Autocrine and paracrine actions of intestinal fibroblast-derived insulin-like growth factors

JAMES G. SIMMONS,1,2 JOLANTA B. PUCILOWSKA,1,2 AND P. KAY LUND1,2

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Simmons, James G., Jolanta B. Pucilowska, and P. Kay Lund. Autocrine and paracrine actions of intestinal fibroblast-derived insulin-like growth factors. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G817–G827, 1999.—Paracrine and autocrine actions of the insulin-like growth factors (IGFs) are inferred by local expression within the bowel. CCD-18Co cells, IEC-6 cells, and immunoneutralization were used to analyze whether IGFs have direct autocrine or paracrine effects on proliferation of cultured intestinal fibroblasts and epithelial cells. Growth factor expression was analyzed by ribonuclease protection assay and RT-PCR. Extracellular matrix (ECM) was analyzed for effects on cell proliferation. CCD-18Co cells express IGF-I mRNA and low levels of IGF-I mRNA. Conditioned medium from CCD-18Co cells (CCD-CM) stimulates proliferation of IEC-6 and CCD-18Co cells. Neutralization of IGF immunoreactivity in CCD-CM reduced but did not abolish this effect. RT-PCR and immunoneutralization demonstrated that other growth factors contribute to mitogenic activity of CCD-CM. Precultivation of CCD-CM with ECM prepared from IEC-6 or CCD-18Co cells reduced its mitogenic activity. ECM from CCD-18Co cells enhanced growth factor-dependent proliferation of IEC-6 cells. IEC-6 cells ECM inhibited IGF-I action on CCD-18Co cells. We conclude that IGF-II is a potent autocrine mitogen for intestinal fibroblasts. IGF-II interacts with other fibroblast-derived growth factors and ECM to stimulate proliferation of intestinal epithelial cells in a paracrine manner.

intestinal epithelial cells; intestinal fibroblasts; extracellular matrix; conditioned medium

IN THE INTESTINAL MUCOSA, connective tissue, comprising fibroblasts or myofibroblasts, lies beneath the epithelial cells, and the two cell types are separated by a basement membrane composed of extracellular matrix (ECM) (10, 25, 26). Normal morphogenesis and differentiation of the intestinal epithelium during development require interactions between epithelial cells and these neighboring mesenchymal cells (10, 25). It is generally accepted that the intestinal mesenchymal cells provide trophic factors that regulate crypt cell proliferation and continuous renewal of the intestinal epithelium. The precise mesenchymal cell-derived growth factors that regulate these processes are not well defined. Little is known about the growth factors that regulate proliferation of intestinal fibroblasts or myofibroblasts themselves. The insulin-like growth factors (IGFs) are good candidates as mesenchymal cell-derived growth factors that may modulate proliferation of intestinal crypt epithelial cells in a paracrine manner and/or have autocrine effects on intestinal mesenchymal cells. Accumulating evidence indicates that the IGFs are trophic factors for the small intestine (11, 13). IGF-I and IGF-II are widely expressed in many tissues, including the small and large intestine (5, 12, 33). Available evidence indicates that the primary sites of expression of the IGFs within the small and large bowel are mesenchymal cells within the lamina propria of normal bowel (5, 33) or mesenchymal cells within lamina propria, submucosa, or smooth muscle layer of bowel during experimentally induced inflammation and damage (15, 35, 37). These sites of IGF expression support a hypothesis that IGF-I derived from intestinal mesenchymal cells may exert paracrine actions to regulate growth and function of neighboring epithelial cells. An alternative, and not mutually exclusive, possibility is that IGFs regulate proliferation and function of intestinal mesenchymal cells themselves. These hypotheses are supported by observations that levels of locally expressed IGF-I mRNA within the intestine correlate with changes in growth induced by fasting and refeeding (33), bowel resection, damage, or disease (14, 15, 23, 36, 37). More direct evidence for autocrine actions of IGF-I on intestinal mesenchymal cells derives from recent observations in transgenic mice that have germ line transmission of a chimeric transgene composed of the α-smooth muscle actin promoter and a rat IGF-I cDNA (32). In these mice, the transgene is expressed at high levels in intestinal smooth muscle cell layers and this leads to increased mass of enteric smooth muscle and increased bowel length (32). The present study used model in vitro cell culture systems to test whether IGF-I or IGF-II expressed in intestinal fibroblasts have direct proliferative actions on intestinal epithelial cells or on fibroblasts themselves.

MATERIALS AND METHODS

Cell lines. IEC-6 cells, an epithelial cell line derived from neonatal rat intestine (20), and CCD-18Co cells, a fibroblast cell line derived from human colon (30), were used as model systems. IEC-6 cells at passage 9 were kindly provided by Dr. Andrea Quaroni (Cornell University Medical Center, Ithaca, NY). IEC-6 cells (CRL 1592) at passage 13 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). CCD-18Co cells (CRL 1459, normal human colon fibroblasts) at passage 6 were obtained from ATCC. Both cell types were propagated in 75- or 150-cm² flasks at 37°C in 5% CO₂-95% humidity. IEC-6 cells were propagated in growth medium (DME), supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin), and ITS (insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), Boehringer Mannheim, Indianapo-
lis, IN]. CCD-18Co cells were grown in the same medium without ITS. All experiments were performed on IEC-6 cells between passage 14 and 20 and on CCD-18Co cells between passage 8 and 13. For experiments, both cell types were switched to serum-free DMEM supplemented with 0.1% BSA and 5 µg/ml transferrin. For experiments with conditioned medium (CM), the CM was collected from subconfluent cells after 4 h of incubation in serum-free medium and used immediately or stored at 4°C until use. CM was used routinely within 1 wk of collection.

