CMLS, Cell. Mol. Life Sci. 54 (1998) 158–166
1420-682X/98/020158-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Production of insulin-like growth factor I (IGF-I) and IGF-binding proteins by rat intestinal stromal cells in vitro

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Received 25 August 1997; received after revision 7 November 1997; accepted 20 November 1997

Abstract. To determine if intestinal stromal cells secrete diffusible factors such as insulin-like growth factors (IGFs) capable of regulating epithelial cell growth in vitro, stromal cells were isolated by enzymatic digestion of rat intestine. Incorporation of [^{3}H]thymidine into DNA and [^{14}C]leucine into protein of IEC-6 cells, a model intestinal epithelial cell line, was significantly increased (two- to threefold) when the IEC-6 cells were co-cultured with stromal cells, relative to IEC-6 cells grown alone. Medium conditioned by stromal cells stimulated DNA synthesis of IEC-6 cells in a dose-dependent manner. Analysis of the conditioned medium revealed that intestinal stromal cells secreted IGF-I, but little IGF-II, in addition to an M_r 32,000 IGF-binding protein (IGFBP-2) and an IGFBP having $M_r \sim$ 24,000. We conclude that rat intestinal stromal cells secrete one or more diffusible factors, which may include IGF-I and IGFBPs, capable of stimulating proliferation of IEC-6 cells in vitro.

Key words. Insulin-like growth factors; IGFs; IGFBP; stroma; epithelium; stromal-epithelial interactions.

Insulin-like growth factors (IGFs) are mitogens for several types of mammalian cells [1, 2]. Although the liver is the major source of circulating IGF-I, IGFs are also synthesized by fibroblasts and other cell types throughout the body and may have paracrine and autocrine as well as endocrine mechanisms of action [1, 2]. Important elements of the IGF axis in responsive cells are the IGF-binding proteins (IGFBPs) [3, 4]. To date, six high-affinity IGFBPs have been purified and sequenced, and recently Oh et al. have demonstrated that mac25 is a low-affinity IGFBP, designated IGFBP-7 [5]. The role of IGFBPs in modulating the metabolic and mitogenic actions of IGFs on most cell types is still under intensive investigation, but it is well established that IGFBPs can inhibit or enhance IGF responses. The degree and direction of IGFBP effects on IGF action depend on whether the IGF-IGFBP complexes are soluble or bound to the cell surface or extracellular matrix [3, 4, 6]. In addition, IGFBPs, particularly IGFBP-3, can have IGF-independent effects [7, 8].

Recent studies support the notion that IGFs play an important role in regulating intestinal cell growth and differentiation. Parenteral administration of IGFs to rats promotes proliferation of the small intestine [9] and enhances mucosal adaptation after jejunoileal resection [10]. Oral administration of IGF-I caused intestinalweight gain due to an increase in villus height in neonatal pigs [11] and stimulated brush border enzyme activities, but not intestinal growth, in suckling rats [12]. Northern blot, quantitative

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reverse transcriptase–polymerase chain reaction and in situ hybridization analyses have revealed the presence of mRNAs for IGF-I and IGF-II in human and rat intestine [13–16]. In addition, mouse intestinal explants release IGF-I into the surrounding medium [17]. Utilizing IEC-6 cells, an epithelial cell line derived from rat intestinal crypts [18, 19], we have shown that IEC-6 cells produce IGF-II and IGFBP-2 [20]. Furthermore, we reported increased incorporation of [^{3}H]thymidine into DNA by IEC-6 cells in response to insulin and IGFs [20]. Receptors for both IGF-I and IGF-II have been found in human and ruminant intestinal crypt cells analysed in vivo [21], n plasma membranes isolated from rat intestinal mucosa [12], in primary epithelial cell cultures established from rat jejunum [22] as well as in IEC-6 cells [23]. Collectively, these findings suggest the possibility that the actions of IGFs may be exerted locally in the intestine near the site(s) of secretion.

In the small intestine, the bases of the crypts of Lieberkuhn are surrounded by stromal cells [24, 25], which have been implicated in regulating proliferation and differentiation of the adjacent epithelial cells [24–26]. Analysis of in vitro models for intestinal stromal-epithelial interactions has permitted identification of some of the matrix components and diffusible factors that mediate these effects [27–29]. Transforming growth factor β (TGF-β) was able to supplant the need for fibroblast co-cultures in support of T84 intestinal epithelial cell proliferation [29], and fibroblast modulation of T84 secretory responses in vitro was dependent on release of cyclooxygenase products by the fibroblasts [27]. IEC-6 cells cultured on extracellular matrix laid down by fetal intestinal fibroblasts or with medium conditioned by such fibroblasts showed increased sucrase activity indicative of a more differentiated phenotype [30]. Despite the wealth of evidence for the importance of IGFs in regulating intestinal epithelial cell proliferation, few studies have addressed the possibility that fibroblast production of IGFs may contribute to stromal-epithelial interactions in the intestine.

