Proliferation and Differentiation of a Human Colon Cancer Cell Line (CaCo2) Is Associated with Significant Changes in the Expression and Secretion of Insulin-Like Growth Factor (IGF) IGF-II and IGF Binding Protein-4: Role of IGF-II*

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ABSTRACT

The extent to which the insulin-like growth factor (IGF) system contributes to the initiation and progression of colon cancer remains poorly defined. We recently reported that a majority of human colon cancers express and secrete the potent mitogen IGF-II and at least two inhibitory binding proteins, IGFBP-2 and IGFBP-4. In the present study we measured the expression and secretion of IGF-II, IGFBP-2, and IGFBP-4 in relation to growth and differentiation of CaCo2 human colon cancer cells, which undergo spontaneous enterocytic differentiation in culture. Under the conditions of the present study, CaCo2 cells demonstrated an initial rapid phase of growth between Day 2 through Days 7-9 of culture, followed by a significant retardation in the growth between Days 9-13. Alkaline phosphatase (ALP) activity, a marker of enterocytic differentiation, progressively increased between Days 7-13 in culture, temporally correlating with post-confluent phase of negligible growth. These changes in growth and differentiation were accompanied by >80% decline in the relative concentration of IGF-II messenger RNA (mRNA) between Days 2-13. In contrast, the relative mRNA concentrations of inhibitory binding proteins (IGFBP-2 and IGFBP-4) increased rapidly to 200% of Day 2 values by Days 5-7 before returning to baseline levels by Day 13. The relative protein concentrations of the three factors measured in the conditioned media of the cells followed a pattern very similar to that measured for the mRNA levels. While the changes in the relative protein concentrations and mRNA levels of IGF-II and IGFBP-4 were statistically significant, the changes measured in the RNA and protein levels of IGFBP-2 were not, as a result of large inter experimental variations. Thus these results suggested that CaCo2 cell differentiation may require an attenuation of IGF-II effects.

To confirm the latter possibility, additional studies were conducted with a specific neutralizing antibody against IGF-II. Incubation of CaCo2 cells with anti-IGF-II antibodies from Day 0 through Day 7 significantly retarded the growth of the cells and was accompanied by a significant increase in the concentration of Alkaline phosphatase activity per 10^6 cells. Recently, we reported a potent inhibitory role of IGFBP-4 in the growth of colon cancer cells. In the present studies, a possible important role of IGF-II is illustrated not only in the growth but also in the differentiation of colonic cells. Our studies thus suggest that differential expression of IGF-II and IGFBPs may be playing a critical role in both proliferation and differentiation of colonocytes. (*Endocrinology* **137:** 1764–1774, 1996)

Insulin-like growth factors (IGFs) consist of two related polypeptides, IGF-I and IGF-II, which play an important role in the proliferation and differentiation of many cell types (1). Membrane receptors for IGFs and soluble binding proteins (IGFBPs) (secreted extracellularly) modulate the action of IGFs (1). IGFs, IGF-receptors, and IGFBPs thus constitute the three components of the IGF system (2). Besides having an important role in the development and function of various tissues, IGFs have also been implicated in neoplastic trans-

formation and proliferation of cancer cells (reviewed in Reference 2). IGF-I and IGF-II are equally effective as mitogenic agents, with only slight differences in their potencies (2). Neoplastic conversion of epithelial cells is associated with over-expression of IGF-II (3, 4). The importance of IGF-II expression in neoplastic transformation has been experimentally confirmed in some cell types (5, 6).

Recent evidence suggests that the IGF system may play an equally important role in the proliferation of colon cancer cells (2). Primary human colon cancer tumors and colon cancer cell lines express and secrete the potent mitogen IGF-II (7, 8). A majority of the human colon cancers also express functional Type I receptors (IGF-I-R) (8–10), suggesting that IGF-II may function as an important autocrine growth factor in this disease. More recently, human colon cancers have also been found to express several IGF-binding proteins (IGFBPs) (8) capable of modulating the mitogenic potency of IGFs on colon cancer cells (11–13). Of the six IGFBPs identified and cloned to date (14), most of the human colon cancers express IGFBP-2 and IGFBP-4 (8); less than 50% also express IGFBP-3,

IGFBP-5, and IGFBP-6; and none express IGFBP-1 (8). Since colon cancer cell lines express both the mitogen IGF-II and the modulatory IGFBPs, it can be expected that IGFBPs may be playing an important role in the growth and differentiation of colon cancer cells in response to endogenous IGF-II. At the present time it is not known if IGF-II plays a role in the differentiation of colonic cells. Therefore to examine a role of IGF-II and IGFBPs in both the growth and differentiation of colonic cells, we chose a human colon cancer cell line, CaCo2, that expresses IGF-II and several of the IGFBPs (8, 15, 16) and is known to undergo spontaneous enterocytic differentiation in culture (17).

It is now known that both IGFBP-2 and IGFBP-4 inhibit the mitogenic effects of IGFs (12, 13, 18, 19). These inhibitory effects have been attributed primarily to competitive scavenging of the IGF peptides away from the IGF-I-receptors (20, 21). Either IGF-independent effects or potentiation of IGF effects by some IGFBP proteins have also been reported (21–24). We recently reported a possible IGF-independent inhibitory effect of IGFBP-4 on the growth of the human colon cancer cell line HT-29 in response to IGFs (13). Because IGFBP-2 and IGFBP-4 function primarily as inhibitory proteins, it can be expected that secretion of the mitogen (IGF-II) would be inversely related to that of the inhibitory binding proteins during cell growth. Therefore to test a complementary role of binding proteins we measured IGFBP-2 and IGFBP-4 (at the messenger RNA (mRNA) and protein levels) in relation to the expression and secretion of IGF-II during both the proliferative and differentiation stages of CaCo2 cell growth in culture. Our results suggest that a decrease in growth and/or an increase in differentiation may require an attenuation of IGF effects. To test the latter possibility we conducted further studies with neutralizing antibodies against IGF-II, the results of which confirm a possible important role of IGF-II in both growth and differentiation of colonic cells.

Materials and Methods

Cell culture

The CaCo2 human colon cancer cell line was obtained from Dr. Jing Yu, Tufts School of Medicine (Medford MA). The cells were grown as monolayer cultures in a humidified atmosphere of 95% air/5% CO_2 in high glucose (25 mM) DMEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), at 37 C. The cells were monitored regularly for mycoplasma contamination using commercial kits. Stock cultures of the cells were subcultured at appropriate intervals to maintain the cells at subconfluent densities. For cell counting and subculturing, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

Cell growth assay

Serial measurements of cell number were used to establish growth curves and to define exponential and reduced (plateau) phase growth. Exponentially-growing cells were seeded at 5×10^3 cells/cm² into replicate wells of a 24-well culture plate (Falcon, Fisher, Houston, TX) and assayed at sequential time points over a 13-day period. Cell numbers were determined indirectly by the semiautomated tetrazolium-based clonogenic assay (MTT assay), as described previously (25, 26).

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured in whole cell lysates and served as a marker of enterocytic differentiation (27). At the designated time points, triplicate cultures from each treatment group were washed three times with cold PBS (pH 7.4), collected in 2 mM Tris-HCl/50 mM mannitol (pH 7.2) after scraping with a rubber policeman (final volume of 1 ml), and homogenized. After addition of 18 mM $CaCl_2$, the homogenates were sonicated on ice and assayed (25 μl) in duplicate. All enzyme assays were carried out at 37 C. ALP activity was measured by kinetic determination of p-nitrophenol phosphate hydrolysis using a commercially-available kit (Sigma Diagnostics Procedure No. 245, Sigma, St. Louis, MO). Results are expressed in either milliunits per mg protein or U/L/10^6 cells. Cell protein was measured by the BCA method (Pierce, Rockford, IL).