Primary cultures of human intestinal fibroblasts. A subset of experiments was performed on early passage human intestinal fibroblast cultures. These cells were derived from primary cultures of grossly normal tissue at the margins of diseased small intestine obtained from a patient with Crohn's disease after necessary surgical resection. Cells were prepared according to methods described by Stallmach et al. (29). Briefly, the fibroblast-containing lamina propria was dissected away from other layers of the resected bowel after extensive washing in PBS (pH 7.4) plus 0.5% sodium hypochlorite at 4°C. Minced fragments of the lamina propria were incubated in DMEM containing collagenase, neuraminidase, and hyaluronidase for 60 min at 37°C. After enzyme treatment, tissue fragments were cultured on plastic dishes with DMEM plus 10% FBS and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml amphotericin B). Monolayers surrounding explants were subcultured after 10–14 days. Subconfluent cells (HIF-4) were studied at passages 4–6. Cells were deprived of serum for 24 h, and CM was collected as previously described for CCD-18Co cells. Serum-deprived HIF-4 cells were also collected for preparation of total RNA (see RNA analysis).

Growth factors, IGFBP-3, and antibodies. Human recombinant insulin-like growth factor I (IGF-I) was obtained from Genentech (San Francisco, CA) and human recombinant insulin-like growth factor II (IGF-II) was obtained from GIBCO BRL (Kalamazoo, MI). Basic fibroblast growth factor (bFGF, from bovine pituitary gland), human recombinant keratinocyte growth factor (KGF or fibroblast growth factor-7 (FGF-7)), human recombinant epidermal growth factor (EGF), and human recombinant hepatocyte growth factor (HGF) were obtained from Sigma Chemical (St. Louis, MO). Human recombinant IGF binding protein 3 (IGFBP-3) was obtained from Upstate Biotechnology (Lake Placid, NY). A mouse monoclonal antibody (Sm 1.2) that immunoneutralizes human IGF-I and IGF-II (31) was kindly provided by Dr. J. Van Wyk (University of North Carolina at Chapel Hill, NC). Immunoneutralizing mouse monoclonal antibody to human bFGF was purchased from Calbiochem (San Diego, CA), and immunoneutralizing monovalent complexes of human KGF-FGF-7 and human HGF were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies to human vimentin and human α-smooth muscle actin were obtained from Dako (Carpenteria, CA). An anti-actin polyclonal antibody to the conserved COOH terminus of cytoskeletal-contractile actin was purchased from Sigma and used as a negative control in immunoneutralization assays.

Immunocytochemistry. Phenotype of CCD-18Co cells was examined by immunocytochemistry for vimentin and α-smooth muscle actin. Vimentin is a cytoskeletal marker expressed in fibroblasts (4, 17). α-Smooth muscle actin is expressed in cells with myofibroblast or smooth muscle phenotype (4, 27, 30). Cells were grown on plastic slides and serum deprived for 24 h and then washed in PBS and fixed in 100% methanol (−20°C). Slides were blocked with PBS containing 1% horse serum for 30 min at room temperature and then incubated for 1 h at room temperature with 1:100 dilutions of antibodies to vimentin or α-smooth muscle actin. Slides were then incubated with a Texas red–labeled goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (Nikon Diaphot inverted fluorescence microscope).

Assays of cell proliferation. CCD-18Co cells or IEC-6 cells were seeded into 24-well plates at densities of 1 × 10^5 per well and grown for 16–24 h in medium with FBS. Cells were switched to serum-free medium for 24 h, and then medium was aspirated and replaced with fresh serum-free medium with or without growth factors or CM. For assays of DNA synthesis, medium was supplemented with 2 µCi/ml [3H] thymidine for the entire duration (16–24 h) of treatment with serum-free medium with or without growth factor or CM. Thymidine incorporation into DNA was measured by washing cells twice in PBS, fixing with 10% TCA, and harvesting of precipitated DNA in 0.2 N NaOH and 0.1% SDS. Radioactivity was quantified by scintillation counting. Assays were performed in triplicate or quadruplicate and were replicated in at least two separate experiments. To quantitate cell number, serum-deprived cells were incubated with or without growth factors or CM for 24 or 48 h and then washed once with PBS plus 50 µg/ml EDTA and trypsinized (0.25% trypsin-50 mM EDTA). Cell numbers were assessed using a hemocytometer.

RNA analyses. We previously have analyzed IEC-6 cells for expression of IGF-I and IGF-II and found no detectable expression of IGF-I or IGF-II mRNAs using highly sensitive RNase protection assays on IEC-6 cell polyadenylated RNA (24). To evaluate expression of the IGFs in CCD-18Co cells and HIF-4 cells, the cells were grown to subconfluence (~70%) in 150-cm² flasks in DMEM plus 10% FBS, serum-deprived for 24 h, and then washed in PBS and harvested into guandine thiocyanate (12). Total RNA was pelleted by centrifugation over 5.7 M CsCl as previously described (12). Expression of IGF-I and IGF-II was measured by RNase protection assays (33, 37) using [32P]-labeled human IGF-I and IGF-II antisense RNA probes (6, 16). RT-PCR reaction was performed to assess expression of bFGF, HGF, and KGF mRNAs in CCD-18Co and HIF-4 cells. Sense and antisense oligomers used for RT-PCR of these mRNAs and control glyceraldehyde-3-phosphate dehydrogenase mRNA are shown in Table 1. Total RNA was reverse-transcribed into cDNA using oligo(dT) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and then amplified by PCR using the GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Conditions for PCR reactions were denaturation for 2 min at 94°C, followed by 40 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and amplification at 72°C (1 min). Table 1. Oligonucleotide sequences used in RT-PCR