The objective of the present studies was to test the hypothesis that stromal cells underlying intestinal epithelium may produce IGFs and IGFBPs, because our previous work showed that IGFs stimulate IEC-6 cell proliferation. In the present studies, IEC-6 cells exhibited a proliferative response when co-cultured with intestinal stromal cells or upon exposure to medium conditioned by intestinal stromal cells. Fractionation and analysis of stromal cell-conditioned medium revealed that the cells produced IGF-I and several IGFBPs, including IGFBP-2.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (F12), dialysed fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin were obtained from GibcoBRL (Grand Island, NY, USA). Tissue culture dishes and six-well plates were purchased from Becton Dickinson (Lincoln Park, NJ). [^{3}H-Methyl]thymidine and [U-^{14}C]leucine were obtained from ICN (Costa Mesa, CA, USA). Transferrin was obtained from Collaborative Research (Lexington, MA, USA). Radioimmunoassay-grade bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Porcine insulin and recombinant human IGF-I and IGF-II were provided by Mrs. M. H. Niedenthal (Lilly Research Laboratories, Indianapolis, IN, USA). ^{125}I–IGF-I (specific activity, 2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA). ^{125}I–IGF-II (specific activity, 2000 Ci/mmol) was a gift of Dr. Ronald R. Bowsher (Lilly Laboratory, Indianapolis, IN, USA). Rabbit anti-IGF-I serum was obtained from the National Hormone and Pituitary Program (Baltimore, MD, USA). IEC-6 cells, originally established by Quaroni et al. [18], were purchased from the American Type Culture Collection (ATCC CRL 1592, Rockville, MD, USA).

IEC-6 cell culture. IEC-6 cells were maintained and subcultured as described by Quaroni and May [19]. For routine maintenance, the monolayers of IEC-6 cells were grown in tissue culture dishes (100 mm) at 37 °C in a humidified CO_2 incubator (94% air/6% CO_2). Cells between the 16th and 19th passages were used. The complete medium for cell maintenance consists of DMEM/F12 containing 5% FBS, 10 μg/ml insulin, 20 U/ml penicillin and 20 μg/ml streptomycin.

Isolation and culture of intestinal stromal cells. Primary cultures of stromal cells were prepared from theleum of 25-day-old male Sprague-Dawley rats (Sasco, Omaha, NE, USA) by a modification of the method of Ferretti et al. [30]. The rats were sacrificed by carbon dioxide asphyxiation. The small intestine from the ligament of Treitz to the ileocecal junction was dissected and divided into two pieces (jejunum and ileum). After rinsing with sterile saline, ileal mucosa was lightly scraped with a glass slide and discarded. The remaining intestinal segment was chopped with a pair of scissors, and 20 ml of enzyme mixture was added. The enzyme mixture contained 100 mM Hepes buffer, pH 7.4, containing 120 mM NaCl, 50 mM KCl, 5 mM D-glucose, 1.5% BSA, 1 mM $CaCl_2$, 1.6 mg/ml collagenase, 0.3 mg/ml elastase and 0.27 mg/ml hyaluronidase. Tissue was digested for 60 min at 37 °C in a metabolic shaking incubator. The digest

was brought to 50 ml with DMEM/F12 plus 10% FBS and filtered through 240 μm and 100 μm mesh nylon screens to eliminate undigested debris. Cells were washed twice with DMEM/F12 plus 10% FBS by centrifuging at 200*g* for 10 min. Cells were resuspended in DMEM/F12 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and plated in tissue culture dishes. After a 2-h attachment period, the cultures were washed three times with DMEM/F12 and changed to fresh complete medium. During the 2-h attachment period, fibroblasts adhered to the culture dish, while smooth muscle cells, capillary segments and debris did not. Therefore, after washing there remained an essentially pure population of intestinal fibroblasts. Although light microscopic examination failed to reveal the presence of other cell types, we cannot rule out the possibility of a small degree of contamination by smooth muscle cells. For this reason, these cells will hereafter be referred to as 'stromal cells'. Cultures were subcultured 1:3 every 7–10 days by treatment with trypsin-EDTA solution. The medium was changed 24 h after passing, then every 3 days thereafter. Cells of the fourth passage were frozen in liquid nitrogen. Cells were routinely used between the 6th and 10th passages.