Collection of condition media (CM)

Conditioned media (CM) were prepared for measurement of secreted IGF-II and IGFBPs essentially as previously described (8). Cells were seeded into either 175 cm 2 flasks or 100 mm dishes at a concentration of 5×10^3 cells/cm² and cultured in standard growth medium for 2–19 days. At designated time points the cells were washed twice with serum free medium and cultured in serum-free medium for 24 h. The spent media was then discarded and replaced with fresh serum-free medium (20 ml/175 cm² flask or 10 ml/100 mm dish). The CM were harvested 24 h later and centrifuged at 2000 $\times$ g for 10 min to remove cellular debris. The supernatants were collected and stored at −70 C in the presence of 1 mM phenylmethylsulfonyl fluoride until further processing. Cells remaining in the flask/dish were counted to express the results on the basis of cell number.

Western blot analysis of IGFBPs in the CM samples

CM samples (collected from at least 0.3–0.8 $\times$ 10^8 cells/time point) were concentrated in an Amicon (Beverly, MA) stir cell concentration unit containing 10-kDa Diaflo (Amicon) ultra filters. Concentrated CM samples were subjected to 9.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions to fractionate proteins, which were then electroblotted onto nitrocellulose or polyvinylidene fluoride (PVDF, Millipore, Bedford, MA) membranes and processed for either ligand blotting or immunoblotting as described previously (8). Gels were stained to confirm complete transfer of the proteins. For ligand blotting, the membranes were incubated in a heat-sealable plastic bag with 2 $\times$ 10^6 cpm ^{125}[I]IGF-I (Amersham, Arlington Heights, IL) for 24 h at 4 C, washed extensively, and developed autoradiographically. For immunoblotting, the membranes were incubated with antihuman antibodies against either IGFBP-2 or IGFBP-4 (UBI, Lake Placid, NY) for 2 h at 22 C at the suggested dilutions and processed for chemiluminescence immunodetection using the ECL kit (Amersham) as per the manufacturer's instructions.

Quantitation of IGFBP-2 concentration in CM samples

IGFBP-2 concentrations were measured in CM samples using a Western immunoblotting technique recently described by us (8). Briefly, 1–500 ng of rhIGFBP-2 (kindly provided by Dr. J. Schwander, Basel, Switzerland) were subjected to Western blot analysis as described above for IGFBPs in CM. Autoradiograms of the known concentrations of rhIGFBP-2 were then scanned using the UltroScan LX laser densitometer to generate a standard curve as defined by area under the peaks [GelScan XL software (Pharmacia, LKB Biotechnology, Uppsala, Sweden)]. The concentration of IGFBP-2 in the unknown samples was then determined by linear regression analysis and expressed in ng per number of cells. The standard curve was linear from 1-500 ng rhIGFBP-2. In all cases IGFBP-2 standards were run with the unknown samples on the same blot; a representative autoradiogram of the IGFBP-2 standards is presented in Fig. 1. Densitometeric analysis of autoradiograms of the Western ligand blots rather than immunoblots was used to measure relative concentrations of secreted IGFBP-4, because of problems with cross-reactivity with currently available anti-IGFBP-4 antibodies, as discussed previously (8).
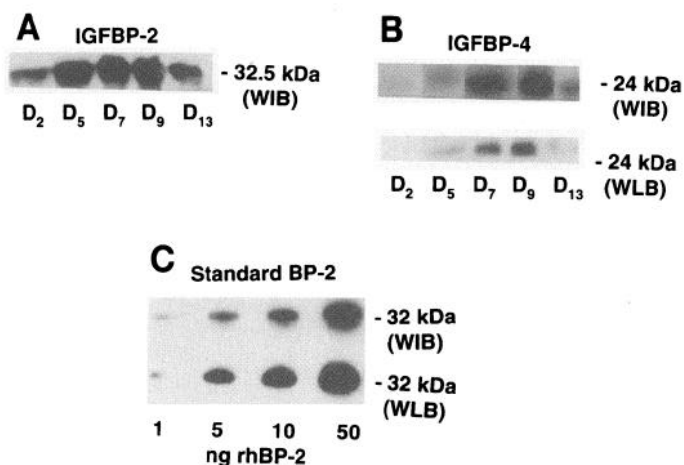
FIG. 1. Autoradiographs of immunoblots of CM samples from CaCo2 cells. Serum-free CM was collected from CaCo2 cells on Days 2–13 of culture (as indicated) and subjected to either immunoblot analysis (WIB) or Western ligand blot analysis (WLB). Recombinant human IGFBP-2, at indicated concentrations, was corun with the unknown IGFBP-2 samples to measure ng concentrations of secreted IGFBP-2. The molecular mass of the bands is indicated on the right-hand side of the figures. CM samples from CaCo2 cells, equivalent to 10^7 cells, were loaded in the lanes and subjected to either immunoblot analysis with specific anti-BP-2 antibody (A) or with anti-BP-4 antibody (B). The IGFBP-4 samples were also analyzed by a ligand blot analysis (lower panel B). IGFBP-2 standards were analyzed by either WLB or WIB, and representative autoradiographs from one of many experiments is presented in C.

Quantitation of IGF-II by RIA

CM samples were first processed for extraction of the IGF peptides (from the bound form) as described previously (8). Briefly, CM samples were dialyzed against deionized water, lyophilized, and reconstituted in acidified water, followed by filtration through Amicon microconcentrators (Centricon 30, W.R. Grace, Beverly, MA). The filtrate was then further washed in Amicon filtration units (Centricon 3) with 0.1 M Tris (hydroxymethyl)aminomethane (Tris buffer) to concentrate and clear the samples of HCl. Samples of the 3–30 kDa fraction were adjusted to pH 7.4 with l m Tris and RIA buffer containing 1% gelatin. As described previously (8), the 3–30 kDa samples were free of contaminating IGFBPs (which was confirmed by ligand blot and immunoblot analysis [data not presented]). ^{125}[I]IGF-II and a monoclonal antibody directed against IGF-II (Amano, Troy, VA) were used to measure IGF-II in the specific RIA. The monoclonal antibody demonstrated less than 10% cross-reactivity with human IGF-I. Nonspecific binding in the presence of excess unlabeled hIGF-II (250 ng/ml) was 9–10%. All samples to be compared were analyzed in the same assay. Interassay variation was less than 10%.

Isolation of RNA

CaCo2 cells were seeded into either 175 cm 2 flasks or 100 mm dishes and cultured in standard growth medium for 2–13 days. On the day of harvesting the cells were washed with ice-cold serum-free medium and processed for RNA extraction, essentially as described by Chomczynski and Sacchi (28), with the help of the commercial reagent TrisOL (BioTexc Laboratories, Houston, TX) as per the manufacturer's instructions. Total cellular RNA was further recovered by ethanol precipitation and quantitated by measuring absorbance at 260 nm. RNA purity was determined by comparing absorbance at 260/270/280 nm. RNA integrity was assessed by fractionating on 1% formaldehyde-agarose gels and observing the intensities of 28S and 18S RNA bands stained with ethidium bromide.