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<th>mRNA</th>
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<tr>
<td>GAPDH Sense</td>
<td>5'-CTA CTG GCC CTG CCA AGG CTG T-3'</td>
</tr>
<tr>
<td>GAPDH Antisense</td>
<td>5'-GCC ATG AGG TCC ACC ACC CTG TTG-3'</td>
</tr>
<tr>
<td>GAPDH bFGF Sense</td>
<td>5'-CTT CAC ATC AAG CTA CAA CTT CA-3'</td>
</tr>
<tr>
<td>GAPDH KGF Sense</td>
<td>5'-TCT GTC GAA CAC AGT GGT ACC TCC-3'</td>
</tr>
<tr>
<td>GAPDH HGF Sense</td>
<td>5'-CCA TGA TAC CAC ACG AAC ACA GC-3'</td>
</tr>
<tr>
<td>GAPDH Glyceroldehyde-3-phosphate dehydrogenase</td>
<td>5'-GTC AAG AGT ATA GCA CCA TGG CCT-3'</td>
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GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bFGF, basic fibroblast growth factor; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor.
Immunoneutralization assays. To assess whether IGFs or other growth factors contribute to mitogenic activity in CM from CCD-18Co cells (CCD-CM), [3H]thymidine incorporation was measured in IEC-6 cells or CCD-18Co cells that were treated with CCD-CM or CCD-CM plus 6 µg/ml of anti-IGF monodonal Sm 1.2 or CCD-CM plus neutralizing doses of antibodies to bFGF (0.6 µg/ml), HGF (3 µg/ml), or KGF (3 µg/ml) (Sigma), as recommended by the manufacturer. CCD-CM plus an anti-actin polyclonal antibody (3 µg/ml) was used as a negative control. As an additional test for IGF bioactivity in CCD-CM, IGFBP-3, an IGF binding protein that binds IGF-I and IGF-II with high affinity (7), was added to CCD-CM before assays of effects on thymidine incorporation in IEC-6 cells. Similar neutralization assays with Sm 1.2 and IGFBP-3 were performed on CM from HIF-4 cells to assess if IGFs are mitogenic factors in CM from early passage cultures of human intestinal fibroblasts as well as the CCD-18Co cell line.

Isolation of ECM. IEC-6 and CCD-18Co cells were seeded in 12-well plates and grown to confluence and then serum deprived for 24 h. Cells were lysed, and ECM was prepared according to Kramer et al. (8). Briefly, cell monolayers were washed once with PBS plus 1% BSA, followed by two 5-min washes with hypotonic buffer [10 mM Tris·HCl, 0.1% BSA, 0.1 mM CaCl₂, pH 7.5, plus protease inhibitors, 1 mM phenylmethylsulfonyl fluoride and 1 mM N-ethylmaleimide]. Cell membranes were dissolved in hypotonic solution plus 0.5% nonidet P-40 (NP-40, Sigma). This was followed by two 2-min extractions in hypotonic buffer with 0.2% sodium deoxycholate. As described by Kramer et al. (8) and confirmed in this study by light microscopy, ECM at this point was free of membranes, nuclei, and cellular debris. For experiments, cells were plated on freshly prepared ECM immediately after preparation.

Statistics. All data are expressed as means ± SE; statistical comparisons were made by Student’s t-test or one-factor ANOVA for comparisons among multiple groups. Statistical significance is set at P < 0.05.

RESULTS

Characterization of CCD-18Co cells for phenotype and IGF expression. Subconfluent, serum-deprived CCD-18Co cells uniformly stain positive for vimentin, but only a few scattered cells (less than 5%) show fibrillar immunostaining for α-smooth muscle actin (Fig. 1). Primarily negative immunostaining for α-smooth muscle actin indicates fibroblast phenotype. The presence of a small subset of α-smooth muscle actin-positive cells is a common feature of fibroblast cell lines, even clonally derived cell lines, indicating plasticity of phenotype and ability to spontaneously transform into myofibroblasts (3, 4, 22).

Mitogenic effects of IGF-I, IGF-II, and other peptide growth factors in CCD-18Co and IEC-6 cells. Effects of different doses of IGF-I and IGF-II on DNA synthesis were compared in CCD-18Co and IEC-6 cells. Both IGFs exert mitogenic effects on CCD-18Co and IEC-6 cells. IGF-I was more potent than IGF-II in CCD-18Co cells, but maximum responses to both growth factors were observed at doses between 5 and 10 ng/ml (Fig. 2A). IGF-I and IGF-II were equipotent in IEC-6 cells.
with maximum effects also observed at doses between 5 and 10 ng/ml (Fig. 2B). IGF-I, rather than IGF-II, was used in subsequent assays of cell proliferation because it is more readily available. In addition, all known mitogenic effects of IGF-I and IGF-II are mediated by the type 1 receptor, which interacts with both IGFs with similar affinity (11). IGF-II, and not IGF-I, can interact with a type 2 IGF receptor, which is identical to the cation-independent mannose-6 phosphate receptor (11). At present, the biological role of the type 2 IGF receptor is not known. By using IGF-I rather than IGF-II in subsequent assays of cell proliferation, we excluded any possible type 2 IGF receptor-mediated effects. Effects of maximal doses of IGF-I and IGF-II (10 ng/ml) on DNA synthesis in CCD-18Co and IEC-6 cells are shown in Fig. 3. Effects of maximal doses of IGF-I and IGF-II (10 ng/ml) on DNA synthesis in CCD-18Co and IEC-6 cells are shown in Fig. 3. Effects of the IGFs also were compared with several other growth factors. All growth factors were used at doses shown to elicit maximum responses in preliminary experiments. Growth factors were tested alone and in combinations. In CCD-18Co cells, IGF-I and IGF-II had more potent effects on DNA synthesis than bFGF or EGF (Fig. 3A). HGF and KGF did not significantly stimulate DNA synthesis above background. Responses to IGF-I in combination with EGF or bFGF did not differ significantly from responses to IGF-I alone. Responses to EGF and bFGF were less than additive when the two growth factors were added in combination.

