Constitutive Insulin-like Growth Factor-II Expression Interferes with the Enterocyte-like Differentiation of CaCo-2 Cells*

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In this study we have examined the role of insulin-like growth factor-II (IGF-II) in the differentiation of the CaCo-2 human colon carcinoma cell line. We have shown previously that IGF-II is an autocrine growth factor for CaCo-2 cells. IGF-II expression is high in proliferating, undifferentiated CaCo-2 cells and markedly decreases when cells become confluent and start to differentiate. To evaluate whether differentiation of CaCo-2 cells depends on an IGF-II related pathway, we treated cells with a blocking antibody to the IGF-I receptor that mediates most IGF-II biological effects. Treatment of preconfluent CaCo-2 cells with this antibody decreased by 40% autonomous cell proliferation and induced differentiation as shown by an increase in sucrase-isomaltase activity and apolipoprotein A-I (apoA-I) mRNA levels. To examine the significance of autocrine IGF-II production in CaCo-2 cell differentiation, we generated stable CaCo-2 cell lines that constitutively express rat IGF-II under the control of a Rous sarcoma virus promoter. Sustained expression of IGF-II resulted in: (a) increased proliferative rate; (b) high IGF-I receptor number, even after reaching confluence; (c) increased capability of anchorage-independent growth; (d) inhibition of the expression of apoA-I and SI mRNAs. Analysis of several independent IGF-II-transfected clones showed an inverse correlation between IGF-II mRNA levels and expression of the differentiation markers, the cells expressing the higher levels of the transfected IGF-II being the less differentiated ones. Our data suggest that perturbation of IGF-II-mediated cell proliferation interferes with the enterocyte-like differentiation pathway of CaCo-2 cells.

The molecular mechanisms that govern cell growth and differentiation of normal and neoplastic human intestinal epithelium are poorly understood. In the small intestine and colon, epithelial cells arise from stem cells located in crypts at the base of villi (small intestine) or near the base of the colonic glands. As intestinal epithelial cells migrate along the crypt-villus axis, they exit from the cell cycle and enter into an alternative pathway in which a specialized phenotype is assumed (1, 2). Several in vitro models of colon cancer cells have been used to study these processes. Cultured colon cancer cells are reversibly growth inhibited and induced to differentiate by treatment with sodium butyrate (3, 4), phorbol esters (4), and polar planar solvents (5) as well as by changes in cell density (6, 7) and glucose concentration (7). Also, the mechanisms of the balance between proliferation and differentiation of the enterocytes has been studied in an in vivo model of transgenic mice in which the expression of SV40 T Ag is restricted to differentiated enterocytes (8). Expression of the transgene in villus-associated enterocytes causes them to reenter the cell cycle without producing detectable changes in the state of differentiation (8).

Investigation of molecules that regulate cell location along the crypt-villus axis and the time span necessary for the cells to move from the proliferative to the differentiative compartment has also focused on the role of polypeptide growth factors (2). In particular, insulin-like growth factors (IGF-I and IGF-II) regulate proliferation of intestinal mucosa (9–10). IGFs are secreted as small peptides that are structurally related to insulin and display multiformal effects on cell growth and metabolism (9–10). IGF-I and IGF-II both exert their mitogenic activity through type I receptor (IGF-I receptor) (9). In rodents, IGF-II is expressed at high levels in the fetus and at lower levels in the adult and functions primarily as a prenatal growth regulator (11–13). In man, IGF-II is highly expressed during the fetal life, and its synthesis has been demonstrated in adult liver and adult extrahepatic tissues (13).