Preparation and characterization of stromal cell-conditioned medium. Stromal cells were grown to confluency with complete medium. The monolayers were washed three times and serum-deprived for 24 h in the basal medium (DMEM/F12 plus 0.5 mg/ml BSA, 5 μg/ml transferrin and 25 mM Hepes). The medium was replaced with the fresh basal medium, and conditioned medium was collected after an additional 48 h.

To study the effects of stromal cell–conditioned medium on IEC-6 cell growth, IEC-6 cells were grown to approximately 2×10^5 cells/well (8.6 cm^2) in DMEM/F12 containing 5% FBS and 10 μg/ml insulin. The monolayers were washed three times with serum-free DMEM/F12 and serum-deprived for 24 h in the basal medium. After the 24-h serum starvation, the cells were incubated for 24 h in the basal medium in the absence or presence of stromal cell–conditioned medium. During the last 3 h of incubation, [^{3}H]thymidine was included in the medium. The monolayers were washed twice with each of following: ice-cold saline, cold 5% trichloroacetic acid (TCA) (10 min each) and deionized water. The TCA-insoluble residues were dissolved in 0.4 N NaOH, neutralized with acetic acid, and the radioactivity was quantified in a liquid scintillation counter (LKB/Pharmacia, Gaithersburg, MD).

To determine if intestinal stromal cells produce diffusible factors that stimulate IEC-6 cell proliferation, IEC-6 cells and stromal cells were grown on individual tissue culture coverslips (24 mm × 30 mm) in separate eight-well plates to 30 and 90% of confluence, respectively, with DMEM/F12 supplemented with the appropriate addition of FBS or insulin as described above. The cell monolayers were then serum-starved for 24 h in the basal medium. Coverslips bearing the cells were then removed from the eight-well plates and combined, two per well in several combinations in fresh four-well plates, and incubated for 48 h in the basal medium. [^{14}C]Leucine (0.25 μCi/well) and [^{3}H]thymidine (0.25 μCi/well) were added, and the incubation was continued for another 3 h. The incorporation of [^{3}H]thymidine into TCA-precipitable material (DNA) and [^{14}C]leucine into protein was measured [20].

To obtain large volumes of conditioned medium, stromal cells were grown to confluency in roller bottles in complete medium supplemented with 25 mM Hepes. The cells were washed three times with serum-free DMEM/F12, serum-deprived for 24 h and then incubated in the basal medium. The conditioned medium was collected every 48 h for 10 consecutive days, centrifuged (1000*g*; 10 min) to remove debris and unattached cells, and stored at −20 °C in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF). The conditioned medium was dialysed for 72 h against 0.1 M acetic acid in Spectrapor 3 dialysis tubing (molecular weight cutoff = 3500). The dialysed medium was lyophilized and then reconstituted with a small volume of 1 M acetic acid. The solution was clarified by centrifugation at 100,000*g* for 45 min, and the supernatant fraction was applied to a column (1.6 × 40 cm) of Bio-Gel P-10 (200–400 mesh, Bio-Rad) equilibrated with 1 M acetic acid. The column was eluted at 22 °C with 1 M acetic acid, and aliquots of each fraction were dried at room temperature in a Speed Vac Concentrator (Savant) prior to the measurement of IGF-I and IGF-II. The radioimmunoassay (RIA) for IGF-I and the radioreceptor assay (RRA) for IGF-II were carried out as described previously [20].

Characterization of type I and type II IGF receptors in intestinal stromal cells. Stromal cells were grown in 100-mm dishes to 80% confluency in DMEM/F12 plus 10% FBS. The monolayers were washed three times with serum-free DMEM/F12 and serum-starved for 24 h in the basal medium. After the 24-h serum-starvation, the cells were chilled to 3 °C and scraped, and the membranes were prepared as described previously [23]. The final pellets were resuspended in a small volume of the same buffer and stored at −20 °C until needed. Protein was assayed by the Bio-Rad method using human gamma globulin as standard. Affinity labelling of the IGF-I and IGF-II receptors in the membranes was done with the cross-linker disuccinimidyl suberate (DSS) as previously described [23].

Characterization of IGFBPs. The proteins in conditioned media (20 μl) were separated by SDS-PAGE (12.5% acrylamide) under nonreducing conditions as described by McCusker et al. [31]. Western ligand blots were prepared by transferring the proteins onto 0.05-μm BA75 nitrocellulose sheets (Schleicher and Schuell, Keene, NH) by electroblotting. Nonspecific binding sites

Table 1. Effect of intestinal stromal cells on the incorporation of [^{3}H]thymidine and [^{14}C]leucine by IEC-6 cells.