Consistent with prior observations, IGF-I and IGF-II alone had relatively small stimulatory effects on DNA synthesis in IEC-6 cells, much less than the effects observed with EGF or bFGF (Fig. 3B) (24). However, when added in combination with either EGF or bFGF, IGF-I showed synergistic effects on DNA synthesis in IEC-6 cells. In contrast, EGF and bFGF added in combination did not show synergistic or even additive mitogenic effects on DNA synthesis in IEC-6 cells. HGF and KGF had weak mitogenic effects on IEC-6 cells when added alone and had only additive effects when added in combination with IGF-I.

CCD-CM has mitogenic effects on IEC-6 and CCD-18Co cells. We examined the mitogenic effects of CCD-CM on IEC-6 and CCD-18Co cells to assess potential paracrine or autocrine effects on cell proliferation. Inclusion of 25% CCD-CM in serum-free medium stimulated DNA synthesis in both IEC-6 and CCD-18Co cells. The effect observed in IEC-6 cells was much greater (23.4 ± 3.6-fold is maximum response) than in CCD-18Co cells, which showed maximum responses of only 2.8 ± 0.1-fold. CM from IEC-6 cells had no effect on DNA synthesis in either IEC-6 or CCD-18Co cells (data not shown). As shown in Fig. 4A, the effect of CCD-CM on DNA synthesis in IEC-6 cells was dose dependent. CCD-CM also stimulated proliferation and/or survival of IEC-6 cells. IEC-6 cells plated in serum-free medium survive for 2 days but then begin to detach and die by day 3 of culture (Fig. 4B). Addition of CCD-CM promoted an increase in cell number by day 2 of incubation and then maintained survival of cells after 3 days in culture in serum-free medium, a time when IEC-6 cells grown in serum-free medium alone began to die. The mitogenic activity of CCD-CM was still evident, although diminished, after storage for up to 4 wk at 4°C, indicating that the mitogenic activity is reasonably stable.

IGFs are a component of complex mitogenic activity secreted by intestinal fibroblasts. To assess if IGFs are expressed in CCD-18Co cells or primary cultures of human intestinal fibroblasts, RNA protection analyses were performed on total RNA. Both CCD-18Co and HIF-4 cells express significant levels of IGF-II mRNA and low levels of IGF-I mRNA (Fig. 5).

Antibody Sm 1.2, a neutralizing IGF antibody, was tested on IEC-6 and CCD-18Co cells for effect on basal or CCD-CM-stimulated DNA synthesis. Antibody Sm 1.2 modestly inhibited basal DNA synthesis in CCD-18Co cells but was without effect on basal DNA synthesis in IEC-6 cells (Fig. 6). Antibody Sm 1.2 partially inhibited CCD-CM-mediated DNA synthesis in CCD-
18Co and in IEC-6 cells, although the inhibitory effect was smaller in IEC-6 cells (17.6 ± 6.5% inhibition) than in CCD-18Co cells (37.2 ± 5.0% inhibition; Fig. 6). The partial neutralization of the mitogenic effects of CCD-CM with antibody Sm 1.2 could indicate that the antibody cannot completely neutralize the IGFs present in CCD-CM. Addition of increasing amounts of antibody Sm 1.2 or preincubation of antibody Sm 1.2 with CCD-CM before addition to cells did not, however, enhance the inhibitory effects of the antibody (data not shown), providing some evidence against this possibility. Antibody Sm 1.2 has relatively low affinity for the IGFs (31), and so we tested an alternative inhibitor of IGF-action, IGFBP-3. IGFBP-3 binds both IGF-I and IGF-II with high affinity and with similar affinity as the type I IGF receptor (7). In IEC-6 cells, IGFBP-3 had more potent inhibitory effects on CCD-CM-stimulated DNA synthesis than antibody Sm 1.2 (Table 2). In CCD-18Co cells, IGFBP-3 exhibited similar inhibitory effects on CCD-CM-stimulated DNA synthesis (36.0 ± 3.2%) as observed with Sm 1.2 (37.2 ± 5.0%). For IEC-6 cells, less than complete inhibition of mitogenic activity in CCD-CM by Sm 1.2 or IGFBP-3 may be because the mitogenic effects of CCD-CM reflect interactions be-

Fig. 4. A: graph to show dose-dependent effects of conditioned medium from CCD-18Co cells (CCD-CM) on [3H]thymidine incorporation (means ± SE, n = 6) measured in IEC-6 cells. Amount of added CCD-CM is expressed as percentage of total volume of medium. B: graph to indicate cell number in IEC-6 cell cultures incubated in serum-free medium with or without 25% CCD-CM. Time points on abscissa reflect total number of days in serum-free medium ± CCD-CM. Data represent means ± SE of 8 independent observations. *P < 0.05 for cells incubated with CCD-CM compared with controls incubated in serum-free medium minus CCD-CM.

Fig. 5. RNase protection assays of IGF-I, IGF-II, and control glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. Shown is autoradiogram of RNase protection assays on total RNA extracted from adult human liver (positive control) and serum-deprived CCD-18Co cells (CCD) and HIF-4 cells. P, undigested probes as indicated at left; P + R, RNase-treated probes. Lanes I, II and G show protected bands with IGF-I, IGF-II and GAPDH (RNA) probes, respectively, in liver, CCD-18Co cells, and HIF-4 cells. Multiple bands occur with IGF-I and IGF-II probes because of alternate splicing (11). Two protected bands typically are observed with commercially purchased human GAPDH probe. Sizes of undigested probe in nucleotides (nt) are shown at left, and sizes of protected fragments are shown at right.