Little is known about the physiological role of IGFs during intestinal differentiation. A large percentage of primary human intestinal tumors express IGF-II (10, 13), and almost all primary human colon cancers and colon cancer cell lines are positive for IGF-I receptors (10). Recently, expression of IGF-II and of type I and II receptors has been shown in CaCo-2 cells, a human colon carcinoma cell line that spontaneously differentiates in culture with features similar to mature small bowel enterocytes (6, 14–17). We have shown previously that IGF-II is an autocrine growth factor for CaCo-2 colon carcinoma cells (14). Also, IGF-II expression is high in proliferating, undifferentiated CaCo-2 cells and decreases by more than 10-fold when cells become confluent and start to differentiate (14). In the present study, we investigated whether IGF-II might inhibit the differentiation of CaCo-2 cells. We showed that the blockade of IGF-I receptor, through which most IGF-II related

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The abbreviations used are: IGF, insulin-like growth factor; apoA-I, apolipoprotein A-I; SI, sucrase-isomaltase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; mAb, monoclonal antibody; RSV, Rous sarcoma virus.
IGF-II and CaCo-2 Cell Differentiation

8109

events are mediated, stimulates CaCo-2 cell differentiation. We also altered the normal pattern of IGF-II expression associated with CaCo-2 cell differentiation by introducing an exogenous IGF-II cDNA under a constitutive promoter. The effect of constitutive IGF-II overexpression in the CaCo-2 cells resulted in unregulated growth and inhibition of the enterocyte-like differentiation of the cells. These data suggest that IGF-II interferes with the differentiation of CaCo-2 cells.

EXPERIMENTAL PROCEDURES

Materials—32P-Labeled isotopes, 125I-IGF-I, and (methyl-3H)thymidine were from Amersham Corp. (Buckinghamshire, United Kingdom). Recombinant human IGF-I and IGF-II were obtained from Sigma (Milano, Italy). Monoclonal antibody (mAb) to rat IGF-II (18) was obtained from Amano Pharmaceutical (Nagoya, Japan) and was a generous gift of Dr. P. Nissley (NCI, National Institutes of Health); α-IR3 mAb to IGF-I receptor (19) was from Oncogene Science (Manhasset, NY); MOPC-21 (IgGk-1) control isotypic mAb was from Sigma. All other materials were of the best grade commercially available.

Construction of the Prepro-IGF-II Expression Vector—A 379-base pair EcoRI-Sacl cDNA fragment spanning exons 4, 5, and the 5′ end of exon 6 of rat IGF-II (20) was fused to a plasmid carrying a 3600-base pair Saci-HindIII genomic fragment isolated from B272 phage containing the 3′ sequences of exon 6 and the downstream polyadenylation site of rat IGF-I gene (21). A 578-base pair HindIII-NdeI fragment containing the sequence of the Rous sarcoma virus promoter (RSV) (22) was subcloned in the EcoRI site of the resulting plasmid after linearization and fill-in of the recessed 3′ ends by the Klenow enzyme. The preparation vector plasmid was checked by DNA sequence analysis to ensure that the correct coding sequences of the prepro-IGF-II hormone was inserted downstream of the RSV promoter (data not shown).

Generation of Constitutive IGF-II-expressing CaCo-2 Cells—CaCo-2 cells were cultured as described previously (6, 14, 17). CaCo-2 cells were cotransfected with a 10:1 ratio of the rat prepro-IGF-II expression vector carrying the neomycin resistance gene under the control of the RSV promoter using the calcium phosphate technique (22). Stable transfecants were selected in the presence of the neomycin analog G418 (0.6 mg/ml). Between 2–3 weeks, single clones or pools of clones were isolated with cloning cylinders and expanded. Stable transfecants were further maintained in the presence of 0.4 mg/ml G418. All the experiments with neomycin- or IGF-II-transfected cells were performed in the presence of 0.4 mg/ml G418.

Cell Proliferation Assay—Cells were seeded at a density of 40,000 cells/35-mm dish in DMEM supplemented with 10% fetal calf serum (FCS). In the experiments evaluating the effects of α-IR3 and MOPC-21 mAb, cells were preincubated for 1 at 37 °C with mAbs and then incubated for 48 h in serum-free medium alone or with 10 ng/ml IGF-II. Cell growth was assessed at selected times by hemocytometric cell count, followed by trypsinization. Viability of cells was assayed by trypan blue dye exclusion test.

