Expression of insulin-like growth factor I receptors and binding proteins by colonic smooth muscle cells

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Zeeh, Jörg M., Helena S. Ennes, Peter Hoffmann, Frank Procaccino, Viktor E. Eysselein, William J. Snape, Jr., and James A. McRoberts. Expression of insulin-like growth factor I receptors and binding proteins by colonic smooth muscle cells. Am. J. Physiol. 272 (Gastrointest. Liver Physiol. 35): G481–G487, 1997.—We recently demonstrated upregulation of insulin-like growth factor I (IGF-I) binding sites in the smooth muscle layer of inflamed rat colon. The increase in binding sites was due to increased expression of IGF binding proteins (IGFBPs), which modulate the effects of IGF. To further study the role of IGF in the colon, we investigated whether cultured colonic smooth muscle cells (SMC) express IGF-I receptors and IGFBPs. SMC were isolated by collagenase digestion from rat colonic smooth muscle and grown in primary culture. Equilibrium binding studies with IGF-II and insulin, together with our in vivo studies in the rat, support a role for IGF in tissue fibrosis and stricture formation during chronic intestinal inflammation.

CROHN’S DISEASE is an idiopathic chronic inflammatory condition that can involve any part of the gastrointestinal tract. Two of the common long-term consequences of this disease are tissue fibrosis and stricture formation. These pathological processes are associated histologically with hyperplasia of smooth muscle cells (SMC) and increased collagen deposition within the bowel wall (8). The role of cytokines and growth factors in tissue fibrosis and stricture formation is still largely unknown. Zimmerman et al. (29) recently reported a role for insulin-like growth factor I (IGF-I) in the development of fibrotic lesions in rats with an experimental model of enterocolitis that has pathological features similar to Crohn’s disease. IGF-I and a structurally related peptide, designated IGF-II, are the product of separate genes that have important functions in regulating cell growth, differentiation, and metabolism (21). These peptides have structural homology with insulin and are synthesized principally by the liver but can also be produced by a variety of extrahepatic tissues. Both forms of IGF are potent mitogens for a variety of cells (21, 24) and have important roles in tissue repair (14, 25). Furthermore, local levels of IGF-I are increased in several inflammatory conditions (9, 29), and IGF-I has been shown to stimulate collagen synthesis by a variety of cell types (6, 7). Thus IGF could contribute to the pathological features of chronic inflammatory conditions.

The biological effects of IGF are mediated by high-affinity cell surface receptors. Two types of IGF receptors have been identified on the basis of their distinct biochemical properties and their relative affinities for IGF-I, IGF-II, and insulin (17, 24). The IGF-I or type I receptor is believed to mediate most of the biological effects of IGF. This receptor is structurally homologous to the insulin receptor with a tetrameric α2β2 structure and endogenous phosphotyrosine kinase activity. In most tissues, the IGF-I receptor has higher affinity for IGF-I than IGF-II and will also bind insulin with ~100-fold lower affinity (17). The IGF-II or type II receptor is structurally and functionally dissimilar to the IGF-I receptor, being composed of a single large transmembrane protein that lacks phosphotyrosine kinase activity (17). This receptor is identical to the mannose-6-phosphate cation-independent receptor that directs proteins containing mannose-6-phosphate to lysosomes. The IGF-II receptor binds IGF-II with very high affinity and IGF-I with much lower affinity; it does not bind insulin (17). The IGF-II receptor is believed to interact with GTP-binding proteins; however, its role in mediating biological responses is presently unclear (17).