Northern blot analysis

For Northern blot analysis, RNA samples were electrophoresed through formaldehyde-agarose gels, transferred to nylon membranes,

and cross-linked either with ultraviolet light or baking, as previously described (29). Partial or full-length human complementary DNA (cDNA) probes obtained from other investigators (as detailed under the legend for Fig. 2) were used for analysis of mRNA levels for IGF-II, IGFBP-2, and IGFBP-4. The cDNA probes were labeled by nick translation using commercial kits from Promega (Madison, WI), and the samples were subjected to Northern hybridization as described previously (8, 29).

The relative expression of two housekeeping genes, β-actin and cyclophilin, was also measured during Days 2–9 of culture to assess the extent to which baseline gene expression fluctuated during CaCo2 cell differentiation. mRNA levels of β-actin exhibited extreme variability between Days 2–9 (data not shown); cyclophilin mRNA levels exhibited less variability but also fluctuated with an apparent increase from Days 3–9 of culture (Fig. 2). Because of this variability, the relative density of ethidium bromide staining of the 28S band was employed as marker of RNA loading (as shown in Fig 2).

Measurement of the effect of IGF-II antibody (Ab) on the growth and differentiation of CaCo2 cells in culture

Effect of IGF-II-Ab treatment on growth was measured in an MTT assay, while the levels of ALP activity were measured as a marker of differentiation. Rat IGF-II-Ab from UBI (Lake Placid, NY), which was used in the studies, has been shown to neutralize the mitogenic effects of all forms of rat and human IGF-II (30). Rabbit IgG against mouse IgG (Organon Teknika, Cappel Research Products, Durham, NC) was used as a control at equal protein concentrations. For the MTT assay CaCo2 cells were plated at densities ranging from $1–3.5 \times 10^3$ cells in 3 different experiments in 96 well plates in a total volume of 100 μl normal growth medium containing 10% FCS. Growth factors (IGF-II, IGF-I [GIBCO]),
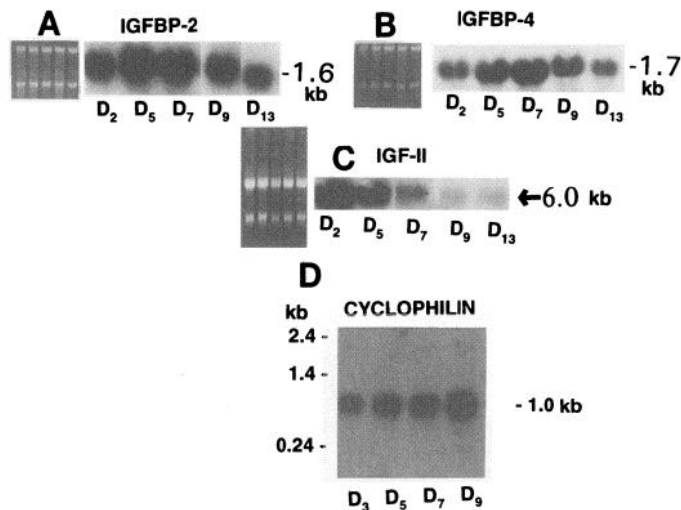

FIG. 2. Autoradiographs of Northern blots of IGFBPs and IGF-II measured in the CaCo2 cells. Total RNA was extracted from CaCo2 cells on the indicated days of culture (Days 2–13) and subjected to Northern blot analysis with the specific human cDNA probes for IGFBP-2, IGFBP-4, IGF-II, and cyclophilin. The ethidium bromide staining of the 28S and 18S RNA bands of the corresponding blots is presented to the left of each autoradiograph for IGFBP-2, IGFBP-4, and IGF-II in panels A, B, and C, respectively. Based on the relative mobility of the RNA ladder, co-run with the samples, the approximate molecular mass of the bands is indicated on the right-hand side of each autoradiograph in kb. Each autoradiograph is representative of 4–5 blots from 2–3 separate experiments. In each lane a total of 30 μg of total RNA was loaded. The RNA blots were hybridized with ^{32}P-labeled human cDNA probes. The human cDNA probes used were the following: A 450 bp ECORI and HindIII insert of pHBP2–501 was used for IGFBP-2; A 500 bp ECORI and HindIII insert of pHBP4–503A was used for IGFBP-4; A 1.5 kb insert of P1GF2/8–1 was used for IGF-II, and a 500 kb cDNA probe (obtained from Dr. G. Gomez in the Department of Surgery, UTMB) was used for cyclophilin.

and the neutralizing IGF-II-Ab were added in a total volume of 25 μl of normal FCS containing growth medium on Days 0 (day of plating), 3, 5, and 7. Cell growth was analyzed by an MTT assay on Day 9 and expressed as OD readings/well. We have previously reported that IGF-I and IGF-II at concentrations of 15 ng/ml are optimally effective towards exerting a mitogenic effect on the growth of colon cancer cells (9). IGF-I and IGF-II were therefore used at the optimal concentration. In previous studies we reported that colon cancer cells, including CaCo2 cells, secrete approximately 1 ng IGF-II (and no IGF-I)/10^7 cells (8). Based on the information provided by UBI, we chose 2 concentrations of the neutralizing antibody: one which would be sufficient for neutralizing the effect of endogenous IGF-II (10 μg Ab/ml), and another which would be suboptimally effective towards neutralizing the growth effects of 1–10 ng of IGF-II (1 μg Ab/ml). In preliminary studies we confirmed that 10 μg/ml of the IGF-II antibody completely inhibited the growth response of Colo-205 cells (a highly responsive human colon cancer cell line) (8, 9), to 1–10 ng of IGF-II, *in vitro*; one μg Ab/ml, on the other hand, was only minimally effective. For the differentiation studies, CaCo2 cells at densities of 0.5- 2.5 × 10^6 cells were plated in 35 mm dishes in 3 separate experiments in a total volume of 1.0 ml growth medium containing 10% FCS, which additionally contained the indicated growth factor and/or antibody. The growth medium was replaced on Days 3, 5, and 7 with serum containing medium to which the growth factor and/or antibody was added at the indicated concentrations. On Day 9 cells were harvested and ALP activity measured as described above. Duplicate dishes, treated similarly, were used for cell counting with a Coulter counter, and ALP results were expressed per 10^6 cells.

Results

Growth and differentiation status of CaCo2 cells

Our initial experiments were designed to define the growth and differentiation status of CaCo2 cells over a 13-day time period. With respect to growth status, CaCo2 cells were seeded at 5 × 10^3/cm^2 into replicate wells of a 24-well plate (Falcon) and enumerated on Days 0, 2, 5, 9, and 13 by the MTT dye assay. This assay provides a sensitive and reliable estimate of viable cell number (25, 26). As shown in Fig. 3, viable cell number increased exponentially from Days 2–9 and then plateaued (with a significant reduction in growth) between Days 9 and 13. On the basis of these data, Days 2–9 were defined as the exponential phase of growth and Days 9–13 as the plateau phase. Of note, the onset of growth retardation (plateau phase growth) roughly correlated with the onset of confluence by gross inspection.