Thymidine Incorporation—10,000 cells/well were seeded in 24-well plates in DMEM, 10% FCS. At different days of culture, cells were pulse-labeled for 18 h by addition of [methyl-3H]thymidine (1 μCi/well). Medium was removed by aspiration, and after trypsinization, the cells were collected on glass fiber filter mats using an LKB automatic cell harvester prior to liquid scintillation counting. In each experiment, three replicate wells were labeled for each point, and mean values were plotted. All experiments were repeated several times.

Anchorage-independent Growth—The ability of transfected cell lines to grow in the absence of anchorage was determined by scoring the number of colonies formed in 0.3% agarose supplemented with 10% FCS (with a 0.5% agarose overlay). 10,000 cells/60-mm Petri dish were seeded. Colonies larger than 0.5 or 1.2 mm were counted after 3 weeks in culture.

RNA Analysis—Total RNA was isolated from cells by the guanidine thiocyanate acid-phenol procedure (23). RNase protection was performed as described previously (24). The riboprobe for RSV-IGF-II transcripts has been obtained by subcloning a 215-base pair EcoRI-HindIII fragment encompassing pRSV-IGF-II transcription start site into pSP72 (Promega), which was transcribed with Sp6 polymerase, after linearization with EcoRI. Northern analysis was carried out as described previously (25). Probes were generated by random primer extension or by S end labeling (25). The probe used to detect SI was the 0.42-kilobase Sall-EcoRI insert from pH51-1 plasmid (26). A 0.85-kilobase BamHI insert of a full-length human cdNA was used as a probe for apoA-I (27). An oligonucleotide (5′-AAGCATCTAGATGTTGTT-TCACC-3′) complementary to human 28 S rRNA (28) was used as a loading control. Sizes of hybridizing RNAs were calculated by reference to the rRNA bands on the filters after ethidium bromide staining. Estimated sizes for 18 and 28 S rRNA transcripts were 1.9 and 5.0 kilobase, respectively (28). RNA levels were quantified by densitometric scanning of the autoradiographs using a Sony CCD videocamera linked to a Macintosh IIfx computer and the National Institutes of Health IMAGE program (public domain).

Sucrase-Assay—SI activity was assessed in total cell extracts by the Dahlquist’s method as described previously (17).

Collection of Conditioned Medium and IGF-II Immunological Studies—Cells were grown at different times in serum-containing medium. After three times with phosphate-buffered saline, and incubated 12 h in serum-free medium. The medium was then discarded and replaced with fresh serum-free medium (5 ml/10-cm2 dish). Conditioned medium was harvested 48 h later, centrifuged (3000 rpm for 20 min), and concentrated using Centricon 3 microconcentrators. Protein analysis was performed using 15% SDS-polyacrylamide gel electrophoresis under nonreducing conditions. After electrophoresis, proteins were blotted by electrophoret transfer to nitrocellulose filters (BA85; Schleicher & Schull) (29). Immunodetection experiments were carried out using a mouse monoclonal antibody directed against native rat IGF-II (18) and a secondary goat anti-mouse IgG serum conjugated to horseradish peroxidase (29); the bands were visualized using the enhanced chemiluminescence detection system (ECL, Amersham Corp.). Semiquantitative measurements of immunoreactive IGF-II were performed by chemiluminescence on serial dilutions of conditioned media directly spotted on BA85 nitrocellulose filters (30). Immunoreactive signals were analyzed using a Sony CCD videocamera linked to a Macintosh IIfx computer and the National Institutes of Health IMAGE program (public domain). Values for IGF-II immunoreactive values were interpolated from standard curves constructed using serial dilutions of recombinant human IGF-II. IGF-II concentration was expressed as ng/106 cells.

