IGFBP-3 and IGFBP-5 production by human intestinal muscle: reciprocal regulation by endogenous TGF-β1

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Bushman, Toni L., and John F. Kuemmerle. IGFBP-3 and IGFBP-5 production by human intestinal muscle: reciprocal regulation by endogenous TGF-β1. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1282–G1290, 1998.—Insulin-like growth factor-I (IGF-I)-mediated growth of cells can be modulated by specific IGF binding proteins (IGFBPs) that inhibit or augment IGF-I ligand-receptor interaction. IGFBP expression and production by human intestinal muscle cells in culture was characterized in rapidly growing cells (day 3 of culture), in confluent cells (day 7), and in postconfluent cells (day 14). RT-PCR analysis identified IGFBP-3, IGFBP-4, and IGFBP-5 mRNA during all three phases of growth. The production of IGFBP-3 and IGFBP-5 was regulated in reciprocal fashion. IGFBP-5 production was high on day 3 and decreased two- to fivefold by day 14, and IGFBP-3 production was low on day 3 and increased five- to eightfold by day 14. IGFBP-4 production remained constant. IGFBP-3 inhibited and IGFBP-5 augmented IGF-I-induced proliferation. IGFBP-3 and IGFBP-5 production was regulated in reciprocal fashion by transforming growth factor-β1 (TGF-β1). Immunoneutralization of endogenous TGF-β1 decreased the production of IGFBP-3 and increased the production of IGFBP-5. Addition of exogenous recombinant human TGF-β1 had the opposite effect. We conclude that the expression and time-dependent production of IGFBP-3, IGFBP-4, and IGFBP-5 and their regulation by endogenous TGF-β1 represent mechanisms by which human intestinal muscle cells regulate autocrine IGF-I-mediated growth.

Six human insulin-like growth factor-I (IGF-I) binding proteins (IGFBPs) have been identified, sequenced, and cloned (13, 14, 17, 29, 30). This family of proteins, which have no sequence homology with the IGF-I receptor, functions as modulators of IGF-I actions. Human IGFBP-1 through IGFBP-6 share ~50% homology within the family, and they each share ~80% sequence homology with IGFBPs of other mammalian species (13, 14, 15, 17, 29, 30). IGFBP-1 through IGFBP-6 are secreted proteins and have been identified in the circulation and the extracellular space; in addition, all but IGFBP-4, which has been identified in soluble form only, are present also on the cell surface or in association with the extracellular matrix (13, 14, 15, 17, 29, 30). Several mechanisms account for the modulatory effects of IGFBPs on IGF-I actions. Secreted IGFBPs, by binding and sequestering IGF-I, inhibit ligand-receptor interaction. IGFBPs on the cell surface or associated with the extracellular matrix, can either inhibit ligand-receptor interaction (IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-6) or promote ligand-receptor interaction (IGFBP-3 and IGFBP-5) (1, 4, 6, 15, 22, 28). Two recent lines of investigation suggest that this system is more complex than originally thought. Although IGFBPs modulate IGF-I actions, emerging evidence suggests that IGF-I may, in turn, modulate the actions of IGFBP-3, IGFBP-4, and IGFBP-5 through regulation of proteolysis of the binding proteins (6, 7, 28). In addition, IGF-I-independent effects have been reported for IGFBP-1, IGFBP-3, and IGFBP-5 (11, 19, 30).

The expression of IGFBPs by vascular and visceral smooth muscle cells is both species and tissue specific. Porcine vascular and aortic smooth muscle cells express IGFBP-2, IGFBP-4, and IGFBP-5 (9). Rat vascular and aortic smooth muscle cells express IGFBP-2, IGFBP-3, and IGFBP-4 (12) and human aortic smooth muscle cells express IGFBP-3, IGFBP-4, and IGFBP-6 (3). Rabbit airway smooth muscle cells express IGFBP-2 exclusively (24). Human myometrium expresses IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 (32). Smooth muscle cells of the rat colon express IGFBP-3, IGFBP-4, and IGFBP-5 (34, 36). An important role for the IGF-I/IGFBP system in the rat intestine is suggested by the observation that the induction of colitis in rats by treatment with trinitrobenzene sulfonate or the induction of enterocolitis by peptidoglycan-polysaccharide resulted in the upregulation of IGFBP-4 and IGFBP-5 expression (34–36). Exogenous IGF-I is capable of upregulating IGFBP-5 expression in cultures of rat colonic muscle cells (36). The expression and regulation of IGFBP production in the human intestine have not been investigated.