In addition to the two IGF receptors, IGF also binds to a family of structurally homologous binding proteins (2, 21, 22, 24). Six IGF binding proteins (IGFBP-1–6) have recently been cloned and sequenced from both humans and rats (22). These six binding proteins were initially identified by their differing molecular masses on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. All six IGFBPs have a very high affinity for IGF-I and IGF-II and do not bind insulin (2). A variety of tissues have been shown to synthesize and secrete one or more of these binding proteins, and each is present to varying degrees in serum. IGFBPs in serum, principally IGFBP-3, act as carriers for IGF, preventing degradation of the peptide and allowing the maintenance of relatively constant blood levels of IGF (2, 22). The function of IGFBPs in tissues is less clear. Addition of different purified IGFBPs to cultured cells has been reported to both inhibit and potentiate the effects of exogenously added IGF. For example, purified IGFBP-4 inhibits the mitogenic effect of IGF-I in several cell culture systems, whereas IGFBP-5 has the opposite effect, increasing the potency of IGF-I (2, 11, 23). The capacity to potentiate the effect of IGF appears to be at least partly...
mediated by the association of IGFBP-5 with either the cell surface or the extracellular matrix (2, 11). This interaction lowers the affinity of IGFBP-5 for IGF-I and IGF-II to values similar to that of the IGF-I receptor and increases the local concentration of IGF available to bind to cell surface receptors. The growing consensus is that expression and localization of the different tissue IGFBPs act to target IGF to specific cell types.

The role of IGFBPs in inflammatory lesions in the gastrointestinal tract is unknown. We recently reported (26) an increase in IGF-I binding sites in the inflamed colonic mucosa of rats administered triptorexin (3). This increase in binding sites was localized in the muscularis propria. Based on the relative ability of IGF-II and insulin to compete for IGF-I binding, we interpreted this increase in binding sites as an increase in the local production of one or more IGFBPs (26). Indeed, Northern analysis of RNA isolated from inflamed tissues demonstrated a two- to threefold induction of IGFBP-4 and IGFBP-5. These findings suggest that IGFBPs may play an important role in intestinal hyperplasia and excess collagen disposition by targeting IGF-I to the SMC. However, IGF receptor and IGFBP expression by colonic SMC has not yet been characterized. To extend our in vivo studies to in vitro conditions that are more amenable to biochemical manipulation, we adapted a previously described method for the isolation of rabbit colonic SMC to the rat (4, 13). The aim of this study was to investigate if primary cultures of rat colonic SMC express IGF-I receptors and if they produce and secrete IGFBPs.

MATERIAL AND METHODS

Tissue and cell culture preparation. SMC were isolated from the colon of male Sprague-Dawley rats (250–300 g, Harlan, San Diego, CA) by modification of previously described methods (4, 13). Animals were killed by a phenobarbital overdose, and the colon was removed, rinsed briefly with saline, and placed in oxygenated standard incubation buffer (SIB) containing (in mM) 115 NaCl, 2.9 KCl, 0.6 MgCl₂, 2.13 KH₂PO₄, 0.3 CaCl₂, and 25 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), as well as 2.6% (vol/vol) basal Eagle's medium essential amino acids (100×, Irvine Scientific), pH 7.4. The colon was cut longitudinally along the contramecenteric eide and placed in oxygenated SIB containing 100 µg/ml streptomycin, 100 U/ml penicillin, 100 µg/ml gentamycin, and 10 µg/ml amphotericin B. For all subsequent procedures, SIB contained 270 µg/ml streptomycin, 100 µg/ml penicillin, 0.04 µg/ml gentamycin, and 1 µg/ml amphotericin B. With the use of a dissecting microscope, the mesentery, the serosa, and the mucosa were removed with a fine forceps. The remaining smooth muscle layer, which included both circular and longitudinal layers, was incubated twice for 15 min at 34°C in SIB containing 1 mg/ml bovine serum albumin (BSA), 0.5 mg/ml collagenase (CLS type II, Cooper Biomedical, Malvern, PA), and 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical, St. Louis, MO) and rinsed in SIB containing 1 mg/ml BSA. The tissue was sucked through a plastic pipette (2-mm diameter orifice) several times to remove cells. Dispersed cells then were filtered through a nylon filter (600-µm pore size), centrifuged at ~100 g for 20 min, and resuspended in Dulbecco's minimum essential medium (DMEM) containing 1 mM sodium pyruvate, 2 mM glutamine, 10% (vol/vol) heat-inactivated fetal calf serum (Irvine Scientific), 200 U/ml penicillin, 8 µg/ml ampicillin, 90 µg/ml streptomycin, 0.04 µg/ml gentamycin, and 1 µg/ml amphotericin B. Cell number was determined with a standard hemocytometer.