Culture conditions	[^{3}H]Thymidine incorporation (dpm/coverslip)	[^{14}C]Leucine incorporation (dpm/coverslip)
IEC-6 cells + IEC-6 cells	28,100 ± 2,500*	2400 ± 290
IEC-6 cells + stromal cells	88,400 ± 4,300	5800 ± 350

*Mean ± SEM, $n = 4$. Values are significantly different ($P < 0.05$) between different culture conditions for each parameter assessed. Stromal cells and IEC-6 cells were grown on individual tissue culture coverslips (24 mm × 30 mm) in separate eight-well plates to 90 and 30% confluency, respectively, in complete medium. After a 24-h serum starvation, coverslips were incubated for 48 h as pairs within the same well of fresh four-well plates, in the indicated combinations. After incubation with [^{3}H]thymidine or [^{14}C]leucine, the coverslips containing IEC-6 cells were washed and counted individually for incorporation of radioactivity into TCA-insoluble material.

were blocked by incubating the blots sequentially in 1% Nonidet P-40, 0.1% BSA and 2% Tween-20 for 10 min, 2 h and 20 min, respectively. To detect IGFBPs, the blots were incubated overnight at 3 °C with ^{125}I–IGF-I (100,000 cpm/ml). The blots were washed, and IGFBPs were visualized by autoradiography. Relative molecular weights (M_r) were estimated by running prestained molecular weight standards (Amersham, Arlington Heights, IL) in a parallel lane. To detect IGFBPs immunologically, blots were blocked with 3% BSA before being incubated with polyclonal antisera against human IGFBP-I [32] or bovine IGFBP-2 [33]. The blots were probed for 90 min, and immunoreactive bands were detected using an alkaline phosphatase-conjugated second antibody (Sigma) and the Protoblot detecting system according to the instructions of the manufacturer (Promega, Madison, WI).

Analysis of data. The data were expressed as mean ± SEM, and comparisons were made by analysis of variance. The differences among experimental treatments were analysed by Student's t test for independent samples or by Duncan's multiple range test [34].

Results

Stromal cells isolated from rat intestinal mucosa by enzymatic digestion could be cultured for up to the 11th passage and exhibited a doubling time of ~48 h. Light microscopic examination of the monolayers revealed a distinct fibroblast-like cellular morphology, with a flattened appearance and multiple elongated extensions (data not shown). To investigate whether intestinal stromal cells produce diffusible factors that stimulate intestinal epithelial cell growth, co-culture studies were performed. As shown in table 1, the incorporation of [^{3}H]thymidine into DNA and [^{14}C]leucine into protein by IEC-6 cells was significantly increased, by 3-fold and 2.5-fold, respectively, when IEC-6 cells were co-cultured with stromal cells. These results are consistent with those obtained from studies utilizing stromal cell-conditioned medium, which stimulated [^{3}H]thymidine incorporation into the DNA of IEC-6 cells in a dose-dependent manner (fig. 1).

The effect of stromal cells to stimulate IEC-6 cell proliferation could be mediated by several growth factors to which IEC-6 cells are responsive, including EGF [21, 35, 36], TGF-α [35] and IGFs [20, 21, 36]. To determine if stromal cells produced IGFs, serum-free conditioned medium was collected from confluent cultures, concentrated and subjected to gel filtration chromatography on a Bio-Gel P-10 column under acidic conditions (fig. 2). Fractions from this column were assayed for IGF-I by an RIA and for IGF-II by an RRA. Fractionation of medium conditioned by intestinal stromal cells yielded two major peaks of IGF-I RIA-reactive material. Identification of each peak was made by chromatography of purified IGFs and IGFBP-2 isolated from buffalo rat liver cells [37] on this same column. Similar assignments have been made previously using this approach [20, 37, 38]. Based upon this analysis, stromal cells produced mainly IGF-I and very little IGF-II. In addition, stromal cells produced IGFBPs, as

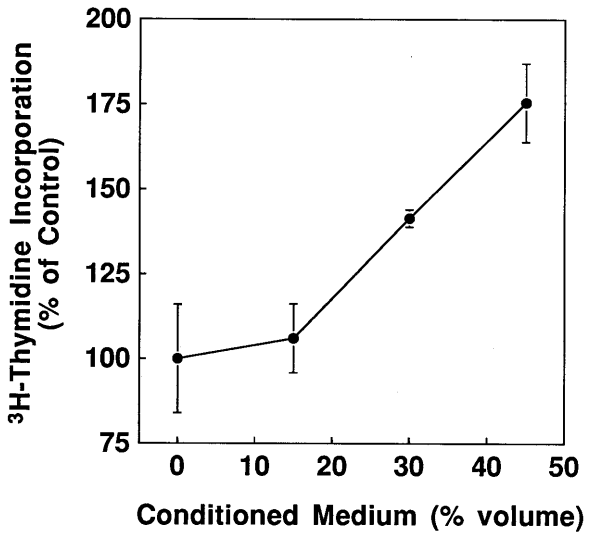

Figure 1. Effect of stromal cell-conditioned medium on the DNA synthesis of IEC-6 cells. Serum-starved IEC-6 cells were incubated for 24 h in the basal medium in the presence or absence of increasing amounts of stromal cell–conditioned medium prior to assay of [^{3}H]thymidine incorporation. Symbols represent means ± SEM ($n = 6$).