Our previous work (16) has shown that human intestinal smooth muscle cells in culture secrete IGF-I and transforming growth factor-β1 (TGF-β1). Although the levels of total IGF-I production remain constant in culture as cells are proliferating (day 3 of culture), attain confluence (day 7 of culture), and become postconfluent (day 14 of culture), the levels of free IGF-I decline with time in culture. This is paralleled by the decreasing ability of exogenous IGF-I to stimulate growth. These findings suggest that changes in IGFBPs may be responsible for the changes in IGF-I levels and its effects on growth. An opposite pattern is seen for TGF-β1: secretion is low early in culture and increases as cells attain postconfluence (16).

In the present study, we examined the expression of IGFBPs by human intestinal smooth muscle cells in culture by RT-PCR. The production of IGFBPs was...
METHODS

Preparation of isolated muscle cells from human jejunum. Muscle cells were isolated from the circular muscle layer of human jejunum, as described previously (16). Briefly, 4- to 5-cm segments of normal jejunum were obtained from patients undergoing surgery for morbid obesity according to a protocol approved by the Institutional Committee on the Conduct of Human Research. The segments were opened along the mesenteric border, the mucosa was dissected away, and the remaining muscle layer was cut into 2 x 2 cm strips. Slices were obtained separately from the circular layer using a Stadie-Riggs tissue slicer. The slices were incubated for two successive 60-min periods at 37°C in 25 ml of medium containing 0.2% collagenase (CLS type II) and 0.1% soybean trypsin inhibitor. The medium consisted of (in mM): 120 NaCl, 4 KCl, 2.6 KH2PO4, 2 CaCl2, 0.6 MgCl2, 25 HEPES, 14 glucose, and 2.1% Eagle's essential amino acid mixture. After the second incubation, the partially digested muscle strips were washed and incubated in enzyme-free medium for 30 min and the cells were allowed to disperse spontaneously.

Culture of human intestinal smooth muscle cells. Primary cultures of human intestinal muscle cells were initiated and maintained as described previously (16). Briefly, muscle cells dispersed from the circular layer were harvested by filtration through 500-µm Nitex mesh and centrifugation at 150 g for 5 min. Cells were resuspended and washed twice in PBS. The suspension was digested twice with 1 mg/ml collagenase for 15 min at 37°C and centrifugation at 150 g for 5 min and resuspension in Ca2+- and Mg2+-free Hanks' balanced salt solution (HBSS) containing 200 U/ml penicillin, 200 µg/ml streptomycin, 100 µg/ml gentamicin, and 2 µg/ml amphotericin B. After washing, the muscle cells were resuspended in DMEM containing 10% fetal bovine serum (DMEM-10) and the same antibiotics. The cells were plated at a concentration of 5 x 105 cells/ml as determined by counting in a hemocytometer. Cultures were incubated in a 10% CO2 environment at 37°C. DMEM-10 medium was replaced every 3 days until the cells reached confluence, at which time they were passaged. All subsequent studies were performed in first passage-cultured cells after 3 days in culture, when cells were proliferating, after 7 days, by which time the cells were confluent, and after 14 days, when the postconfluent cells had attained the "hill-and-valley" configuration characteristic of cultured smooth muscle (16).