Differentiation status was defined on the basis of ALP activity, a commonly employed *in vitro* and *in vivo* marker of enterocytic differentiation (27). ALP activity was selected because it is known to increase relatively early during CaCo2 cell differentiation, whereas increases in the other brush border enzyme activities, such as sucrase-isomaltase, only appear at late post-confluence (31, 32). CaCo2 cells were again seeded at 5 × 10^3 cells/cm^2 into replicate 60 mm dishes (Falcon) and assayed on Days 2, 5, 9, and 13. As shown in Fig. 3B, CaCo2 cells exhibited a modest but insignificant increase in ALP activity between Days 2 and 5; between Days 5 and 13, however, ALP activity steadily increased, achieving a significant 3.3-fold increase by Day 9 and 5.4-fold increase by Day 13. These increases roughly correlated with post-confluent growth. All subsequent experiments were performed at the same seeding density and time course.
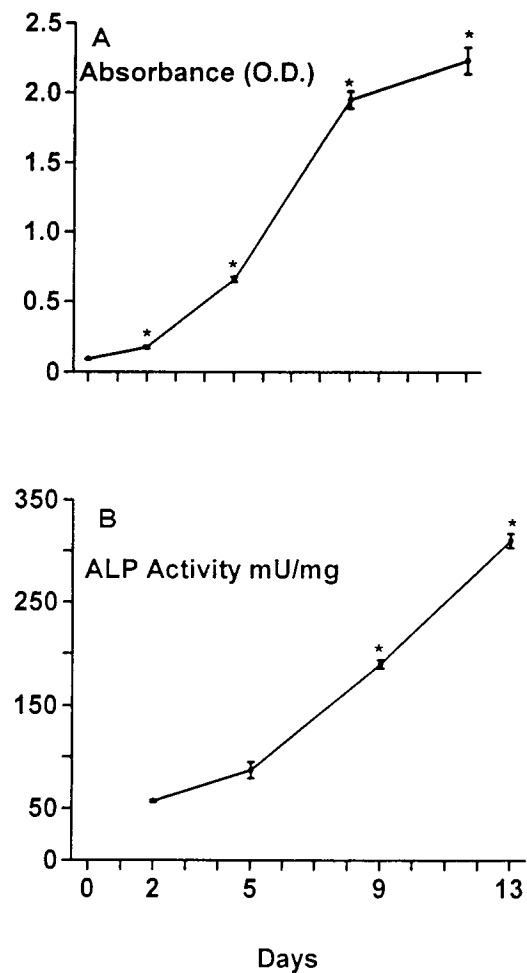

Fig. 3. Growth and differentiation status of CaCo2 cells in culture. CaCo2 cells were seeded at 5 × 10^3 cells/cm^2 and assayed on Days 2, 5, 9, and 13. A, Growth status was determined by changes in viable cell number as measured indirectly by the MTT dye assay. B, ALP activity was employed as a functional marker of enterocytic differentiation and measured by kinetic determination of p-nitrophenyl phosphate hydrolysis. Results are expressed as mean absorbance (OD) ± SE and mean activity (mU per mg protein) ± SE, respectively. Asterisks (*) denote significant differences compared with Day 2 values ($P < 0.05$). The data presented are from a single experiment and are representative of three similar experiments.

Profile of secreted IGFBPs during CaCo2 cell differentiation

Conditioned medium (CM) was collected from CaCo2 cells at sequential time points between Days 3–19 and analyzed by Western ligand blotting and immunoblotting to establish IGFBP profiles. A representative IGFBP profile from one of two experiments is presented in Fig. 4. Western ligand blotting with ^{125}[I]IGF-I revealed two major bands of 24 kDa and 32 kDa and a minor band of 28 kDa (Fig. 4). The 28 kDa band may represent the glycosylated form of IGFBP-4, as previously reported for HT-29 cells (8, 12, 13). The IGFBP-4 antibody, however, did not always detect the 28 kDa protein, for which reason the exact identity of this protein remains to be confirmed. Because in preliminary studies we did not detect the presence of BP-1, BP-3, BP-5, and BP-6 mRNA in Day 7 CaCo2 samples, it appears likely that the 28 kDa protein detected by ligand blot analysis in Fig. 3 is
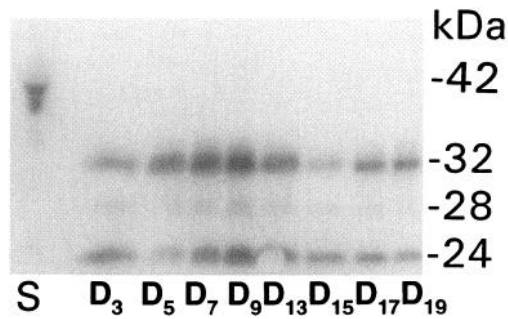
FIG. 4. IGFBP profiles of CaCo2 cells. Serum-free CM was collected from CaCo2 cells as described under *Materials and Methods* on Days 2–19. The CM samples were concentrated and analyzed by WLB analysis as described under *Materials and Methods*. The CM samples were loaded in each lane equivalent to 10^7 cells to compare the data between different days of culture. Samples in S lane represent 0.5% FCS, run as a positive control. The molecular mass of the bands is shown to the right of the figure in kDa. The molecular mass of the unknown samples was determined from the relative mobility of the low mol wt standards that were corun with the samples. Autoradiographs presented are representative of several blots from two similar experiments.

perhaps the glycosylated form of IGFBP-4. The 32 kDa protein was identified as IGFBP-2 and the 24 kDa IGFBP as IGFBP-4 by immunoblot analysis of Day 7 and Day 9 samples (data not shown). As can been seen in Fig. 4, the relative concentrations of secreted IGFBP-2 (32 kDa BP) and IGFBP-4 (24 kDa BP) underwent significant alterations during CaCo2 cell differentiation. The relative concentrations of IGFBP-2 and IGFBP-4 increased by approximately 200–300% from Day 5 through Day 9, declined thereafter, reaching relatively constant levels by Day 15 through Day l9 (Fig. 4).

Measurement of relative concentrations of secreted IGFBP-2, IGFBP-4 and IGF-II in CM of CaCo2 cells

Because the profile of IGFBPs (Fig. 4) did not change significantly after Day 13 (Fig. 3), we measured relative concentrations of the secreted IGFBPs from Days 2–13 in all further studies. The relative concentrations of IGFBP-2 and

IGFBP-4 secreted by the CaCo2 cells into serum-starved CM per 24 h was analyzed by either Western immunoblot (WIB) or by Western ligand blot (WLB) analysis as described under *Materials and Methods*. Autoradiographs of representative blots from one of three separate experiments is shown in Fig. 1. In the case of IGFBP-2, we have previously demonstrated that the IGFBP-2 antibody from UBI is specific for recognizing human IGFBP-2 and cross-reacts insignificantly with all other IGFBP proteins (8). We therefore chose to primarily analyze relative concentrations of IGFBP-2 by Western immunoblot analysis. Recombinant human IGFBP-2, in concentrations of 1–500 ng, were co-run with the CM samples to measure relative concentrations of IGFBP-2 in ng or fmole concentrations from a standard curve as described under *Materials and Methods*. Representative autoradiographs of the rhBP-2 peptide, when analyzed by both Western ligand blot or Western immunoblot analysis is presented in Fig. 1. Since both analyses gave essentially similar results, the relative concentrations of IGFBP-2 presented in Table 1 and Fig. 5 were derived from densitometric analysis of the immunoblots only.

The CM samples for IGFBP-4 were analyzed by both Western immunoblot (WIB) and Western ligand blot (WLB) analysis. Representative autoradiographs from an experiment are shown in Fig. 1. Both analyses gave essentially similar results. Even though the Western immunoblot analysis of IGFBP-4 appeared to be more sensitive, the background labeling was higher compared with the Western ligand blots. Therefore, the relative concentrations of IGFBP-4 presented in Table 1 and Fig. 5 were derived from densitometric analysis of the ligand blots only.

Relative concentrations of IGF-II were also measured in the CM samples by specific RIA as described under *Materials and Methods*, and the results are presented in Table 1 and Fig. 5. Total protein secreted into the CM was additionally measured to compare the secretory activity of the CaCo2 cells on different days of culture with reference to the secretion of specific proteins (IGFBP-4, IGFBP-2, IGF-II) and expressed as $\mu gs/10^7$ cells/24 h (Table 1, Fig. 5).