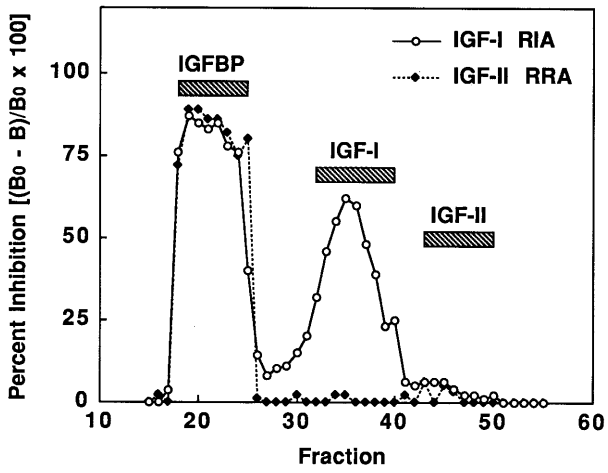

Figure 2. Fractionation of stromal cell-conditioned medium by Bio-Gel P-10 chromatography. Serum-free conditioned medium was collected from confluent cultures and dialysed for 72 h against 0.1 M acetic acid. Samples equivalent to 200 ml of original medium were then chromatographed on a Bio-Gel P-10 column, and 100-µl aliquots of each 1-ml fraction were removed for the determination of IGF-I by RIA and IGF-II by RRA. The horizontal bars denote the elution positions of purified IGFs and IGFBP-2 as determined by separate chromatographic runs using this same column.

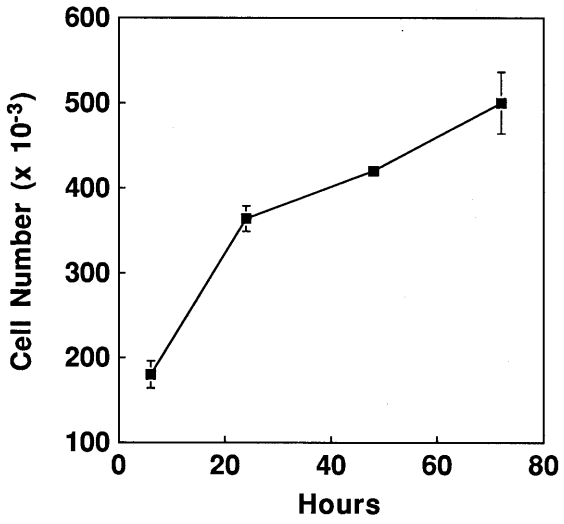

Figure 3. Proliferation of intestinal stromal cells in serum-free medium. Stromal cells (30% confluent) were rinsed three times and serum-deprived for 24 h in the basal medium. The medium was replaced with fresh basal medium, and the number of cells was estimated after an additional 6, 24, 48 and 72 h. Means ± SEM ($n = 6$).

indicated by the material eluting in the void volume that was highly reactive in both the RIA and RRA.

Since intestinal stromal cells produce several growth factors that may be involved in autocrine growth regulation, we investigated the possibility that intestinal stromal cells proliferate in serum-free medium in the absence of added growth factors. As shown in figure 3, they proliferated slowly in the basal medium without any added growth factors.