Preparation of conditioned medium. Conditioned medium was prepared from muscle cells growing in 100-mm plates after 3, 7, or 14 days. At each time point, the cells were washed free of serum and incubated in DMEM in the absence of serum for 48 h. In some experiments the effect of TGF-β1 was examined by incubation of the cells with either recombinant human TGF-β1 (rhTGF-β1) (10 pM to 1 nM) or neutralizing antibody to hTGF-β1 (100 and 1,000 ng/ml) during the 48-h period. The requirement for new IGFBP synthesis was investigated by addition of cycloheximide (1 µg/ml). At the end of the incubation, the conditioned medium was removed and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (0.1 mM) and aprotenin (0.1 µg/ml) were added to the conditioned medium. Cellular debris was removed by centrifugation at 350 g for 5 min at 4°C. Aliquots of the resulting supernatant were concentrated 10-fold in Centricon-10 tubes at 4°C. The resulting concentrated conditioned medium was added to sample buffer containing 62.5 mM Tris (pH 6.8) with 2% SDS, 25% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol to provide samples derived from equal amounts of total cellular protein (200 µg/30 µl).

Preparation of whole cell lysates. Cell lysates were also prepared from the same cultures from which the conditioned medium was prepared. The cultured cells were washed three times in ice-cold PBS and then lysed in PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 µg/ml PMSF, 1% aprotinin, and 1 mM sodium orthovanadate. The cells were incubated for 20 min at 4°C and then DNA sheared by passage through a 21-gauge needle. Cellular debris in the lysates was precipitated by centrifugation at 12,000 g for 20 min at 4°C. Total cellular protein in each lysate was measured using the Bio-Rad protein assay (Hercules, CA). The resulting whole cell lysates were added to a volume of sample buffer containing 62.5 mM Tris (pH 6.8) with 2% SDS, 25% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol to provide samples of equal total cellular protein (30 µg/30 µl).

Preparation of RNA and RT-PCR analysis of IGFBPs. The expression of IGFBPs was investigated by RT-PCR analysis by modification of previously described methods (33). Briefly, total RNA was isolated from cultures of muscle cells growing in 100-mm culture dishes on days 3, 7, and 14 of culture, using Ultraspec RNA isolation reagent. Two micrograms of total RNA from each preparation were reverse transcribed in a reaction volume of 20 µl containing 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM dNTP, 2.5 µM random hexamers, and 200 U of SuperScript II RT. The reaction was carried out in a thermal cycler for 15 min at 25°C, for 50 min at 42°C, and terminated by heating to 70°C for 15 min. The reverse-transcribed cDNA (1 µl) was amplified in a final volume of 50 µl by PCR under standard conditions: 2 mM MgCl2, 125 µM dNTP, and 2.5 U Taq polymerase using specific primer pairs for IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 based on the known sequences of the six human IGFBPs (23, 26) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (18) as internal standard (Table 1). Sense and antisense primers were designed from different exons so that cDNA amplification resulted in PCR products of specific length, whereas genomic DNA would give longer amplified fragments. For each experiment, a parallel control without RT was processed. The amplified PCR products were separated on a 1% agarose gel containing 0.1 µg/ml ethidium bromide. The visualized bands were analyzed semiquantitatively using image scanning densitometry.

Measurement of IGFBP production in conditioned medium and in whole cell lysates by Western blot analysis. Samples prepared from either whole cell lysates from conditioned medium were boiled for 5 min and placed on ice before centrifugation for 5 min at 12,000 g. After centrifugation for 5 min at 12,000 g, each derived from equal amounts of total cellular protein, were separated by SDS-PAGE on 12% polyacrylamide gels. The separated proteins were electrophoresed to 0.2-µm nitrocellulose membranes overnight at 4°C in a buffer containing 25 mM Tris (pH 8.3) and 192 mM sodium bisulfite (55). After transfer, membranes were treated with 250 mM sodium acetate (pH 5.2) containing 0.1% Tween-20. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, and primary antibodies were added to the membranes for 1 h in the blocking buffer. After washing, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. After washing, the membranes were exposed to X-ray film. All samples were analyzed in at least triplicate. After quantitation by scanning density analysis on a Bio-Rad GS 800 Imaging Densitometer, the specific activities were calculated as the amount of labeled protein in each sample, as the amount of labeled protein in each sample, divided by the total cellular protein in each sample.
Table 1. PCR oligonucleotide primers and experimental conditions

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<th>No. of PCR Cycles</th>
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1IGFBP, insulin-like growth factor binding protein. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. S, sense. AS, anti-sense.