Colonic SMC primary culture. For receptor binding and cross-linking studies, cells were plated on 12-well plates at a density of 1.2 × 10⁶ cells per plate. For RNA extraction, cells were plated in 100-mm culture dishes at a density of 1.8 × 10⁶ cells per dish. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was changed every 3 days. The medium used after the initial plating was amphotericin- and gentamycin-free. Serum-free medium contained DMEM and Ham's F-12 (DMEM-F12) (1:1, v/v/wt), 5 µg/ml transferrin, and 25 × 10⁻⁵ M selenium. Purity of the cultures was assessed by indirect immunofluorescent staining with smooth muscle-specific antiactin monoclonal antibodies (COAT 1:50; a gift from Dr. Allen M. Gown, University of Washington, Seattle, WA) or IA4 (1:400, Sigma Chemical) as previously described (4). By this criterion, the smooth muscle cultures contained ~1% non-smooth muscle cells.

Receptor binding. Cells were used for all studies when monolayers approached 70–80% confluence. The medium was aspirated, and the monolayers were rinsed with 1 ml of binding buffer containing (in mM) 26 HEPES, 1.2 MgCl₂, 5 KCl, 15 sodium acetate, 120 NaCl, and 1 EDTA, as well as 1% BSA (wt/wt) and 1 µM aprotinin, pH 7.4. Cells were then incubated with 100 pM ¹²⁵I-IGF-I (Amersham) in the presence and absence of 100 pM to 100 nM cold IGF-I (Intergen), 100 pM to 100 nM IGF-II, or 1 nM to 10 µM insulin for 2–3 h at 4°C. After incubation, the buffer was aspirated and cells were rinsed with 0.1 M phosphate buffer containing 0.9% NaCl (wt/wt) and 1% BSA (wt/wt), pH 7.4. Cells were dissolved by addition of 0.5 ml of 0.5 N NaOH for 1 h, and the cell extracts were counted in a gammacounter. The cell count per well was measured using a standard hemocytometer after trypsinization of representative monolayers.

Chemical cross-linking. Cultured SMC were incubated with 0.1 nM ¹²⁵I-IGF-I in the presence or absence of unlabeled IGF-I (100 nM) or insulin (10 µM) in binding buffer. After 3 h at 4°C, the cells were washed three times with ice-cold BSA-free binding buffer, and cross linking was carried out by addition of BSA-free binding buffer containing freshly prepared 0.2 M disuccinimidyl suberate. After 10 min at 25°C, the reaction was quenched by the addition of 0.75 ml 10 M NaOH for 1 h, and the cell extracts were counted in a gammacounter. Cells were solubilized by addition of SDS sample buffer containing 2-mercaptoethanol, and ~40 µg total cell protein was loaded onto each track of a Laemmli slab gel with a 6% separating gel. After electrophoresis, the gel was fixed, stained with Coomasie blue, dried, and exposed to Kodak X ray film at -70°C with the use of DuPont intensifying screens.