Previously, we have shown that IEC-6 cells have abundant quantities of the IGF-I and IGF-II receptors, suggesting that the mitogenic effect of IGFs is most likely mediated through the IGF-I receptor [23]. In the present studies, the structural characteristics of IGF receptors associated with stromal cell membranes were investigated in affinity-labelling studies. Affinity cross-linking with 1 nM ^{125}I–IGF-I using DSS followed by gel electrophoresis under reducing conditions revealed three labelled bands of apparent M_r of 270,000, 245,000 and 133,000 (fig. 4). The major labelled band was the 133,000 M_r species. In the presence of 20 nM unlabelled IGF-I, labelling of all three bands was coordinately diminished, by approximately 60%. Labelling of these three species was $\geq$80% inhibited by 100 nM IGF-I. IGF-II was less potent than IGF-I in its ability to inhibit labelling of either the 133,000 or 270,000 M_r species, but unlabelled IGF-II inhibited labelling of the 245,000 M_r species more efficiently than IGF-I. Unlabelled insulin was totally ineffective in inhibiting the ^{125}I–IGF-I labelling of any of these three bands. These data suggest that the 133,000 M_r species represents the α-subunit of the IGF-I receptor. The 270,000 M_r species may be a product of partial reduction of the heterotetrameric IGF-I receptor, whereas the 245,000 M_r species exhibits the ligand-binding specificity and molecular size expected of the IGF-II/mannose 6-phosphate receptor. Affinity cross-linking of 1 nM ^{125}I–IGF-II to isolated stromal cell membranes resulted in nearly exclusive labelling of a 245,000 M_r band (fig. 5). Unlabelled IGF-II efficiently inhibited labelling of this species, whereas at identical concentrations IGF-I was much less effective and insulin was without effect. These findings suggest that this species is the IGF-II/mannose 6-phosphate receptor.

Ligand blot and immunoblot analyses of medium conditioned by intestinal stromal cells revealed two ^{125}I–IGF-I binding proteins with M_r estimates of 32,000 for the major species and 24,000 for the minor species (fig. 6). Immunoblot analysis of conditioned medium was performed using antisera to bovine IGFBP-2 and human IGFBP-1. The IGFBP-2 antiserum reacted with the 32,000 M_r protein, whereas neither antibody reacted with the 24,000 M_r IGFBP. Based upon its apparent size and immunological characteristics, the 24,000 M_r IGFBP is probably IGFBP-4 [39].

Discussion

In the present study we report that stromal cells derived from the rat small intestine produced IGF-I-like peptides and IGFBPs. Our principal finding was that IEC-6

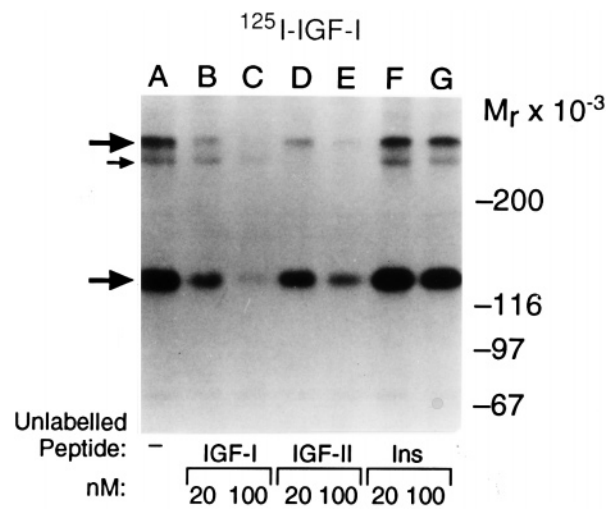

Figure 4. Affinity cross-linking of ^{125}I–IGF-I to intestinal stromal cell membranes. Stromal cell membranes were incubated with 1 nM ^{125}I–IGF-I in the absence of unlabelled peptide (lane A) or in the presence of the indicated concentrations of unlabelled IGF-I, IGF-II or insulin. The receptor–ligand complexes were cross-linked with DSS and electrophoresed on a 5% polyacrylamide gel after treatment with 50 mM dithiothreitol. An autoradiogram of the dried gel is shown. These data are representative of two replicate experiments. Arrows designate the major radiolabelled species migrating at M_r 270,000, 245,000 and 133,000.

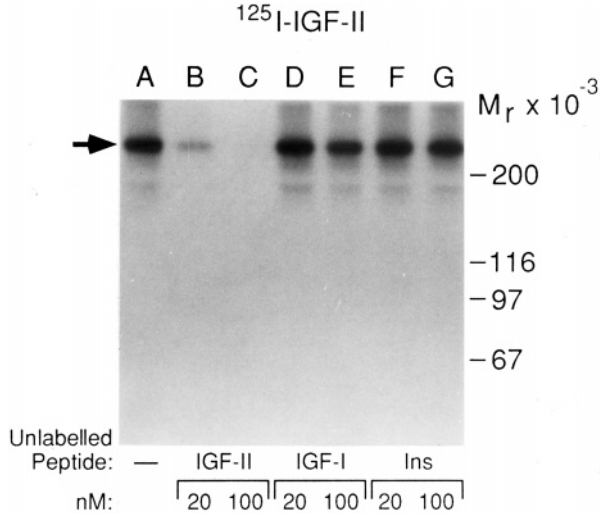

Figure 5. Affinity cross-linking of ^{125}I–IGF-II to intestinal stromal cell membranes. Stromal cell membranes were incubated with 1 nM ^{125}I–IGF-II in the absence of unlabelled peptide (lane A) or in the presence of the indicated concentrations of unlabelled peptides. The receptor–ligand complexes were cross-linked with DSS and electrophoresed on a 6% polyacrylamide gel after treatment with 50 mM dithiothreitol. The arrow designates the major radiolabelled species migrating at M_r 245,000.