RESULTS

Characterization of IGFBPs by RT-PCR. The expression of IGFBPs by human intestinal smooth muscle cells during the three phases of culture was examined using semiquantitative RT-PCR. Total RNA was obtained from rapidly growing cells (on day 3 of culture), confluent cells (on day 7 of culture), and postconfluent cells (on day 14 of culture). RNA (2 µg) was reverse transcribed, and the resulting cDNAs were amplified using PCR with specific primers for each human IGFBP and for GAPDH as a control.

RT-PCR identified transcripts of the predicted size for IGFBP-3 (439 bp) and GAPDH (555 bp) during all three phases of culture (Fig. 1). Transcripts of the predicted size for IGFBP-4 (310 bp) and IGFBP-5 (377 bp) were also identified during all phases of culture (Fig. 1).

The relative mRNA levels for each IGFBP on days 3, 7, and 14 were measured densitometrically and normal-
Fig. 1. Human intestinal muscle cells in culture express insulin-like growth factor (IGF) binding protein-3 (IGFBP-3), IGFBP-4, and IGFBP-5 mRNA. Expression of IGFBP-3, IGFBP-4, and IGFBP-5 by human intestinal muscle cells in culture was investigated, using RT-PCR, during 3 phases of culture: rapid growth (day 3), confluence (day 7), and postconfluence (day 14). Top panel: RT-PCR using IGFBP-3-specific primers yielded a single product of expected 439-bp size. Top middle panel: RT-PCR using IGFBP-4-specific primers yielded a single product of expected 310-bp size. Bottom middle panel: RT-PCR using IGFBP-5-specific primers yielded a single product of expected 377-bp size. Bottom panel: RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers yielded a single product of expected 555-bp size. Total RNA prepared from cells on days 3, 7, or 14 of culture was reverse transcribed, and the resulting cDNA was amplified by PCR with specific primers for each IGFBP and GAPDH, as described in Methods and Table 1. Resulting products were separated on 1% agarose gels and visualized with ethidium bromide staining. Lane 1, αX174 RF DNA/Hae III fragments; lane 2, negative control (−RT); lanes 3 and 4, rapidly proliferating cells (day 3); lanes 5 and 6, confluent cells (day 7); lanes 7 and 8, postconfluent cells (day 14).

Fig. 2. Human intestinal muscle cells produce IGFBP-3, IGFBP-4, and IGFBP-5 protein. Representative Western immunoblot analysis of IGFBP-3, IGFBP-4, and IGFBP-5 produced by human intestinal muscle cells in culture is shown. Serum-free medium conditioned by smooth muscle cells during various phases of culture were examined using Western immunoblot analysis. Conditioned medium was obtained from rapidly growing cells, confluent cells, and postconfluent cells.

Western blot analysis of proteins in conditioned medium using an antibody directed against human IGFBP-3 confirmed the presence of two proteins corresponding to the 43- and 45-kDa glycosylated forms of IGFBP-3 (Fig. 2). Densitometric measurement was used to compare the relative amounts of IGFBP-3 secreted into the culture medium during various phases of culture. The production of IGFBP-3 was time dependent with lower levels secreted by rapidly growing cells and four- to fivefold higher levels secreted by confluent and postconfluent cells (Fig. 3).

The production of IGFBP-5 was also confirmed by Western blot analysis, which identified a protein corresponding to IGFBP-5, a 31-kDa protein. Also identified were the proteolytic fragments characteristic of IGFBP-5 (Fig. 2). Densitometric analysis of the relative amounts of IGFBP-5 (intact 31-kDa species) secreted by the muscle cells also showed time-dependent production. A pattern opposite to that of IGFBP-3 secretion was observed for IGFBP-5. Levels of secreted IGFBP-5 were highest in rapidly growing cells and were 1.5- to twofold lower in confluent and postconfluent cells, respectively (Fig. 4).