DNA synthesis than antibody Sm 1.2 (Table 2). In CCD-18Co cells, IGFBP-3 exhibited similar inhibitory effects on CCD-CM-stimulated DNA synthesis (36.0 ± 3.2%) as observed with Sm 1.2 (37.2 ± 5.0%). For IEC-6 cells, less than complete inhibition of mitogenic activity in CCD-CM by Sm 1.2 or IGFBP-3 may be because the mitogenic effects of CCD-CM reflect interactions be-

Fig. 6. Partial inhibition of CCD-CM-stimulated DNA synthesis by immunoneutralization of IGF-immunoreactivity with monoclonal antibody Sm 1.2. Histograms show [3H]thymidine incorporation in serum-deprived CCD-18Co cells and IEC-6 cells during additional 24 h in serum-free medium without (−) or with (+) CCD-CM and in absence (−) or presence (+) of antibody Sm 1.2. Data represent means ± SE (n = 6). *P < 0.05 vs. identical conditions and no Sm 1.2 antibody.
Inhibition of CCD-CM-stimulated DNA synthesis in IEC-6 cells

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<thead>
<tr>
<th>Inhibitory Factor</th>
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<tr>
<td>IGFBP-3</td>
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</tr>
<tr>
<td>Anti-actin</td>
<td>0.0 ± 3.1</td>
</tr>
<tr>
<td>Anti-Ig (Sm 1.2)</td>
<td>15.0 ± 2.0*</td>
</tr>
<tr>
<td>Anti-bFGF</td>
<td>25.2 ± 4.4*</td>
</tr>
<tr>
<td>Anti-KGF</td>
<td>21.0 ± 3.4*</td>
</tr>
<tr>
<td>Anti-HGF</td>
<td>26.7 ± 2.3*</td>
</tr>
<tr>
<td>Anti-IGF + anti-bFGF</td>
<td>27.7 ± 3.2*</td>
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<tr>
<td>Anti-IGF + anti-KGF</td>
<td>15.0 ± 2.0*</td>
</tr>
<tr>
<td>Anti-IGF + anti-HGF</td>
<td>28.1 ± 2.1*</td>
</tr>
<tr>
<td>Anti-bFGF + anti-KGF</td>
<td>25.6 ± 7.1*</td>
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<td>Anti-bFGF + anti-HGF</td>
<td>41.7 ± 2.9*</td>
</tr>
<tr>
<td>Anti-KGF + anti-HGF</td>
<td>29.9 ± 1.5*</td>
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Values are means ± SE. CCD-CM, conditioned medium from CCD-18Co cells; IGF, insulin-like growth factor; IGFBP, IGF binding protein. Each data point represents mean of at least 6 separate observations obtained from 2 separate experiments performed in triplicate. *P < 0.05 vs. CCD-CM alone.

Table 2.

Inhibitory effects of IGFBP-3 and another growth factor(s). To address this possibility, we tested whether neutralizing antibodies to other growth factors affect the mitogenic activity of CCD-CM on IEC-6 cells. As illustrated in Table 2, anti-KGF, anti-HGF, and anti-bFGF all inhibited CCD-CM-stimulated DNA synthesis in IEC-6 cells. A control antibody to actin was without inhibitory effect (Table 2). No single antibody or IGFBP-3 was able to completely neutralize the mitogenic activity of CCD-CM on IEC-6 cells and, although we tested antibodies to one or more growth factors in combination, we were not able to achieve complete inhibition of the mitogenic activity in CCD-CM. In fact, addition of combinations of antibodies to different growth factors generally failed to elicit greater inhibitory effects than antibodies to individual growth factors alone, with possible exception that the inhibitory effects of anti-bFGF plus anti-HGF were additive (Table 2). IGF-I can exert synergistic mitogenic effects on IEC-6 cells when cells are briefly preexposed to even low doses of other growth factors such as bFGF or EGF followed by subsequent addition of IGF-I (24). It seems possible therefore that immunoneutralization is not able to completely neutralize the synergistic interactions among growth factor combinations present in CCD-CM or that as yet unidentified mitogens are present in CCD-CM. Another possibility is that mitogenic factors in CCD-CM bind to ECM of IEC-6 cells which impedes complete immunoneutralization by the antibodies. Subsequent studies indicate that ECM of IEC-6 cells does bind mitogens (see Effects of ECM on proliferation of IEC-6 and CCD-18Co cells).

Inhibitory effects of anti-HGF, anti-bFGF, and anti-KGF on CCD-CM-stimulated mitogenic responses in IEC-6 cells indicated that these growth factors are expressed by CCD-18Co cells. RT-PCR analysis confirmed expression of each of these growth factors in serum-deprived CCD-18Co cells (Fig. 7).

Because cell lines such as CCD-18Co may not fully reflect the characteristics of intestinal fibroblasts, we analyzed CM from cultured HIF-4 cells isolated from human intestine for mitogenic effects on IEC-6 cells. CM from HIF-4 cells stimulated DNA synthesis in IEC-6 cells, although effects were less potent (4.1 ± 0.2-fold above serum-free control) than CCD-CM. Addition of Sm 1.2 or IGFBP-3 modestly inhibited the mitogenic effects of HIF-4 CM (15.2 ± 3.5% inhibition with Sm 1.2 and 11.3 ± 3.0% inhibition with IGFBP-3), indicating that, as in CCD-18Co cells, IGFs contribute to the secreted mitogenic activity in HIF-4 cells. RT-PCR revealed that, like CCD-18Co cells, HIF-4 cells express HGF, KGF, and bFGF (Fig. 7).