cells co-cultured with these stromal cells responded by an increased incorporation of [^{3}H]thymidine and [^{14}C]leucine. There was also an increase in the DNA and protein synthesis of IEC-6 cells when stromal cell-conditioned medium was added to the incubation medium. Thus, we have provided evidence for elaboration by intestinal stromal cells of diffusible growth factors, including IGF-I and IGFBPs, capable of eliciting a proliferative response in an intestinal epithelial model cell line. To our knowledge, the only previous report of factors produced by intestinal fibroblasts in vitro identified prostaglandin E2 release in response to inflammatory mediators [27]. IEC-6 cells have been employed before as the epithelial cell partner in an in vitro model of fibroblast-epithelial cell interaction, in which Ferretti et al. [30] demonstrated increased sucrase activity in IEC-6 cells exposed to fibroblast-conditioned medium or extracellular matrix. The observation that rat intestinal stromal cells produced IGF-I in vitro is in agreement with previous studies by Lund et al. [13] and Dvorák et al. [16] that demonstrated the presence of IGF-I mRNA in rat intestine. It is noteworthy that in the latter study, IGF-I expression was highest in the crypt region of adult rats, the same area from which the stromal cells used in our study were derived.

In the lamina propria of the small intestine, the bases of the crypts of Lieberkuhn are surrounded by a layer of myofibroblasts referred to as the pericryptal fibroblast sheath [24]. These cells are thought to interact intimately with the adjacent epithelial cells in order to regulate the proliferation and subsequent differentiation of the epithelial progenitor cells located in the lower one-third of the crypts [24, 26]. The proximity and synchrony of these cell populations led to the postulation by Zajicek [40] that they form an intestinal 'proliferon'. Although this hypothesis has largely been discredited [41], current evidence still favours the concept of regulation of epithelial cell function by the pericryptal fibroblast sheath [26]. IGF-I may be one component of the intercellular communication network operating as a paracrine mechanism between these cell populations. However, several issues must be resolved in order to provide definitive support for this hypothesis. First, it is important to determine if the intestinal stromal cells that we have isolated are actually derived from the pericryptal sheath. Second, production of IGF-I by intestinal stromal cells must be demonstrated in vivo. Third, we must examine the possibility that other factors are involved in this interaction. These may include other diffusible factors that have demonstrated effects on growth of intestinal epithelial cell lines, such as EGF, TGFα or TGF-β [21, 35, 36, 42, 43], extracellular matrix component or possibly juxtacrine effects mediated through direct cell–cell contact. Finally, higher-order regulation of intestinal epithelial regeneration by neural and endocrine mechanisms may also be mediated through the fibroblasts [25, 44, 45].

We have previously reported that growth hormone (GH) did not stimulate IEC-6 cell growth [23], even though this anterior pituitary hormone has been shown to enhance mucosal growth in vivo [44]. In addition, our previous results indicate that IEC-6 cells do not possess growth hormone receptors [23]. These results

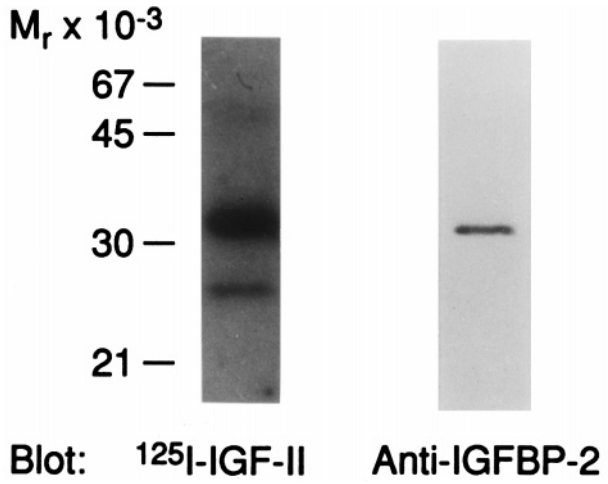

Figure 6. IGFBP secretion by intestinal stromal cells. Left: Ligand blot of intestinal stromal cell-conditioned medium with ^{125}I–IGF-I. Right: Immunoblot of stromal cell-conditioned medium with IGFBP-2 antiserum. The scale at the left indicates the positions of protein molecular weight standards.

suggest that GH does not have a direct action on intestinal epithelial cells, but an indirect action mediated by other cell types is likely. Cultured human dermal fibroblasts secrete immunoreactive IGFs into serum-free medium under the influence of GH [46, 47]. Thus, it is possible that GH stimulates IGF-I production by intestinal stromal cells, which in turn stimulates the proliferation of intestinal epithelial cells. In addition to GH, other factors may stimulate IGF-I production by intestinal fibroblasts. The search for and study of such factors should help elucidate the mechanisms that modulate intestinal epithelial regeneration in normal and disease states.