IGFBP-4 secretion was also confirmed by Western blot analysis. A protein of apparent 24-kDa molecular mass was identified that corresponded to the known molecular mass of intact IGFBP-4 (Fig. 2); however, a proteolytic fragment of IGFBP-2 of similar 24-kDa size has been identified in vascular muscle (10). Although

RT-PCR using IGFBP-2-specific primers (2) failed to identify transcripts of the expected 446-bp size (Table 1) during any phase of culture. A cDNA insert encoding human IGFBP-2 excised from the pHBP2–501 plasmid (the kind gift of Dr. Shunichi Shimasaki), using the appropriate restriction enzymes, served as positive control. With this cDNA as a template, the primers used identified the predicted 446-bp size product.

Characterization of secreted IGF BPs by Western blot analysis. The presence and relative amounts of IGFBP-3, IGFBP-4, and IGFBP-5 secreted by human intestinal muscle cells in culture was investigated, using RT-PCR, during 3 phases of culture: rapid growth (day 3), confluence (day 7), and postconfluence (day 14).
the antibody used displays ~50% cross-reactivity with IGFBP-2, the absence of identifiable IGFBP-2 transcripts by RT-PCR suggests that the protein band identified in these studies corresponded to intact IGFBP-4. While IGFBP-3 and IGFBP-5 secretion was time dependent, IGFBP-4 secretion remained relatively constant during all phases of growth in the cultured muscle cells (Fig. 5).

The requirement for new binding protein synthesis was investigated in confluent (day 7) cultures using the protein synthesis inhibitor cycloheximide (1 µg/ml). Basal production of soluble IGFBPs was strongly inhibited by cycloheximide: IGFBP-3 production was inhibited by 88±64%, IGFBP-4 production was inhibited by 81±64%, and IGFBP-5 production was inhibited by 97±1% vs. control levels. These results implied that, even under basal (unstimulated) conditions, new protein synthesis was required to maintain levels of soluble IGFBPs.

Characterization of IGFBPs in cell lysates. Well-defined roles for cell surface-associated IGFBP-3 and IGFBP-5 in regulating the actions of IGF-I have been described previously (15). The effects of cell-associated IGFBP typically parallel those of soluble binding protein and act in concert with soluble protein to regulate IGF-I ligand-receptor interaction. Accordingly, the presence of IGFBP-3 and IGFBP-5 was examined also in cell lysates derived from cultured cells during all three periods of growth. Cell-associated IGFBP-3, as identified by Western immunoblot analysis, had a similar pattern to that secreted by the cells. Levels of IGFBP-3 were lowest in rapidly growing cells, increasing by threefold in confluent cells and by eightfold in postconfluent cells (Fig. 3).

Similarly, the levels of IGFBP-5 associated with the cells paralleled the levels of secreted IGFBP-5. Levels of IGFBP-5 were highest in rapidly growing cells and were three- to fivefold lower in confluent cells and postconfluent cells, respectively (Fig. 4).

Modulation of IGF-I-induced proliferation by IGFBP-3 and IGFBP-5. The effects of IGFBP-3 and IGFBP-5 on IGF-I-induced proliferation were identified by measurement of [3H]thymidine incorporation. Incubation of confluent (day 7) human intestinal muscle cells for 24 h with a submaximal concentration of IGF-I (1 nM) increased [3H]thymidine incorporation by 147±18% above basal levels. The ability of IGF-I to induce proliferation was significantly inhibited in a concentration-dependent fashion by recombinant human IGFBP-3 (38±5% inhibition from control with 50 nM IGFBP-3; Fig. 6). In contrast, the ability of IGF-I to induce proliferation was significantly enhanced in a concentration-dependent fashion by recombinant human IGFBP-5 (37±5% above control with 50 nM IGFBP-5; Fig. 6).
Reciprocal regulation of IGFBP-3 and IGFBP-5 production by TGF-β1. The pattern of TGF-β1 secretion parallels that of IGFBP-3 in cultured human intestinal muscle cells (16). Overall, the effects of TGF-β1 are predominantly growth inhibitory in these cells. Two complementary methods were used to examine the interplay between TGF-β1 and IGFBP-3 and IGFBP-5. In the first method, the ability of TGF-β1 to alter the production of IGFBP-3 and IGFBP-5 was examined by incubation of the cells with exogenous TGF-β1. In the second method, an autocrine role for endogenous TGF-β1 in the regulation of IGFBP-3 and IGFBP-5 production was examined by immunoneutralization of endogenous TGF-β1.