Effects of ECM on proliferation of IEC-6 and CCD-18Co cells. Mesenchymal cell ECM is known to have profound effects on survival and differentiation of fetal intestinal endoderm (26). We therefore compared growth factor-dependent proliferation of IEC-6 cells cultured on ECM prepared from CCD-18Co cells (CCD-ECM) and cells cultured on plastic. Culture of IEC-6 cells on CCD-ECM enhanced basal DNA synthesis and DNA synthesis stimulated by IGF-I, EGF, or IGF-I and EGF in combination (Fig. 8). One potential concern was whether this effect is merely due to nonspecific effects of ECM to bind or sequester [3H]thymidine and artificially enhance basal or growth factor-stimulated DNA synthesis. We believe that this is unlikely because cells cultured on CCD-ECM show a much greater absolute increase in [3H]thymidine incorporation in the presence of growth factors than in serum-free medium alone (Fig. 8A), the latter presumably reflecting the possible nonspecific effects of CCD-ECM on thymidine uptake and/or stimulatory effects of CCD-ECM on basal DNA synthesis. Furthermore, CCD-ECM-enhanced growth factor-mediated increases in cell number, further suggesting a specific effect on cell proliferation. When IEC-6 cells were grown on plastic, IGF-I and EGF alone and even IGF-I and EGF in combination failed to induce significant increases in cell number (Fig. 8B). In contrast, when cells were grown on CCD-ECM, there was a small but significant increase in cell number with IGF-I or EGF alone relative to serum-free control and IGF-I plus EGF induced a 2.5 ±
A 0.1-fold increase in cell number (Fig. 8B). Interestingly, when CCD-18Co cells were plated on ECM prepared from IEC-6 cells (IEC-ECM), this reduced basal and IGF-I-induced DNA synthesis relative to cells grown on plastic (Fig. 8C).

Effect of preabsorbing CCD-CM on ECM. Stimulatory effects of CCD-ECM on IEC-6 cell proliferation may reflect sequestration of growth factors onto the ECM. We therefore tested the effects of preabsorption of CCD-CM onto ECM prepared from IEC-6 or CCD-18Co cells. CCD-CM was preabsorbed onto ECM prepared from IEC-6 cells or CCD-18Co cells before it was applied to IEC-6 cells. Preabsorption of CCD-CM onto ECM prepared from IEC-6 cells reduced the subsequent effect of CCD-CM on DNA synthesis in IEC-6 cells by 88.7 ± 8.5% (Fig. 9). Preabsorption of CCD-CM onto ECM prepared from CCD-18Co cells reduced the subsequent effect on DNA synthesis in IEC-6 cells by 55.9 ± 6.7% (Fig. 9). This suggests that ECM from both cell types can bind or sequester the mitogenic activity present in CCD-CM.

Fig. 8. A and B: effect of growing IEC-6 cells on extracellular matrix (ECM) from CCD-18Co cells (CCD-ECM). [3H]thymidine incorporation (A) and cell number (B; means ± SE; n = 6) are given. IEC-6 cells were grown to subconfluence on plastic (no ECM) or CCD-ECM. After serum deprivation for 24 h, cells were switched to serum-free medium (no Tx) or serum-free medium plus IGF-I, EGF, or IGF + EGF (I + E), as indicated, for assays of [3H]thymidine incorporation. [3H]thymidine was added at the same time as cells were switched to new medium with or without growth factors, and total incorporation into DNA was measured over 16-h incubation period. Cell numbers were assayed 48 h after switching to serum-free medium with or without growth factors. C: effects of IEC-6 cell ECM on CCD-18Co cells. CCD-18Co cells were grown to subconfluence on plastic (no ECM) or ECM prepared from IEC-6 cells (IEC-ECM). Cells were serum deprived for 24 h and then switched to serum-free medium (no Tx) or serum-free medium plus IGF-I (IGF-I) supplemented with 2 µCi/ml [3H]thymidine. Thymidine incorporation into DNA was measured 16 h later, and means ± SE (n = 7) are shown. *P < 0.05 for specific effect of culture on ECM vs. culture on plastic under otherwise identical conditions in each set of histograms.

Fig. 9. Effects of preabsorbing CCD-CM onto ECM prepared from IEC-6 or CCD-18Co cells. IEC-6 cells were grown to subconfluence on plastic (no ECM) and then serum deprived for 24 h. Cells were then treated with serum-free medium with (+) or without (-) 25% CCD-CM as indicated. Histograms labeled IEC-6 and CCD indicate that CCD-CM was preabsorbed onto ECM prepared from IEC-6 cells or CCD-18Co cells before it was applied to IEC-6 cells. Histograms show [3H]thymidine incorporation (means ± SE, n = 4). *P < 0.05 for significant effect of preabsorption of CCD-CM on mitogenic activity of remaining CCD-CM relative to nonpreabsorbed control.
DISCUSSION

We have used the CCD-18Co intestinal fibroblast cell line and the IEC-6 intestinal epithelial cell line as simple model systems to study the direct and autocrine actions of the IGFs on proliferation of intestinal fibroblasts and to gain insight into the role of IGFs in mediating paracrine interactions between intestinal fibroblasts and epithelial cells. IEC-6 cells are one of few nontransformed epithelial cell lines derived from the small intestine that are used widely to study proliferation of intestinal epithelial cells (18). CCD-18Co cells are fibroblasts derived from human colon that can exhibit fibroblast or myofibroblast phenotype (19, 30). Myofibroblasts and fibroblasts both stain positive for fibrillar actin (4, 19). Under the conditions used here to analyze fibroblast-derived mitogenic factors, the CCD-18Co cells exhibit primarily fibroblast phenotype as evidenced by intense staining for vimentin and the presence of only scattered cells that stain positive for fibrillar actin. We note that Valentich et al. (30) detected a-smooth muscle actin in serum-deprived CCD-18Co cells by Western blot assays. Our results are not inconsistent because Western blot analysis would detect a-smooth muscle actin even in the small population of positively stained cells.