Analysis of medium conditioned by intestinal stomal cells in vitro revealed that these cells produced IGF peptides and IGF-binding proteins. Furthermore, these cells have IGF-I and IGF-II/mannose 6-phosphate receptors, indicating that they are fully equipped to respond to their own endogenously produced IGFs as well as to IGFs secreted by neighbouring cells. The possibility of IGF-dependent autocrine growth regulation within the intestinal mesenchyme is also suggested by the observation that intestinal stromal cells proliferated, albeit slowly, in basal medium lacking exogenously added growth factors. However, it is not yet certain that endogenously produced IGFs provided the driving force for proliferation in these cell cultures. Stromal cells also secreted large amounts of IGFBPs, but the function(s) of these molecules in modulating the IGF-mediated communication between stromal and epithelial cells in the intestine is not known. The particular binding protein which predominates in this system, IGFBP-2, has been found to attenuate cellular responses to IGFs by sequestering the growth factor under most circumstances [2, 4, 20]. However, binding of IGF–IGFBP-2 complexes to heparin and extracellular matrix has recently been demonstrated, and it is not yet known whether this interaction may potentiate the IGF response [48]. Thus, although the role of IGFBP-2 in the intestinal crypt may be to dampen IGF-dependent intercellular communication, further study of its properties and expression is certainly warranted. The stromal cells also secrete a 24,000 M_r IGFBP, which we infer from its size to be IGFBP-4. This species and its relevance to IGF action in this system remain to be definitively identified.

In the present study, we have developed in vitro model systems for the study of intestinal epithelial cell proliferation. Based upon the idea that stromal cells underlying the epithelial progenitor cells in the crypts are intimately involved in regulating epithelial cell growth and differentiation [24, 26, 40], we decided to characterize the two cell types for the elements which control IGF action. This analysis has revealed that IEC-6 cells respond to IGF-I with increased synthesis of protein and DNA [20], and that the IGF-I necessary to invoke this response can be produced by intestinal stromal cells in vitro [this report]. In spite of our efforts to devise as simple a model as possible, we discovered a complex array of IGFs, IGF receptors and IGFBPs expressed by both cell types. These observations serve to underscore the difficulties inherent in tracing the pathways of intercellular communication even in the simplest models for IGF action. Given the present state of our knowledge, extrapolation of our results to the in vivo situation is risky. Nevertheless, our data suggest and we speculate that IGF-I produced by pericryptal stromal cells is an important paracrine factor for stimulation of intestinal epithelial cell proliferation in vivo. There may be a coordinated mechanism whereby IGF-I and IGF-II are secreted by different cell types within the gut, and the magnitude and directionality of cellular responses depend on fine-tuning of IGF peptide availability by the presence of IGFBPs also secreted by these cells.

In summary, affinity cross-linking studies indicate that intestinal stromal cells have abundant quantities of the type I and II IGF receptors. The observations that stromal cell–conditioned medium stimulates DNA synthesis in IEC-6 cells and that DNA and protein synthetic rates in IEC-6 cells are increased when IEC-6 cells are co-cultured with stromal cells suggest that diffusible factors produced by stromal cells may regulate the growth of intestinal epithelial cells by a paracrine mechanism. Characterization of medium conditioned by intestinal stromal cells provided evidence for elaboration of an IGF-I-like peptide, IGFBP-2 and a second IGFBP among those factors.

In addition, the discovery that stromal cells proliferate in serum-free medium suggests that they regulate their own growth rates by an autocrine mechanism.

Acknowledgments. This work was supported by a grant from Hallym University. We are grateful to Mrs. M. H. Niedenthal of Lilly Research Laboratories, Indianapolis, IN, for the gifts of porcine insulin and recombinant human IGFs, to Dr. R. Bowsher for ^{125}I–IGF-II and to the National Hormone and Pituitary Program, Baltimore, MD, for rabbit IGF-I antiserum. Immunoblots of conditioned medium proteins for IGFBP-1 and IGFBP-2 were performed at the University of North Carolina at Chapel Hill using antisera developed by Dr. D. R. Clemmons.

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