Incubation of confluent (day 7) cultures for 48 h in the presence of rhTGF-β1 resulted in concentration-dependent changes in IGFBP-3 and IGFBP-5 production. Production of soluble IGFBP-3 was increased in a concentration-dependent fashion by rhTGF-β1 with an increase of 618 ± 10% above control in response to 1 nM rhTGF-β1 (P < 0.01 vs. control) (Fig. 7). Production of cell-associated IGFBP-3 was also increased in a concentration-dependent fashion by rhTGF-β1 with an increase of 136 ± 27% above control levels in response to 1 nM rhTGF-β1 (P < 0.05 vs. control) (Fig. 8). An opposite pattern of response was observed for IGFBP-5. Production of soluble IGFBP-5 was decreased by TGF-β1 in a concentration-dependent fashion with levels of IGFBP-5 virtually undetectable by Western blot analysis after incubation for 48 h with 1 nM rhTGF-β1 (Fig. 7). The production of cell-associated IGFBP-5 was also decreased in a concentration-dependent fashion by rhTGF-β1 with a 71 ± 7% inhibition from control levels in response to 1 nM rhTGF-β1 (P < 0.05) (Fig. 8). These results imply that endogenous TGF-β1 might act as an autocrine regulator of IGFBP-3 and IGFBP-5 production.

This notion was investigated by examining the effect of immunoneutralization of endogenous TGF-β1 on the production of IGFBP-3 and IGFBP-5. Cultures of confluent (day 7) muscle cells were incubated for 48 h in the presence of increasing concentrations of an immunoneutralizing antibody directed against human TGF-β1. Treatment of cells in this fashion resulted in concentration-dependent changes in IGFBP-3 and IGFBP-5 production (Fig. 9). In the presence of 1,000 ng/ml of antibody the production of IGFBP-3 was inhibited by 51 ± 5% (P < 0.05 vs. control) and the production of IGFBP-5 was augmented by 56 ± 6% (P < 0.05 vs. control). The ability of TGF-β1 immunoneutralization
and increased fivefold as cells attained confluence, by the muscle cells, however, depended on the time in culture (15). Our previous work has described in METHODS. Values represent means ± SE of 3 experiments. *P < 0.05 vs. control levels.

Fig. 9. Endogenous TGF-β1 regulates IGFBP production in reciprocal fashion. Immunoneutralization of endogenous TGF-β1 causes a decrease in IGFBP-3 secretion and a concomitant increase in IGFBP-5 secretion in confluent (day 7) cultures of human intestinal muscle cells. Muscle cells were incubated for 48 h with increasing concentrations of an immunoneutralizing antibody recognizing TGF-β1, and secretion of IGFBP-3 and IGFBP-5 was measured in conditioned medium by Western immunoblot analysis as described in METHODS. Values represent means ± SE of 3 experiments. *P < 0.05 vs. control levels.

to alter IGFBP-3 and IGFBP-5 production implies that endogenous TGF-β1 plays a role in the regulation of IGFBP production.

DISCUSSION

In human intestinal smooth muscle cells, the proliferative effects of IGF-I represent the net effects of IGF-I itself and the effects of various IGFBPs acting either as stimulatory or inhibitory modulators of IGF-I ligand-receptor interaction. Three mechanisms then exist by which these cells can regulate the autocrine growth effects of IGF-I: alteration of IGF-I secretion, alteration in IGFBP expression and/or production, or alteration of IGF-I receptor expression (15). Our previous work has measured levels of total and free IGF-I produced by these cells. Although levels of free IGF-I (unbound to IGFBPs) fell nearly 40-fold from day 3 (proliferating cells) to day 14 (postconfluent cells), total IGF-I production remained constant during all periods of growth (16). Changes in IGF-I receptor expression or in its coupling to intracellular signaling cascades may also occur, but this has not been examined.