Western ligand blot of conditioned medium. Subconfluent SMC cultures were grown in serum-free medium (DMEM-F12 containing 5 µg/ml transferrin and 25 nM selenium) for 96 h. The conditioned medium was removed and centrifuged at low speed to remove cell debris. Aliquots of the supernatant were dried using a Savant Speedvac Concentrator and then resuspended in nonreducing SDS sample buffer (Novex). The samples were boiled for 5 min and then centrifuged at 1,000 g for 5 min before loading onto Novex Precast 12% SDS PAGE gels. Electrophoresis was carried out under nonreducing conditions, and the gel was electroblotted onto nitrocellulose membranes. The ligand blotting method for detection of IGFBPs followed that of Hossenlopp et al. (10) as modified by Rosenfeld et al. (20). All blocking, binding, and washing steps were carried out at 4°C with agitation. The blot...
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was incubated for 2 h each in three successive blocking buffers containing 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.5 mg/ml sodium azide, and either 3% Nonidet P-40, 1% BSA, or 0.1% Tween-20. The blot was cut into appropriate strips, and ligand binding was done in the same buffer containing 1% BSA, 0.1% Tween-20, and 0.05 nM 125I-IGF-I in the presence or absence of 10 nM unlabeled IGF-I. After an overnight incubation, the blot strips were washed two times in the blocking buffer and three more times in wash buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.5 mg/ml sodium azide) for 15 min each. The blot strips were air-dried and exposed to Kodak film at -70°C with intensifying screens.

Identification of IGF I receptors on cultured colonic SMC. To identify IGF-I receptors on cultured rat SMC, equilibrium receptor binding experiments were performed using radioiodinated human recombinant IGF-I. Subconfluent SMC monolayers (70—80% confluence) were incubated with 100 nM 125I-IGF-I in the presence of increasing concentrations of unlabeled IGF-I. The resulting "cold saturation" binding data were processed using the EBDA and LIGAND computer programs and fit to both a one-site and a two-site model (16). The results indicated a single class of receptors with an apparent dissociation constant of 1.96 ± 0.27 nM and 52 ± 6 × 10^3 receptors per cell (Fig. 1). The ability of unlabeled IGF-II and insulin to compete for binding of 125I-IGF-I binding was also determined and compared with IGF-I. IGF-II had slightly lower affinity than IGF-I with a calculated inhibition constant (K_i) of 3.1 nM, whereas insulin competed ~170-fold less, with a K_i of 560 nM (Fig. 2). These results are consistent with the presence of an IGF-I receptor on the cell surface of colonic SMC.

To positively identify the IGF-I receptor on colonic SMC, chemical cross-linking studies were carried out. SMC cells were incubated with 100 nM 125I-IGF-I in the presence or absence of unlabeled 100 nM IGF-I or 10 µM insulin, using the same equilibrium binding conditions as above. The monolayers with the bound peptide were then rapidly washed and incubated in BSA-free binding buffer containing 0.2 nM disuccinimidyl suberate for 10 min at room temperature. This bireactant chemical cross-links the radioiodinated peptide to any proteins to which it is closely associated. The labeled peptide-protein complexes were identified by SDS-PAGE and autoradiography. As shown in Fig. 3, 125I-IGF-I labeled protein bands with apparent molecular weights of 140 × 10^3, ~280 × 10^3, and >350 × 10^3. Labeling was eliminated in the presence of excess...
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Fig. 2. Competition of monoiodo-IGF-I binding by IGF-I, IGF-II, and insulin. Binding of \( ^{125}\)I-IGF-I to cultured SMC was determined in the presence of graded concentrations of IGF-I (○), IGF-II (▲), or insulin (■). Nonlinear regression analysis of data gave inhibition constant values for IGF-II and insulin of 3.1 ± 0.5 and 560 ± 220 nM, respectively. Points are mean of 2–3 experiments, each carried out in triplicate. Standard errors (<20%) have been deleted from the figure to simplify presentation. In these experiments, the fraction of total label bound averaged 1.4%, with 85% specific binding.

unlabeled IGF-I, indicating that the labeling was specific. When the molecular weight of the cross-linked peptide was taken into account, the deduced molecular weight of the lower molecular mass protein was \( \sim 132 \times 10^3 \). This value is in reasonable agreement with those of the α-subunit of the IGF-I receptor described in other tissues (15, 17). The higher molecular mass bands most likely represent oligomers of the receptor (i.e., \( \alpha_2 \alpha_2 \beta_2 \)), which become cross-linked to each other as well as to the iodinated peptide (15). A very large excess of unlabeled insulin eliminated cross-linking to all of these bands, supporting the conclusion that all three represent the IGF-I receptor. There was no evidence for cross-linking of IGF-I to the IGF-II receptor, which would have an apparent molecular weight of \( 220-240 \times 10^3 \) on these gels and whose labeling would not be competed by excess unlabeled insulin (15).