TABLE 1. Relative concentrations of IGFBP-2, IGFBP-4, and IGF-II in conditioned media (CM) in relation to corresponding mRNA levels on different days of culture of CaCo2 cells

Days	Protein Concentration in CM/10^7 cells/24 h		IGFBP-4 in CM	IGFBP-4 mRNA	IGFBP-2 in CM		IGFBP-2 mRNA	IGF-II in CM		IGF-II mRNA
	μgs (n = 6)	% of D_2^a (n = 6)	% of D_2^a (n = 6)	% of D_2^a (n = 2–4)	ng^b (n = 6)	% of D_2^a (n = 6)	% of D_2^a (n = 2–4)	pgs^c (n = 6)	% of D_2 (n = 6)	% of D_2^a (n = 2)
2	181 ± 33	100.0	100.0	100.0	12.3 ± 4.5	100.0	100.0	729 ± 41.5	100.0	100.0
5	$335 ± 62^d$	196 ± 36	$211 ± 15^d$	$180.0 ± 18^d$	23.0 ± 2.0	243 ± 112	195 ± 43	$546 ± 29.7^d$	$75.6 ± 8.5^d$	$80.0 ± 3.0^d$
7	$563 ± 103^d$	$311 ± 37^d$	$309 ± 14^d$	$269.0 ± 31^d$	$28.0 ± 2.5^d$	330 ± 176	165 ± 30	$360 ± 48.5^d$	$51.6 ± 9.0^d$	$37.0 ± 7.5^d$
9	$560 ± 89^d$	$309 ± 31^d$	$280 ± 37^d$	$153.0 ± 12^d$	$26.0 ± 1.8^d$	259 ± 122	115 ± 10	$209 ± 40.5^d$	$28.0 ± 4.7^d$	$15.2 ± 6.2^d$
13	$505 ± 107^d$	$279 ± 42^d$	137 ± 40	67.6 ± 15	9.6 ± 2.3	83 ± 28	100 ± 13	$52 ± 6.5^d$	$5.0 ± 2.3^d$	$5.3 ± 1.2^d$

CaCo2 cells cultured for Days 2–13 in regular growth medium were harvested for either RNA extraction or processed for 24 h CM collection under serum-free conditions as described under *Materials and Methods*. Each value is the mean ± SEM of the indicted number (n) of observations from two to three separate experiments.

[a] Day 2 (D_2) values were arbitrarily assigned a 100% value, and readings for all other days are presented as a percentage of the Day 2 values.

[b] Relative concentrations of IGFBP-2 in the CM, measured by a Western immunoblot method from a standard curve of rhBP-2 as described under *Materials and Methods*. Relative concentrations of IGFBP-4 were determined by densitometric analysis of the Western ligand blots as described under *Materials and Methods*. The relative mRNA concentrations were determined by densitometeric analysis of Northern blots, after correcting for differences in loading, as described under *Materials and Methods*.

[c] IGF-II concentrations in the CM, measured by a specific RIA.
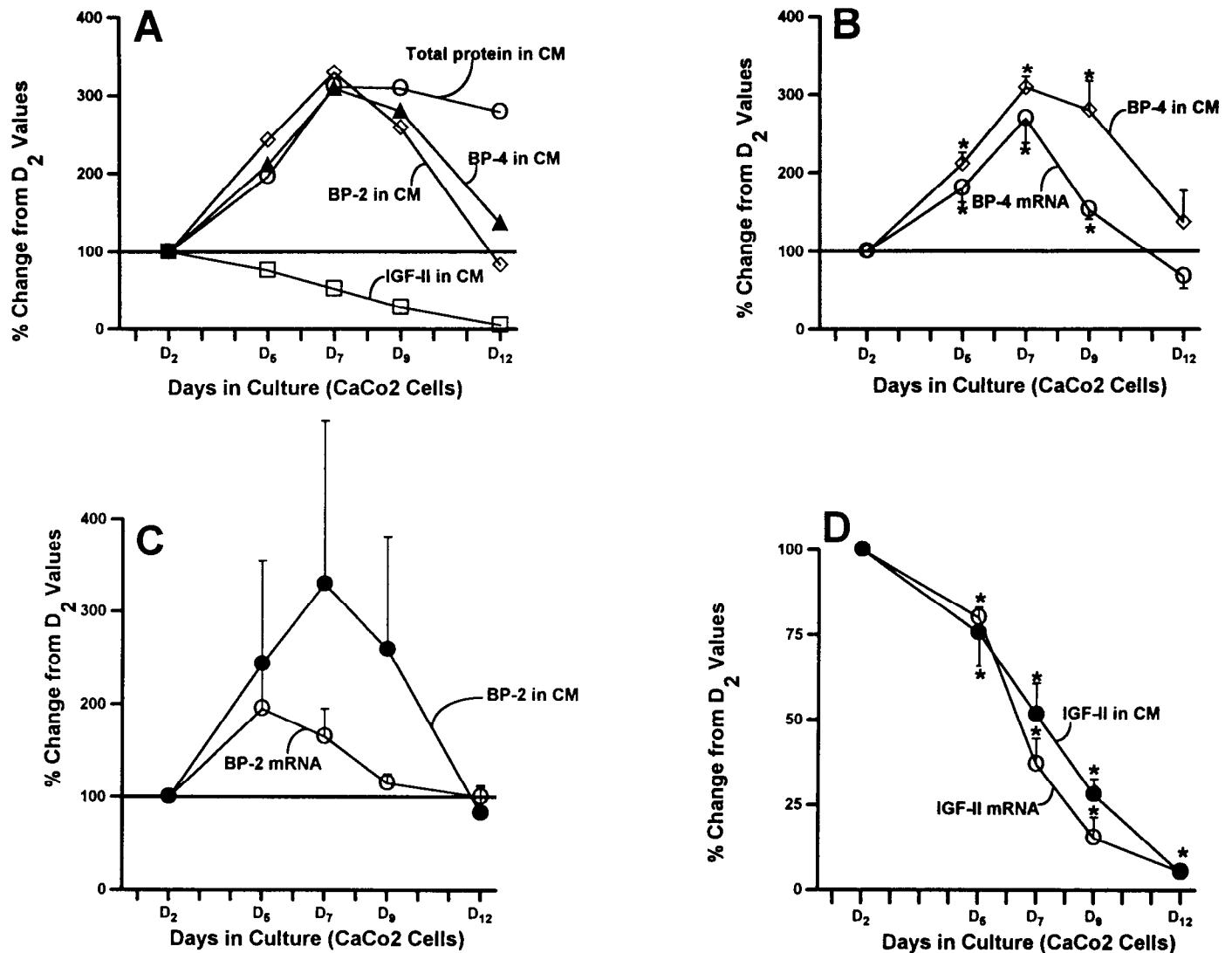
[d] $P < 0.05$ *vs.* Day 2 values.

FIG. 5. Relative expression (at the mRNA level) and secretion (into the CM) of IGF-II, IGFBP-2, and IGFBP-4 by CaCo2 cells in culture. CaCo2 cells were cultured for Days 2–13 and processed for RNA extraction and CM collection as described under *Materials and Methods*. Each data point is mean ± SEM of 4–6 observations from 2–3 separate experiments (numerical details are given in Table 1). All the data are presented as a per cent change from Day 2 values/10^7 cells, which was arbitrarily assigned a 100% value. In Panel A, the relative concentrations of IGFBP-4, IGFBP-2, and IGF-II in the CM of the cells is presented in relation to total protein secreted by the cells into the CM. Only the mean values are presented in Panel A to avoid confusion; the SEM values for these data are presented in Table 1. Panels B, C, and D show the relative mRNA and secreted protein concentrations of IGFBP-4, IGFBP-2, and IGF-II, respectively. $*$ = $P < 0.05$ *vs.* Day 2 values.