In vivo data indicate that the predominant sites of expression of IGF-I or IGF-II in intestine are mesenchymal cells and that epithelial cells in normal small or large bowel are not major sites of IGF expression (5, 33, 37). Our findings that IGF-II is expressed at high levels in CCD-18Co fibroblasts and prior findings that IGF-II mRNA is undetectable in IEC-6 cells (24) indicate that these two cell lines retain phenotypic characteristics of the normal bowel in vivo with respect to IGF expression and therefore represent suitable model systems for the present studies. Early passage fibroblasts cultured from human small intestine (HIF-4 cells), like CCD-18Co cells, express IGF-II, indicating that the CCD-18Co cell line shares features in common with fibroblasts isolated from human small intestine. IGF-I is expressed at only very low levels in CCD-18Co cells and HIF-4 cells. This is typical of cultured cells because few cells maintained in culture express IGF-I (11). Available evidence indicates that IGF-I and IGF-II share identical mitogenic effects mediated by the type 1 IGF receptor (11).

Comparisons between growth factor responses of CCD-18Co intestinal fibroblasts and IEC-6 intestinal epithelial cells provide evidence for a cell-specific pattern of responsiveness to IGFs and other peptide growth factors in the intestine. IGF-I and IGF-II were the most potent mitogens for CCD-18Co cells when compared with a number of other growth factors tested. In contrast to IEC-6 cells, CCD-18Co cells show no synergistic mitogenic response when the IGFs are added in combination with EGF or bFGF and are poorly responsive to the fibroblast-derived growth factors HGF and KGF. Together with observations that CCD-18Co cells express IGF-II and that a neutralizing IGF antibody inhibits basal DNA synthesis in CCD-18Co cells, these observations suggest that IGF-II can serve as a major autocrine growth factor for intestinal fibroblasts.

IEC-6 cells differ from CCD-18Co cells in that IGF-I and IGF-II added alone have only modest stimulatory effects on DNA synthesis and are weaker mitogens in these cells than EGF or bFGF. Immunoneutralization of IGF-II has no significant effect on basal DNA synthesis in IEC-6 cells, indicating that IGF-II does not serve as a major autocrine growth factor for IEC-6 cells. This observation is consistent with prior observations that IGFs are not expressed at significant levels in IEC-6 cells (24). IGF-I does, however, elicit synergistic mitogenic effects in IEC-6 cells when present in combination with EGF or bFGF and has additive mitogenic effects with HGF and KGF. We have reported previously that the synergy between EGF and IGF-I reflects in part effects of EGF to maintain expression of the type 1 IGF receptor and to downregulate an inhibitory IGF binding protein, as well as distinct effects of EGF and IGF-I on pathways leading to transcriptional activation of the transcription factor AP-1 (24). It remains to be established whether synergy between bFGF and IGF-I reflects similar mechanisms of interaction. Support for this possibility stems from the current observations that EGF and bFGF in combination produce less than additive effects on DNA synthesis in IEC-6 cells, indicating that they share a common mechanism of action.

The interactions among exogenous IGF-I, bFGF, HGF, and KGF in mitogenic effects on IEC-6 cells and prior observations in vivo that the mRNAs encoding these growth factors are expressed predominantly in mesenchymal cells in intestine or other systems (1, 11, 13, 28) indicates that intestinal fibroblasts may secrete a complex combination of mitogens that exert potent paracrine activity on neighboring epithelial cells. Localization of IGF-I (5, 13), KGF (1), and HGF (28) mRNAs to intestinal mesenchyme in vivo only infers and does not directly prove a paracrine action of these growth factors. Our current findings provide direct evidence that the CCD-18Co intestinal fibroblast cell line and early passage intestinal fibroblast cultures from human small intestine (HIF-4 cells) synthesize multiple growth factors that exert paracrine mitogenic effects on intestinal epithelial cells. One major goal of our study was to test the hypothesis that IGF synthesized by intestinal fibroblasts serves as a paracrine mediator of proliferation of intestinal epithelial cells. A number of observations support this hypothesis. Two separate inhibitors of IGF bioactivity, a neutralizing monoclonal antibody (Sm 1.2) and IGFBP-3, reduce the mitogenic effects of CCD-CM on IEC-6 cells. Preliminary data in SMP8-IGF-I transgenic mice that express IGF-I under the control of an a-smooth muscle actin promoter also indicate that paracrine effects of mesenchymal cell-derived IGF-I are operative in vivo. Recent preliminary data demonstrate that the SMP8-IGF-I transgene is expressed in mucosal subepithelial cells, as well as in smooth muscle cells, and that the SMP8-IGF-I transgenics exhibit overgrowth of intestinal mucosa as well as
smooth muscle (P. K. Lund, K. L. Williams, and J. A. Fagin, unpublished data). The current RT-PCR and immunoneutralization data indicate that in addition to the IGFs, bFGF, HGF, and KGF all are produced by CCD-18Co cells and contribute to the mitogenic effects of CCD-CM on IEC-6 cells. HGF mRNA has been localized to embryonic intestinal mesenchymal cells in vivo, in proximity to epithelial cells that express the HGF receptor c-met (28). The mRNA encoding keratinoocyte growth factor (KGF/FGF-7), a member of the FGF family of growth factors, has been localized to lamina propria fibroblasts in adult human bowel, especially inflamed bowel (1). To our knowledge, bFGF mRNA has not been previously localized in intestine or identified as a secretory product of intestinal fibroblasts. Our current findings demonstrate also that early passage fibroblast cultures isolated from adult human small intestine also express IGF, HGF, KGF, and bFGF mRNAs, indicating that this property of the CCD-18Co cell line is shared by normal intestinal fibroblasts.

We recognize that RT-PCR analysis is not quantitative and that detection of mRNA by RT-PCR does not directly prove that the encoded protein is synthesized or secreted. However, immunological approaches to assay growth factors at the protein level are problematic because of rapid secretion, high affinity for ECM, and the existence of soluble binding proteins (1, 11–13, 28). The majority of information about growth factor expression derives from mRNA analyses (1, 11–13, 28). In the present study therefore as well as RNAse protection assays and RT-PCR to demonstrate mRNAs, immunoneutralization was used to functionally inhibit the action of secreted protein. The mRNA and immunoneutralization data provide two independent pieces of evidence that bFGF, HGF, and KGF are synthesized and secreted by cultured intestinal fibroblasts even though secreted protein was not definitively identified.