IGF-I Receptor Assay—Cells were seeded in 16-mm tissue culture wells, at a density of 25,000 cells/dish. At different days of culture, cells were washed three times with cold phosphate-buffered saline, then incubated in a total volume of 0.25 ml of K R medium (Kreb’s-Ringer-Hepes containing 0.5% bovine serum albumin and adjusted to pH 7.4) for 18 h with 0.06 nmol/liter 125I-IGF-I with or without various concentrations of unlabeled IGF-I. At the end of incubation, cells were washed three times with phosphate-buffered saline, then lysed in the same buffer containing 1% Triton X-100 and 10% glycerol, and cell-associated radioactivity was counted on a Bedman Gamma 5500 counter. Nonspecific binding, determined by the radioactivity bound in the presence of 10−6 M IGF-I, was subtracted from total binding to obtain specific binding. Nonspecific binding represented about 5% of total binding of 125I-IGF-I.

Statistical Analysis—Correlation was evaluated by regression analysis. Significance of differences was evaluated by analysis of variance followed by Student’s t test for impaired data. Differences were considered significant when p < 0.05.

RESULTS

Effect of IGF-I Receptor-blocking Antibody α-IR3 on Differentiation of Preconfluent CaCo-2 Cells—We have shown previously that the effects of IGF-I on cell growth are mediated by the IGF-I receptor (14). To investigate whether the blockade of IGF-I receptor has any effect on differentiation of CaCo-2 cells, we have treated these cells with a monoclonal antibody that blocks the binding sites of IGF-I receptor (19). α-IR3 mAb inhibited basal cell proliferation to 40% of maximal growth (Fig. 1A) and stimulated differentiation of preconfluent CaCo-2 cells, as assessed by determination of sucrase isomaltase activity (Fig. 1B) and apoA-I mRNA expression (Fig. 1C). The same concentration of the isotopic control antibody MOPC-21 had no inhibitory effect on CaCo-2 cell growth and did not affect sucrase isomaltase activity and apoA-I mRNA expression (Fig. 1, A–C).

Generation of Constitutive IGF-II-expressing CaCo-2 Cell Lines—To evaluate whether IGF-II interferes with differentiation of CaCo-2 cells, we stably transfectted cells with a eukaryotic expression vector carrying rat IGF-II cDNA sequences.
fused to the RSV promoter. A pool of IGF-II-transfected clones and several independent clones were isolated and characterized for the expression of ectopic RSV-IGF-II by an RNase protection assay. A specific transcript originating from the RSV promoter was detected in IGF-II-transfected pool of clones or in four independent clones expressing different amounts of the transfected IGF-II transcripts (Fig. 2, lanes 4–8). Neither the neomycin-transfected, nor the parent cell line, expressed detectable levels of rat IGF-II message (Fig. 2, lanes 1–3). A cross-hybridizing band originating from the endogenous human IGF-II gene was instead present in all analyzed samples.

IGF-II Protein Release in the Conditioned Media of Parental, neomycin- and IGF-II-transfected CaCo-2 Cells—Conditioned media from the parent cell line and from cells transfected with the RSV-IGF-II or with the vector alone were analyzed either by Western blot (Fig. 3) or by immunoenzymatic analysis (Table I). In parental and neomycin-transfected pool of clones and one independent clone, two IGF-II immunoreactive peptides of M, 7500 and M, 15,000 were secreted at high levels in the medium collected at day 4 of culture; the levels were reduced in the medium collected at days 14 and 21 of culture (Fig. 3, lanes 1–3, 4–6, 10, respectively). On the contrary, high levels of IGF-II immunoreactive peptides were detected in the media collected from preconfluent (day 4) or postconfluent (days 14 and 21) IGF-II-transfected pool of clones and from one independent IGF-II-transfected clone (Fig. 3, lanes 7–9, 11, respectively). Immunoenzymatic analysis of conditioned media showed that IGF-II protein secretion was decreased by 10-and 20-fold in postconfluent parental or neomycin-transfected cells, respectively. In contrast, IGF-II peptide levels in media from postconfluent IGF-II-transfected cells were similar to those found at day 4 of culture (Table I). IGF-II peptide levels in the media collected from cells at day 14 and 21 of culture were significantly higher in IGF-II-transfected cells than in parental and neomycin-transfected control cells (p < 0.001).