Measurement of relative mRNA expression of IGFBP-2, IGFBP-4, and IGF-II

Representative autoradiographs of Northern blots from one of two or three experiments, used to measure mRNA levels for IGF-II, IGFBP-2, and IGFBP-4 are depicted in Fig. 2. We have previously reported that CaCo2 cells express various transcripts of IGF-II mRNA, of which >80% is present as the 6.0 kilobases (kb) transcript (8). In CaCo2 cells we similarly measured the 6.0 kb transcript to be the major IGF-II transcript (>90%) (Fig. 2). We therefore measured relative concentrations of the 6.0 kb IGF-II transcript on different days of culture by densitometric analysis of several blots from two separate experiments. The major transcript for IGFBP-2 was approximately 1.6–1.8 kb (Fig. 2), while the

major transcript measured for IGFBP-4 was approximately 1.7–2.0 kb (Fig. 2) as reported previously (8). The combined data from three experiments is presented in Table 1 and Fig. 5 as described below.

Comparison of the expression and secretion of IGFBP-4, IGFBP-2, and IGF-II during CaCo2 cell differentiation

To compare the relative expression and secretion of IGF-II, IGFBP-4, and IGFBP-2 during CaCo2 cell proliferation/differentiation, the data obtained from Western blots (Fig. 1) and Northern blots (Fig. 2) from three separate experiments are summarized in Table 1. To visually compare the relative expression of IGFBP-2, IGFBP-4, and IGF-II at the mRNA and

TABLE 2A. Effect of IGF-II antibody (Ab) on growth of CaCo2 cells in culture

Experiment	OD Readings								
	Control FCS (10%)	IGF-I (15 ng/ml)	IGF-II (15 ng/ml)	Ab-1 (1 μg/ml)	Ab-2 (10 μg/ml)	IgG (10 μg/ml)	IGF-I + Ab 2	IGF-II + Ab-2	IGF-II + IgG
I	0.059 ± 0.004 (100%)	ND	0.081 ± 0.007^a (137%)	ND	0.037 ± 0.005^a (62.7%)	ND	ND	0.061 ± 0.003 (103.4%)	ND
II	0.65 ± 0.08 (100%)	0.63 ± 0.09 (97%)	0.58 ± 0.06 (89.2%)	0.53 ± 0.04 (81.5%)	0.33 ± 0.06^a (51%)	ND	ND	0.68 ± 0.09 (105%)	ND
III	0.82 ± 0.05 (100%)	0.94 ± 0.06 (115%)	0.93 ± 0.06 (113%)	0.95 ± 0.07 (116%)	0.67 ± 0.03^a (81.7)	0.83 ± 0.04 (101%)	0.92 ± 0.03 (112%)	0.82 ± 0.05 (100%)	0.90 ± 0.08 (110%)

Each value, presented for the three separate experiments, is the mean ± SEM of 4–8 separate observations within an experiment; number of cells plated/well in 96 well plates were 1000, 2500, and 3500 in experiments I, II, and III, respectively. The percent of change on treatment, on an average, is presented in parenthesis, wherein the control values were arbitrarily assigned a 100% value.

Ab-1 and Ab-2, IGF-II-Ab; IgG, rabbit IgG against mouse IgG; ND, Not Done.

^{a} $P < 0.05$ vs. control.

TABLE 2B. Effect of IGF-II antibody (Ab) on differentiation of CaCo2 cells in culture

Experiment	ALP Units/L/10^6 Cells					
	Control FCS (10%)	IGF-II (15 ng/ml)	Ab-2 (10 μg/ml)	IgG (10 μg/ml)	IGF-II + Ab-2	IGF-II + IgG
I	2.84 ± 0.21 (100%)	2.07 ± 0.18^a (73%)	4.58 ± 0.28^a (163.3%)	ND	3.15 ± 0.23 (110.9%)	ND
II	1.98 ± 0.06 (100%)	1.83 ± 0.21 (92.4)	4.82 ± 0.12^a (243%)	ND	2.3 ± 0.17 (116%)	ND
III	14.4 ± 0.14 (100%)	3.6 ± 0.11^a (81.8)	6.4 ± 0.15^a (145%)	4.6 ± 0.12 (104%)	5.6 ± 0.12^a (127%)	3.3 ± 0.09^a (75%)

Each value, presented for three separate experiments, is the mean ± SEM of three separate measurements within an experiment; number of cells plated/35 mm dishes were 0.05 × 10^6, 0.15 × 10^6 and 0.25 × 10^6, in experiments I, II, and III, respectively. Cellular samples from either four dishes (Experiment 1), two dishes (Experiment 2) or one dish (Experiment 3) were pooled per measurement. Readings were taken at 2' after the start of the reaction for measuring ALP activity per pooled sample. The percent change on treatment is presented in parenthesis, wherein the control values were arbitrarily assigned a 100% value.

Ab-2, IGF-II-Ab; ND, Not Done; IgG, rabbit IgG against mouse IgG.

^{a} $P < 0.05$ vs. control.

protein levels, the data in Table 1 are also presented as line graphs in Fig. 5.

The relative concentrations of secreted IGFBP-2, IGFBP-4, and total protein per 10^7 cells per /24 h increased rapidly from Day 2 through Day 7 (Table 1 and Fig. 5). Thereafter, however, while the total protein concentrations did not change significantly, the relative concentrations of IGFBP-2 and IGFBP-4 decreased rapidly between Days 7–13. The concentration of IGF-II peptide demonstrated a significantly different profile wherein the relative concentrations of IGF-II decreased rapidly from Day 2 through Day 13 of cell culture. These data thus suggest that IGF-II, IGFBP-2, and IGFBP-4 secretion are independently regulated during CaCo2 cell differentiation.

The relative expression at the mRNA and protein levels of the three IGF factors, IGFBP-4, IGFBP-2, and IGF-II were complementary (Fig. 5, Table 1). The relative concentrations of IGFBP-4 mRNA increased significantly and rapidly from Day 2 through Day 7 and declined steeply thereafter to Day 2 levels (Fig. 5B and Table 1). Similarly, the relative concentrations of IGFBP-4 in the CM samples increased rapidly and significantly from Day 2 through Days 7–9 and declined rapidly thereafter. The maximum increase in mRNA and protein concentrations for IGFBP-4 was 250–300% compared with Day 2 values (Fig. 5B, Table 1). IGFBP-2 exhibited a similar pattern to that of IGFBP-4, but because of interexperimental variability, none of the changes were determined to be statistically significant (Fig. 5C, Table 1). In contradistinction, the expression of IGF-II both at the mRNA level and at the peptide level declined rapidly from Day 2 through Day 13, achieving statistical significance by Day 5 (Fig. 5D, Table 1).