The current study shows that human intestinal smooth muscle cells in culture express IGFBP-3, IGFBP-4, and IGFBP-5. Similar mRNA levels for individual IGFBPs, IGFBP-3, IGFBP-4, and IGFBP-5, were detected in cells during all phases of culture: in proliferating cells (day 3 of culture), in confluent cells (day 7 of culture), and in postconfluent cells (day 14 of culture). The levels of IGFBP-3 and IGFBP-5 protein produced by the muscle cells, however, depended on the time in culture: IGFBP-3 levels were low in proliferating cells and increased fivefold as cells attained confluence, whereas IGFBP-5 levels were high in proliferating cells and decreased twofold as cells attained confluence. The levels of IGFBP-4 protein, in contrast, did not change with time in culture. The levels of IGFBP-3 and IGFBP-5 production are regulated in reciprocal fashion, at least in part, by endogenous TGF-β1.

IGFBP-5 promotes IGF-I-mediated proliferation in human intestinal muscle cells. In addition to cellular mechanisms regulating IGFBP-5 mRNA and protein abundance, the ability of IGFBP-5 to bind IGF-I and to facilitate IGF-I ligand-receptor interaction can also be regulated by the proteolysis of IGFBP-5 (7, 22). Proteolysis of IGFBP-5 yields fragments that are capable of binding IGF-I, but due to the reduced affinity of the fragments for IGF-I, proteolysis, in effect, acts to promote IGF-I ligand-receptor interaction (1). The binding of IGF-I to intact IGFBP-5 also protects the binding protein from proteolysis. The highest levels of intact IGFBP-5 production by human intestinal muscle cells were detected in rapidly proliferating cells with lower levels in confluent and postconfluent cells, as the rate of growth slowed. Increase in the levels of TGF-β1 production by postconfluent human intestinal muscle cells (16) results, in part, in the concomitant decrease in the production of growth stimulatory IGFBP-5. This may represent one mechanism by which human intestinal muscle cells augment IGF-I-mediated growth during periods of rapid growth and diminish the growth effects as the rate of growth declines.

IGFBP-3 can act to either augment or inhibit IGF-I-mediated proliferation depending on the cell type. When it acts as an inhibitor of IGF-I-mediated proliferation, as in human intestinal muscle cells, soluble IGFBP-3 is a potent competitive inhibitor by virtue of its affinity for IGF-I being higher than that of IGF-I for the IGF-I receptor, thereby effectively inhibiting ligand-receptor interaction (28). Membrane- and extracellular matrix-associated IGFBP-3 also contribute to the modulation of IGF-I actions; the affinity of membrane-associated IGFBP-3 for IGF-I, however, is 10-fold lower than that of soluble IGFBP-3, which, in net, is twofold lower than that of IGF-I for the IGF-I receptor (21). Similar to IGFBP-5, the activity of IGFBP-3 is also regulated by proteases. Although intact IGFBP-3 inhibits IGF-I actions, the proteolytic fragments of IGFBP-3, because of their reduced affinity for IGF-I, act to potentiate its actions (6). IGFBP-3-specific proteolytic enzymes and mechanisms to regulate their activity have been identified in a number of cell types (5, 8).

Production of intact IGFBP-3 protein was lowest in rapidly proliferating human intestinal muscle cells. As cells attained confluence and postconfluence and the rates of growth declined, the levels of the growth inhibitory IGFBP-3 increased. In cultured human fibroblasts, addition of exogenous TGF-β has been shown to increase IGFBP-3 production (20). Increase in the levels of TGF-β1 production by postconfluent human intestinal muscle cells also results, in part, in the concomitant increase in the production of growth inhibitory IGFBP-3. This may represent a second complementary mechanism by which these cells minimize the...
growth-stimulatory effects of IGFl as cells reach confluence and the rate of growth declines.

