mRNA in RISM is interesting. It suggests an important role for synthesis of one or more intermediate proteins in maintaining IGFBP-5 integrity. In vascular smooth muscle, cycloheximide abolished only the IGFBP-5 mRNA induced by IGF-I (9), whereas, in RISM, all IGFBP-5 mRNA was abolished by cycloheximide. This difference may be related to differences between regulation of IGFBP-5 mRNA in vascular and intestinal smooth muscle and may indicate important regulatory proteins affecting intestinal IGFBP-5 mRNA.

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