IGF-I Receptor Expression in Neomycin- and IGF-II-transfected CaCo2 Cells—Since we have shown previously that down-regulation of IGF-I binding sites in differentiated CaCo-2 cells parallels the decrease in IGF-II expression, we evaluated whether constitutive expression of IGF-II in IGF-II-transfected cells was associated with changes in the pattern of expression of the IGF-I receptor. A competitive binding assay using [125I]-IGF-I followed by Scatchard plot analysis showed that parental and neomycin-transfected control cells expressed a 5-10-fold higher receptor number (Bmax 148 and 120, respectively) at day 4 of culture with respect to days 14 and 21 of culture, respectively (Table II). On the other hand, Bmax values of IGF-II-transfected cells were still elevated at day 14 and 21 of culture (63 and 59 fmol/106 cells, respectively), being only 2-fold less compared with those at day 4 of culture (Bmax 110). IGF-I receptor number at days 14 and 21 of culture was significantly higher in IGF-II-transfected cells compared with parental (p <
0.05 and p < 0.001 at days 14 and 21 of culture, respectively) and neomycin-transfected control cells (p < 0.01 and p < 0.001 at days 14 and 21 of culture, respectively). Kd values (1.6 nm) of neomycin- and IGF-II transfected cells were similar at the different days of culture and were in the range of those observed for CaCo-2 untransfected cells (data not shown).

Cell Growth of Neomycin- and IGF-II-transfected CaCo-2 Cells—To determine the effects of constitutive IGF-II expression on cellular proliferation, growth curve and thymidine incorporation of parental, neomycin- and IGF-II-transfected cells were analyzed. IGF-II-transfected cells continued to grow logarithmically up to day 14 of culture, while parental and neomycin-transfected control cells reduced their growth by day 8 of culture (Fig. 4A). The growth fraction of cells after confluence was determined by continuous labeling with [3H]thymidine. In parental and neomycin-transfected cells, thymidine uptake was reduced more than 10-fold at day 14 with respect to day 8 of culture (Fig. 4B). On the other hand, thymidine uptake of IGF-II-transfected cells was still elevated at day 14 of culture, being only 3-fold less compared with that at day 8 of culture (Fig. 4B). Thymidine incorporation at days 8 and 14 of culture was significantly higher in IGF-II-transfected cells compared with parental and neomycin-transfected control cells (p < 0.05 and p < 0.01 at days 8 and 14, respectively). Similar results were obtained when both growth curve and thymidine uptake of IGF-II-transfected pool of clones were compared with those of neomycin-transfected pool of clones (data not shown).

Analysis of Anchorage-independent Growth of the Transfected Cells—To determine the effects of constitutive IGF-II expression on anchorage-independent growth, soft agar cloning assays were carried out using the parental cell line, pools, and two independent clones of neomycin- and IGF-II-transfected cells, IGF-II and CaCo-2 Cell Differentiation.
cells. Parental and neomycin-transfected cells formed few
dones when grown in soft agar (Fig. 5). On the other hand, a
pool and one clone of IGF-II-transfected cells formed a higher
number of colonies (Fig. 5). On the other hand, a
pool and one clone of IGF-II-transfected cells formed a higher
clones when grown in soft agar (Fig. 5). On the other hand, a
neomycin clone and IGF clone grown in agar after 3 weeks. Values
are means ± S.D. of three wells. Data are representative of four similar