Effect of IGF-II antibody treatment on growth and differentiation of CaCo2 cells

CaCo2 cells were treated with or without the growth factors (IGF-I, IGF-II) and/or the neutralizing IGF-II antibody (Ab), and the effect of the treatment was measured on the growth and differentiation of cells as described under *Materials and Methods*. The data from three separate experiments are presented in Tables 2A and 2B. Treatment of CaCo2 cells with optimal concentrations of either IGF-I or IGF-II, in the presence of 10% FCS, did not result in a significant change in the growth of the cells compared with that of the control cells (treated with FCS alone) (Experiments II and III, Table 2A). However, in experiment I, wherein the cells were plated at a very low density, a significant increase in the growth of CaCo2 cells was measured in the presence of exogenous IGF-II. It thus appears likely that in experiments II and III, wherein the cells were plated at 2.5- to 3.5-fold higher density, endogenous IGF-II was maximally effective in the presence of 10% FCS, such that exogenous IGFs had no additional growth effect. However, when the cells were plated at a much lower density, as in experiment I, exogenous IGF-II exerted a significant growth stimulatory effect on CaCo2 cells, confirming an important role of IGFs in the proliferation of CaCo2 cells. In all three experiments, irrespective of the initial plating density, the addition of IGF-II-Ab (10 µg/ml) significantly inhibited the growth of the cells compared

with that measured for control cells. The effect of the neutralizing antibody was specific since control IgG had no effect. The effect of the neutralizing antibody was completely reversed in the presence of exogenous IGF-II. The results of the experiments, as presented in Table 2A, thus confirm an important role of endogenous IGF-II in the growth of CaCo2 cells.

Addition of IGF-II significantly depressed differentiation of CaCo2 cells by 20–27% in 2 out of 3 experiments (Table 2B). On the other hand, addition of IGF-II-Ab (10 µg/ml) significantly increased differentiation of CaCo2 cells in all three experiments by 45 to 145%, compared to that of control cells. The effect of IGF-II-Ab was specific since control IgG had no effect. The addition of exogenous IGF-II reversed the effects of neutralizing antibody on differentiation of the cells in all three experiments.

From the three experiments, the growth and differentiation results with IGF-II (15 ng/ml) and IGF-II-Ab (10 µg/ml) were combined and presented as percent (%) change in Fig. 6. The data from control cells were arbitrarily assigned a 0 value. As can be seen from the figure, on an average, addition of IGF-II had a slight stimulatory effect on the growth of the cells, accompanied by a decrease in the differentiation of the cells; the data, however, were not statistically significant because of the interexperimental variations. Addition of the neutralizing antibody (10 µg/ml) had a significant inhibitory effect on the growth of the CaCo2 cells, accompanied by a significant increase in the differentiation of the cells; simul-
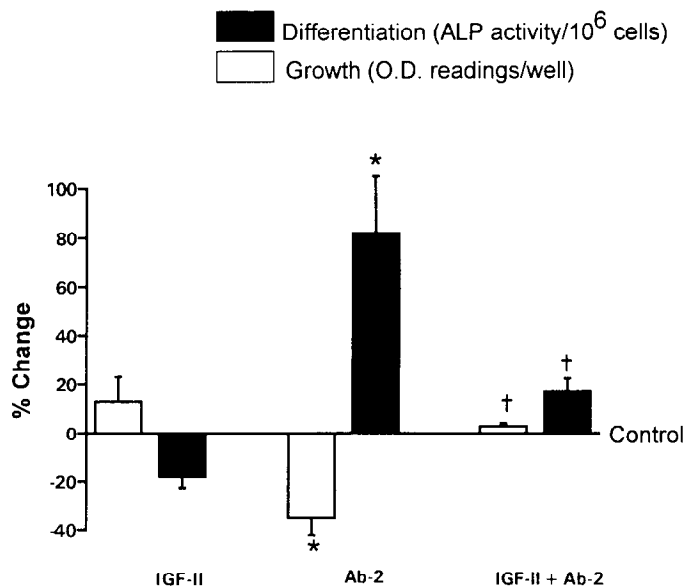

FIG. 6. Effect of IGF-II-antibody on the growth and differentiation of CaCo2 cells. CaCo2 cells were treated with or without IGF-II and/or IGF-II-antibody (Ab-2) as described under *Materials and Methods*. Growth and differentiation of the control and treated cells was measured in three separate experiments, and the data for each experiment is given in Tables IIA and IIB. The per cent changes in growth and differentiation of CaCo2 cells on treatment with IGF-II (15 ng/ml) and/or Ab (10 µg/ml), compared with that of control cells, were combined from all three experiments and are presented as bar graphs. Control values were arbitrarily assigned a 0 value, and the per cent increase or decrease in the growth and differentiation of the cells is presented as mean ± SEM of data from the three experiments. * = $P < 0.05$ *vs.* control; † = $P < 0.05$ *vs.* antibody treated.

taneous addition of IGF-II reversed the effects of the antibody. Thus the results presented in Fig. 6 clearly demonstrate that endogenous IGF-II is importantly involved not only in the growth of CaCo2 cells but also in the differentiation of the cells, wherein neutralization of endogenous IGF-II significantly enhanced the onset of differentiation (irrespective of cell density).

Discussion

In recent years, we and others have reported on a possible autocrine role of IGF-II in human colon cancers (7–11, 16) and in rat intestinal epithelial cells (33). Because human colon cancers also express one or more IGF binding proteins (IGFBPs) (2, 8) that are known to modulate the mitogenic effects of IGFs (12, 13, 18–24), we postulated that the ratio of IGFs and IGFBPs may dictate the growth/differentiation potential of normal and transformed colonic epithelial cells. Support for this hypothesis was derived from previous studies, in which the human colon cancer cell lines HT-29 was transfected with vectors overexpressing either the sense or the antisense mRNA of hIGFBP-4. These studies demonstrated that IGFBP-4 had potent inhibitory effects on the growth of HT-29 cells, which may be mediated by both IGF-dependent and IGF-independent mechanisms (13).

In a previous study we established the fact that a majority of the human colon cancer cell lines express IGF-II and at least two IGFBPs, IGFBP-2, and IGFBP-4 (8). In the present study, we have examined for the first time the expression and secretion of the mitogen IGF-II in relation to that of the inhibitory IGFBPs, IGFBP-2, and IGFBP-4, (2, 13, 18–20) during both the phases of growth and differentiation using a representative human colonic cell line, CaCo2. CaCo2 cells not only express the three factors of interest, but also undergo spontaneous enterocytic differentiation in culture (17), thus providing an *in vitro* model for examining our hypothesis.

In the first set of studies the exponential and the plateau phase of growth were defined for CaCo2 cells. The cells demonstrated an exponential growth phase between Days 3–9, followed by a plateau phase of growth between Days 9–13. The growth curve of the cells was influenced by the initial plating density of the cells. A lower plating density resulted in a shifting of the growth curve to the right, while a higher plating density resulted in a shifting of the growth curve to the left (Singh P., U. Yallampalli, and L. A. Owlia, unpublished data). However, an important observation was that, irrespective of the initial plating density, the cell numbers attained at the end of the exponential phase of growth were similar in all experiments. Thus the surface area of the culture dish dictated the final number of cells that could be accommodated at confluency. For reasons discussed in the *Materials and Methods* section, we chose to measure ALP activity of the cells as a measure of differentiation. Under the conditions of the present study, ALP activity progressively increased between Days 7–13, thus correlating with postconfluent plateau growth phase. However, it is not known if factors that down-regulate proliferation are the same or different from those that up-regulate differentiation.

The expression and secretion of IGF-II changed signifi-

cantly in relation to growth and differentiation of CaCo2 cells. The major IGF-II transcript expressed by cells used in the present study was 6.0 kb (>90%), suggesting that the P3 promoter (34, 35) was the major promoter being used by these cells. Zarrilli *et al.* (16) similarly reported the expression of 6.0 kb IGF-II transcript as the major transcript by CaCo2 cells. Thus existing data suggests that the P3 promoter is primarily used for efficient transcription of IGF-II mRNA by human colon cancer cells (present studies, 7, 8, 16); the P4 promoter is either not used, as in the case of CaCo2 cells used in this study, or inefficiently used, as in the case of some colon cancer cell lines (8, 16).