Expression and secretion of IGFBPs. To determine whether cultured SMC express and secrete IGFBPs, monolayers were switched to serum-free culture conditions, and conditioned medium was collected after 4 days. Portions of the conditioned medium were subjected to SDS-PAGE on 12% gels under nonreducing conditions. The proteins in the gel were then transferred to nitrocellulose membranes, the membranes were incubated with 100 nM \( ^{125}\)I-IGF-I in the presence or absence of excess unlabeled IGF-I, and the blot was autoradiographed to identify the binding proteins present in the conditioned medium. \( ^{125}\)I-IGF-I bound to proteins with molecular masses of 25, 31, and 45 kDa (Fig. 4). Incubation in the presence of 100 nM cold IGF-I led to complete competition of the label from the binding sites, proving that binding is highly specific (data not shown). The 25-kDa IGFBP was present at highest levels in the medium, followed by lower levels of the 31-kDa protein and much lower levels of the 45-kDa protein. Based on the molecular masses of the IGFBPs from rat tissues, the 25-kDa protein can be identified as IGFBP-4, whereas the 45-kDa protein can be identified as IGFBP-3 (22). The identity of the 31-kDa IGFBP cannot be unambiguously determined from its molecular mass, since rat IGFBP-1, -2, and -5 all have similar molecular masses under nonreducing conditions [31–32, 32–33, and 29–31 kDa, respectively (22)].

To identify the 31-kDa binding protein produced by SMC and to confirm that these cells make IGFBP-3 and -4, Northern blot analysis of RNA extracted from cultured SMC monolayers was carried out. cDNA probes specific for all six rat IGFBP transcripts were used to screen for expression of each of the binding proteins. As shown in Fig. 5, transcripts for IGFBP-3 (2.6 kbp), IGFBP-4 (2.6 kbp), and IGFBP-5 (6.0 kbp) were detected in total RNA from rat SMC. The size of these

Fig. 3. Identification of IGF-I receptors by receptor cross-linking. Cultured SMC were incubated with 0.1 nM \( ^{125}\)I-IGF-I in the presence or absence of excess unlabeled insulin or IGF-I before chemical cross-linking with disuccinimidyl suberate as described in MATERIALS AND METHODS. Cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 6% separating gel, and protein bands cross-linked to iodinated peptides were visualized by autoradiography. Incubation conditions were as follows: lane 1, labeled \( ^{125}\)I-IGF-I only; lane 2, labeled peptide plus 100 nM IGF-I; lane 3, labeled peptide plus 10 μM insulin. Position of molecular mass standards (kDa) is shown at left. IGF-I specifically labeled a protein with an apparent molecular mass of 140 kDa. Multimers of IGF-I receptor subunits with molecular mass >280 kDa are apparent at top of gel.
transcripts is in agreement with previously published reports (22). The relative level of expression appeared to be IGFBP-4 > IGFBP-5 > IGFBP-3. No mRNAs coding for IGFBP-1, -2, or -6 could be detected by this method. These results strongly suggest that the 31-, 25-, and 45-kDa IGFBPs detected by Western ligand blotting are in fact IGFBP-5, -4, and -3, respectively.