**Constitutive Expression of IGF-II Inhibits the Enterocyte-like Differentiation of CaCo-2 Cells**—In order to determine whether unregulated IGF-II expression affects the ability of CaCo-2 cells to differentiate, we evaluated the expression of apoA-I and of the brush border associated hydrolase SI. In neomycin-transfected CaCo-2 cells, SI and apoA-I RNA transcripts were expressed at increasingly high levels from day 8 to days 14 and 21 of culture (Fig. 6). In contrast, SI mRNA levels were below the level of detection in a pool (Fig. 6A) and three independent clones of IGF-II-transfected cells or detected at very low levels in one IGF-II-transfected clone, even several days after reaching

Constitutive expression of IGF-II inhibits the enterocyte-like differentiation of CaCo-2 cells. In this study, we showed that blockade of IGF-I receptor inhibited cell growth and stimulated differentiation of preconfluent CaCo-2 cells. Thus, induction of CaCo-2 cell differentiation is paralleled by the inhibition of cell growth, obtained by blocking an IGF-II-mediated pathway. It has been shown that CaCo-2 cells differentiate spontaneously, depending on cell density (6). Our data indicate that differentiation is dependent on the growth status of the cells rather than on the acquisition of cell confluence. Conversely, constitutive IGF-II overexpression in transfected cells resulted in the inhibition of CaCo-2 enterocyte-like differentiation, and this effect was directly related to the levels of exogenous IGF-II transcripts. IGF-II ectopic expression resulted in an unregulated stimulus of cell growth as indicated by delayed inhibition of growth at confluence and by higher thymidine incorporation of IGF-II-transfected cells compared with parental and neomycin-transfected control cells. Thus, the inhibition of the enterocyte-like differentiation of CaCo-2 cells in IGF-II-transfected cells might be mediated through a continuous stimulus of cell growth. Also, IGF-II-transfected CaCo-2 cells showed changes in the malignancy of cell phenotype as assessed by increased clonogenicity in soft agar. The inhibition of differentiation in IGF-II-transfected cells could also be explained by the acquisition of
a more malignant phenotype, since it has been shown that transformation of CaCo-2 cells by polyoma middle T or by activated ras oncogenes inhibited enterocyte-like differentiation of the cells (39).

We have also shown that constitutive IGF-II expression altered the physiological down-regulation of IGF-I receptor associated with CaCo-2 cell differentiation, since receptor number remained elevated in IGF-II-transfected post-confluent cells. IGF-I receptor plays an important role in neoplastic transformation of mammalian cells (40) and high IGF-I receptor expression inhibits muscle differentiation of cultured myoblasts (35, 36). Therefore, the continuous mitogenic stimulus, the increased capability of anchorage-independent growth, and the inhibition of differentiation of IGF-II-transfected cells might all be mediated by the high IGF-I receptor number.

All these data suggest that, at least in vitro, IGF-II-regulated growth and differentiation programs of intestinal epithelial cells are inversely related events. The scenario is more complex in vivo, since transgenic animals expressing SV40 T Ag under the control of an enterocyte-specific promoter showed increased proliferative activity but no changes in the differentiation pattern of the enterocytes (8). However, this mouse model is not comparable with our and other in vitro experimental systems, since the transgene begins to be expressed when the enterocytes are already committed to differentiate.

Regulation of the balance between growth and differentiation of normal and transformed intestinal epithelial cells appears to be a complex phenomenon, depending on several mechanisms. Based on our data, down-regulation of IGF-II and IGF-I receptor expression seems to play a role in the differentiation process to take place. The mechanisms by which IGF-II interferes with the differentiation program are still unclear. It has been shown that activation of the signal transduction pathway of IGF-I receptor by a mitogenic stimulus leads to the nuclear translocation of protein kinase C (41, 42). Since we have shown previously that protein kinase A-mediated differentiation of CaCo-2 cells is negatively regulated by the activation of protein kinase C (17), one can speculate that constitutive IGF-II expression interferes with differentiation through sustained activation of protein kinase C. Further studies are needed to address this issue.

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