The mRNA levels of the 6.0 kb IGF-II transcript were inversely related to the differentiation curve of the CaCo2 cells in culture (Figs. 3 and 5). During the time period when significant concentrations of IGF-II were still being expressed and secreted (Days 2–7), CaCo2 cells had yet not completely entered into the exponential phase of differentiation; however, during the time period when the cells had entered the exponential phase of differentiation (Days 9–13), the cells were expressing and secreting negligible concentrations of IGF-II. The exponential growth phase of the cells was measured to last between Days 2–9. Because both the expression and secretion of IGF-II were significantly attenuated by Day 9, it appears likely that IGF-II, secreted at earlier time points, provided the required mitogenic signal that allowed the cells to continue proliferating at an exponential rate.

We recently reported that a majority of human colon cancer cell lines and primary human colon cancers express IGFBP-4 and IGFBP-2; a subset of colon cancers also express IGFBP-3, IGFBP-5, and IGFBP-6, but none express IGFBP-1 (8). While a subset of CaCo2 cells express significant concentrations of IGFBP-3 (8, 15), CaCo2 cells from Dr. Jing Yu's laboratory mainly express IGFBP-2 and IGFBP-4 (present data). We now know that IGFBP-4 and IGFBP-2 primarily function as inhibitory IGFBPs for several cell types including colon cancers (8, 12, 13, 18, 19). Therefore, to examine the role of inhibitory IGFBPs relevant to human colon cancer, we used the CaCo2 cells from Dr. Yu for the present studies. The relative concentrations of mRNA for IGFBP-4 and IGFBP-2 increased by more than 200% on Days 5–7 of culture compared with Day 2 values. The relative concentrations of IGFBP-4 and IGFBP-2, secreted into the CM of the cells, similarly increased rapidly by 300% on Days 7–9 of culture, compared with Day 2 values. After attaining peak levels around Days 5–7, the mRNA and protein concentrations of both IGFBP-4 and IGFBP-2 declined rapidly to Day 2 values by Days 9–13 in culture. Because the protein concentrations secreted into the CM for both IGFBP-4 and IGFBP-2 followed a pattern very similar to that measured with mRNA levels for the two IGFBPs, it suggested that expression of the two proteins might be regulated at the transcriptional level. The changes in the mRNA and CM concentrations of IGFBP-4 were determined to be statistically significant, whereas the changes with IGFBP-2 were not because of very large interexperimental variations. Therefore, at this point it is difficult to make any concrete assessment regarding the role of IGFBP-2 in the growth and differentiation of CaCo2 cells.

In recent years, specific proteolytic degradation of the IGFBPs has been reported for several cell types (36–38),
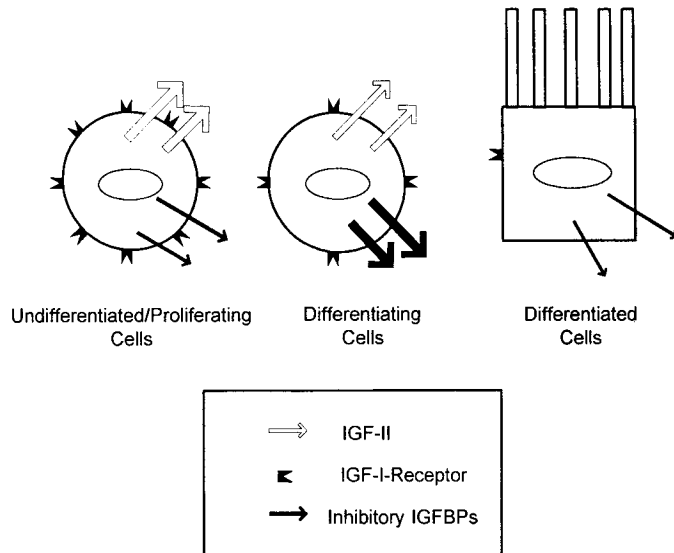
FIG. 7. Hypothetical model, presented in a simplistic manner, depicts the relative expression/secretion of IGF-II, IGF-I-receptor, and IGFBP-4 in CaCo2 cells in relation to the growth and differentiation states, based on the results of the present studies and the studies of Zarrilli et al. (16).

which can dictate the final concentrations of the IGFBP proteins available to the cells. IGFs reportedly promote proteolytic degradation of IGFBP-4 in the CM of fibroblasts by receptor-independent mechanisms (36, 37). The present studies were not designed to specifically measure proteolytic degradation of the IGFBPs. However, it appears unlikely that IGFBPs were subjected to proteolytic degradation during the course of this study, because protein concentrations of IGFBPs secreted into the CM reflected the expression profile of the IGFBPs at the mRNA level. Furthermore, no other proteolytic bands of proteins (< 24 kDa) were detected by either ligand blot or immunoblot analysis with IGFBP-2 and IGFBP-4 antibodies (data not presented). To the best of our knowledge proteolytic degradation of IGFBPs has not been reported for CaCo2 cells or any other colon cancer cell lines. It is possible that colon cancers either express mutated forms of IGFBPs that are not degraded by proteases (39) or do not express IGFBP proteases.

The majority of the studies reported to date indicate that IGFBP-4 functions primarily as an inhibitory IGFBP (8, 19). In recent studies, we similarly confirmed that IGFBP-4 primarily functions as an inhibitory IGFBP for human colon cancer cells via both an IGF-independent and IGF-dependent mechanism (13). We measured an initial up-regulation of IGFBP-4 expression by CaCo2 cells, at a time when the cells were exponentially growing. Since the human colon cancer cell line CaCo2 is unique in its ability to spontaneously differentiate, it is possible that up-regulation of inhibitory IGFBPs, correlated with an attenuation of IGF-II expression, may provide the initial signal for differentiation.

Down-regulation of IGF-II expression has been suggested to induce differentiation of human osteosarcoma cells (39) and of myoblasts (40). It is not known if endogenous IGF-II plays a role in the differentiation of colonocytes. Therefore, to test a role of endogenous IGF-II in growth and differen-

tiation of CaCo2 cells, we conducted studies with neutralizing IGF-II-Ab. The growth of the antibody treated cells was significantly reduced in all three experiments conducted compared with the untreated control cells (Fig. 6). Additionally, the levels of activated ALP (differentiation marker) were significantly increased in treated vs. control cells, irrespective of the cell density (Table 2B, Fig. 6), suggesting for the first time that attenuation of IGF-II effects might be equally important in differentiation of colonocytes.

An equally important finding was that post-confluent, differentiated, CaCo2 cells continued to express significant concentrations of IGFBP-4 and IGFBP-2 during plateau phase growth associated with negligible expression of IGF-II. The latter observation suggests a possible role of inhibitory IGFBPs in maintaining the differentiation status of the cells, which needs to be examined in future studies. Thus our results so far support the hypothesis that relative expression of the mitogen IGF-II and the inhibitory IGFBPs might play an important role in dictating the proliferative/differentiation potential of CaCo2 cells in culture. It is speculated that commitment of the cells along an enterocytic pathway requires an attenuation of not only the mitogenic effects of IGFs (as supported by the present studies), but perhaps also continued expression of the inhibitory IGFBPs, as presented in the hypothetical model in Fig. 7. Future studies will help us to further delineate the specific role of these factors in growth and differentiation of the intestinal cells.

Acknowledgments

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