In contrast to IGFBP-3 and IGFBP-5, IGFBP-4 has been identified only in a soluble form that inhibits IGFl ligand-receptor interaction (4). The effects of IGFBP-4 are also regulated by the production of specific IGFBP-4 proteases in a number of cells, including vascular smooth muscle cells (6). The resulting fragments of IGFBP-4 are virtually incapable of binding IGFl and provide a method to increase free IGFl levels and potentiate IGFl actions. Human intestinal smooth muscle cells express IGFBP-4 during all phases of culture, but the levels of protein secretion do not appear to depend on the phase of culture as do those of IGFBP-3 and IGFBP-5.

Time-dependent production of IGFBPs by human intestinal muscle cells in culture may in part explain the previous findings of constant total IGFl levels but time-dependent changes in free IGFl levels. High levels of free IGFl were measured in cultures of proliferating cells; levels of free IGFl were decreased in cultures of confluent and postconfluent cells. The net effect of growth stimulatory IGFBP-5 and growth inhibitory IGFBP-3 in cultures of proliferating cells, when IGFBP-5 levels are high and IGFBP-3 levels are low, is to promote the autocrine growth effects of IGFl. In contrast the net effect of IGFBP-3 and IGFBP-5 in cultures of confluent and postconfluent muscle cells, when growth inhibitory IGFBP-3 levels are high and growth stimulatory IGFBP-5 levels are low, is to reduce the autocrine growth effects of IGFl. The pletrophic growth factor, TGF-β1, contributes to the regulation of the relative levels of IGFBPs produced. The increasing levels of TGF-β1 produced as cells attain confluence and postconfluence can augment the production of growth inhibitory IGFBP-3 and inhibit the production of growth stimulatory IGFBP-5. The net effect of these events would be to decrease the growth-stimulating effects of IGFl in these cells as they become confluent and postconfluent.

IGFl-independent effects of IGFBP-3 and IGFBP-5, which parallel their individual effects on IGFl ligand-receptor interaction, have also been described (19, 22).

Specific receptors for IGFBPs have been identified in Hs 578T breast cancer cells (25), in prostatic adenocarcinoma cells (PC-3) (27), and in mink lung epithelial Hs 578T breast cancer cells (25), in prostatic adenocarcinoma cells (PC-3) (27), and in mink lung epithelial cells (25), which mediate an IGFl-independent inhibition of growth. This receptor appears to be the TGF-β-V receptor (19). IGFBP-5 binds to specific receptors in a variety of bone cells and stimulates proliferation through an IGFl-independent mechanism (1). The IGFl-independent effects of IGFBP-1 appear to be mediated by binding to the α5β1-integrin receptor (11).

In the normal rat colon, IGFl receptors and IGFBP-3, IGFBP-4, and IGFBP-5 have been identified (34, 36). Infusion of IGFl in neonatal rats increases growth of the intestine, including the muscularis propria, in a tissue-specific manner (31). IGFl is capable of upregulating IGFBP-5 expression (36). Inflammation caused by either trinitrobenzene sulfonate instillation or subserosal injection of peptidoglycan-polysaccharide results in increased levels of IGFl binding and IGFBP-5 and IGFBP-4 in the muscularis propria (35, 36). These findings suggest that the IGFl system and its regulatory binding proteins serve an important function in the response of the colon to inflammation and in the development of fibrosis and stricture formation. The findings of the current study demonstrate both the role and the interplay of endogenous IGFl binding proteins that serve to regulate the growth of human intestinal muscle in culture. Taken together, the existing evidence suggests mechanisms by which the IGFl system may regulate not only the initial response to inflammation but also the limitation of the intestinal response to prevent excessive growth and stricture formation.

In summary, the production of individual IGFBPs, as well as the interplay of IGFBPs having either stimulatory or inhibitory effects on proliferation, contribute to the regulation of IGFl-mediated growth of human intestinal muscle cells. The endogenous growth factor, TGF-β1, acting in an autocrine fashion contributes to the regulation of IGFBP production and thereby to the modulation of IGFl actions in these cells.

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