Major problems in analyses of the biology of intestinal epithelial cells are the limited life span and limited proliferative capabilities of nontransformed intestinal epithelial cells isolated from normal bowel. This has led to the reliance of most investigators on transformed colon cancer cell lines or the limited numbers of available nontransformed epithelial cell lines derived from small intestine (18, 21). CM from mesenchymal cell lines such as CCD-18Co cells may prove useful in overcoming some of the prior difficulties associated with the limited life span of normal intestinal epithelial cells because it represents a source of multiple growth factors with potent mitogenic activity.

A series of elegant studies has defined the important role of ECM components derived from intestinal mesenchyme in morphogenesis and differentiation of intestinal epithelium (36). Little is known about the role of these components in regulating cell proliferation. In addition, a growing body of evidence indicates that growth factors themselves interact with ECM components (7). Findings that preabsorption of CCD-CM onto ECM prepared from IEC-6 cells or ECM prepared from CCD-18Co cells reduced its subsequent mitogenic effects on IEC-6 cells demonstrate that mitogenic factors present in CCD-CM can bind to ECM of both IEC-6 cells and CCD-18Co cells. The inhibitory effect was greater when CCD-CM was preabsorbed onto IEC-6 cell-ECM compared with preabsorption onto ECM from CCD-18Co cells. This may indicate some preferential affinity of mesenchymal cell-derived mitogenic factor(s) for ECM from intestinal epithelial cells. It is also possible that CCD-18Co cell ECM already contains these factors and as a result has a reduced capacity to bind additional activity in CCD-CM. Observations that culture of IEC-6 cells on ECM prepared from serum-deprived CCD-18Co cells enhances DNA synthesis in response to IGF-1, EGF, or IGF-1 plus EGF further suggest that ECM of CCD-18Co cells may contain or bind mitogens. In addition, IGF-1 and EGF in combination were able to support proliferation of IEC-6 cells when cultured on ECM from CCD-18Co cells but not when the cells were cultured on plastic. This effect seems to be distinct from other matrices such as matrigel isolated from a mouse sarcoma which inhibits growth factor responsiveness of IEC-18 cells (34) or, in some instances, has been reported to stimulate differentiation of IEC-6 cells (2). In our studies, culture of IEC-6 cells on ECM from CCD-18Co cells did not alter cell morphology (data not shown). IEC-6 cells have limited capacity for differentiation, and in the present study we have used them primarily as a model to study growth factor-dependent proliferation. We therefore cannot draw conclusions as to whether ECM from CCD-18Co cells can modulate differentiated function of intestinal epithelial cells. It will be of interest, however, to establish if ECM from CCD-18Co cells alters proliferation or differentiated phenotype of other intestinal epithelial cells such as human H-4 cells that have been shown to differentiate on matrigel (21).

A recent report indicates that clonally derived mesenchymal cells from rat fetal intestine exhibit two distinct phenotypes that differentially modulate proliferation and differentiation of fetal endoderm (4). One intestinal fibroblast cell line (F1:G9) exhibits fibrillar α-smooth muscle actin immunostaining in response to transforming growth factor-β (TGF-β) and has low capacity to support growth of fetal endoderm but effectively stimulates morphogenesis and differentiation (4). Another fibroblast cell line (A1:F1) does not exhibit fibrillar immunostaining with α-smooth muscle actin even in the presence of TGF-β and supports growth but not morphogenesis or differentiation of fetal endoderm (4). CCD-18Co cells share phenotypic characteristics common to both of these rat fibroblast cell lines, dependent on the culture conditions. Serum-deprived CCD-18Co cells are α-smooth actin-negative like A1:F1 cells. It will be of interest to establish if the growth-promoting properties of A1:F1 cells reflect a common profile of secreted growth factors as serum-deprived CCD-18Co cells. TGF-β stimulates transformation of CCD-18Co cells into myofibroblast, α-smooth muscle actin-expressing phenotype (19), a characteristic shared by F1:G9 intestinal fibroblasts that stimulate differentiation of fetal intestinal endoderm (4). Future studies will establish if TGF-β-treated CCD-18Co cells share the differen-
Paracrine signals may presumably pass in either direction between intestinal mesenchymal cells and epithelial cells. The juxtaposition of epithelial cell ECM and mesenchymal cells should permit effects of epithelial cell ECM on proliferation or function of intestinal mesenchymal cells. When CCD-18Co cells were cultured on ECM from IEC-6 cells, this reduced basal and growth factor-stimulated DNA synthesis. These observations indicate that ECM from intestinal epithelial cells may inhibit growth of intestinal mesenchyme. We note that this warrants further study because it may be relevant to understanding the mechanisms of mesenchymal responses or fibrosis in response to epithelial damage as occurs, for example, in inflammatory bowel disease.

In conclusion, we have demonstrated that the IGFs are potent autocrine mitogens produced by intestinal fibroblasts. We present direct evidence that IGFs derived from intestinal fibroblasts interact with other fibroblast-derived growth factors to stimulate proliferation of intestinal epithelial cells in a paracrine manner. We also present evidence that ECM from the CCD-18Co intestinal fibroblast cell line enhances survival and growth factor-dependent proliferation of intestinal epithelial cells and that binding or sequestration of fibroblast-derived growth factors may contribute to this effect. Finally, ECM from IEC-6 cells inhibits basal or IGF-stimulated DNA synthesis in CCD-18Co cells, supporting the concept that there may be bidirectional communication between intestinal epithelial cells and underlying mesenchyme and that ECM from intestinal epithelial cells may modulate proliferation of intestinal mesenchymal cells.

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