DISCUSSION

The results of this study show that primary cultures of colonic SMC express IGF-I receptors that are pharmacologically and structurally similar to IGF-I receptors found in a variety of other tissues (17, 24). The IGF-I receptor in colonic SMC has an α-subunit with a molecular mass of 132 kDa and binds IGF-I with high affinity, IGF-II with slightly lower affinity, and insulin at very high concentrations. Although this report did not investigate the function of IGF-I receptors on SMC, in a separate study we found that IGF-I is mitogenic for SMC both in vitro and in vivo (28). IGF-I added to cultured SMC grown in a defined serum-free medium was poorly mitogenic by itself but acted synergistically with epidermal growth factor and fibroblast growth factor to promote growth. In vivo, exogenous IGF administered continuously to rats over the prolonged course of 2 wk resulted in significant growth of SMC in the external muscle layer of the colon. Together these results suggest that IGF is mitogenic for colonic SMC,
although other factors are likely to play a role in this response. The present study demonstrates that SMC have IGF-I receptors and supports the idea that IGF-I mediates its effects through these receptors. The fact that insulin effectively competes for binding of 

$^{125}$I-IGF-I to the IGF-I receptor in cultured colonic SMC also supports our previous conclusion (26) that inflammation-induced increased insulin-resistant binding to the external smooth muscle layer in tissue slices is due to enhanced expression of IGF-I receptors but rather to upregulation of IGFBPs.

The results of our studies also demonstrate that IGFBP are also synthesized and secreted by colonic SMC in culture. Northern analysis of total RNA revealed that cultured SMC express the transcripts for IGFBP-4, -5, and -3. Western ligand blots of conditioned medium demonstrated the secretion of three binding proteins with molecular masses of 25, 31, and 45 kDa. By virtue of their known molecular mass in other systems and the expression of the mRNA transcripts, the 25- and 45-kDa proteins can be identified as IGFBP-4 and IGFBP-3, respectively. On the basis of the expression of a transcript for IGFBP-5, but not for IGFBP-1 or -2, the 31-kDa protein most likely represents IGFBP-5. This profile of IGFBP expression is unique among cultured SMC. Porcine vascular SMC produce IGFBP-2 and -4, whereas rat vascular SMC produce IGFBP-2, -3, and -4, and rabbit airway SMC produce only IGFBP-2 (3, 12, 18). However, the profile of IGFBPs produced is similar to that previously described by us in total RNA from rat colonic tissue (26). This study showed that IGFBP-4 and -5 were increased more than twofold after induction of experimental colitis, and this upregulation of IGFBPs was correlated with an increase in binding sites for $^{125}$I-IGF to the smooth muscle layer. In further studies in this rat model of colitis employing the technique of in situ hybridization, we have shown that IGFBP-5 mRNA was increased specifically in smooth muscle layer, whereas IGFBP-4 mRNA was increased in lamina propria, submucosa, and smooth muscle (27). This study also found that IGFBP-3 mRNA did not change in response to inflammation and was localized to the lamina propria. Thus changes in the relative amounts of IGFBP-4 and -5 synthesized by colonic SMC could have a profound influence on the efficacy of IGF produced locally or distally. Indeed, using in situ hybridization we have shown that IGF-I mRNA was intensely upregulated in the lamina propria, submucosa, and smooth muscle of animals with experimental colitis (27). These data are consistent with previous studies suggesting a role of IGF-I and IGFBP-5 in another model of intestinal inflammation. Zimmermann et al. (29) showed that IGF-I mRNA levels were preferentially elevated in fibroblast-like cells in the fibrotic periphery of granulomatous lesions in peptidoglycan-induced enterocolitis in rats and that IGFBP-5 was also expressed and upregulated (30). Together these studies suggest that the differential expression and localization of IGFBP-5 may serve in vivo to target the effect of IGF-I to the SMC layer. This response could account for SMC hyperplasia, tissue fibrosis, and stricture formation associated with chronic intestinal inflammatory conditions, such as Crohn’s disease.

In summary, this is the first study demonstrating that colonic SMC express IGF-I receptors and IGFBP. This model system should be amenable to further experiments directed at identifying the inflammatory mediators modulating IGFBP expression and defining the functional significance of IGFBP expression in colonic SMC.

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