Journal of Biochemistry and Molecular Biology, Vol. 32, No. 3, May 1999, pp. 266–272

JOURNAL OF
Biochemistry
and
Molecular Biology

Growth Stimulation and Inhibition of Differentiation of the Human Colon Carcinoma Cell Line Caco-2 with an Anti-Sense Insulin-Like Growth Factor Binding Protein-3 Construct

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Received 29 December 1998, Accepted 19 February 1999

The insulin-like growth factor (IGF) system consisting of IGF-I, IGF-II, IGF-receptors, and IGF-binding proteins (IGFBP) regulates the proliferation of a variety of cancer cell types. To examine whether a decrease in endogenous IGFBP-3 stimulates proliferation or inhibits differentiation, Caco-2 cells, a human colon adenocarcinoma cell line, were stably transfected with an anti-sense IGFBP-3 expression construct or pcDNA3 vector as control. Accumulation of IGFBP-3 mRNA and secretion of IGFBP-3 into serum-free conditioned medium, 9 days after plating, were significantly lower in Caco-2 cell clones transfected with anti-sense IGFBP-3 cDNA compared to the controls. The anti-sense clones grew at a similar rate to the controls for 8 days after plating, but achieved a higher final density between days 10 and 12. The levels of sucrase-isomaltase mRNA, a marker of enterocyte differentiation of Caco-2 cells, were lower in the anti-sense clones examined on day 9. In conclusion, proliferation of Caco-2 cells can be stimulated by lowering endogenously-produced IGFBP-3.

Keywords: Caco-2, IGFBP-3, Insulin-like growth factors.

Introduction

The insulin-like growth factors (IGFs), IGF-I and IGF-II, are powerful polypeptide mitogens for a variety of cell types (Cohick and Clemmons, 1993; Jones and Clemmons, 1995). Current evidence indicates that most of the cellular effects of IGFs are mediated by the type I IGF receptor and that IGF action is also modulated by interactions with IGF-binding proteins (IGFBPs). In addition to the six high-affinity IGFBPs, IGFBP-1 through -6, four IGFBP-related proteins have been found to have a relatively low affinity for IGFs (Baxter *et al.*, 1998). IGFBPs can potentiate or inhibit IGF action (Jones and Clemmons, 1995), depending on whether the IGF-IGFBP complexes are soluble or bound to the cell surface or extracellular matrix (Conover, 1992; Rechler, 1993; Jones and Clemmons, 1995).

Effects of peptide growth factors, including IGF-I and IGF-II, on cancer cell proliferation have been studied extensively because inappropriate expression of these growth factors has been implicated in the etiology of cancer (Aaronson, 1991; Macauley, 1994; Westley and May, 1995). IGF-II mRNA has been shown to be expressed in human and mouse colon carcinoma cell lines (Guo *et al.*, 1995; Lamonerie *et al.*, 1995). In addition, IGF-II mRNA was found to be overexpressed in human colon carcinomas compared to normal adjacent tissues (Tricoli *et al.*, 1986; Lambert *et al.*, 1990). Furthermore, colon tumor tissues and tumor cell lines have higher IGF-I receptor numbers compared to normal mucosa (Guo *et al.*, 1992). These findings suggest that the IGF system may be important for colon cancer cell proliferation. The human colon adenocarcinoma cell line, Caco-2, has IGF-I and IGF-II receptors, and expresses mRNAs for IGF-II and IGFBP-2, -3, -4, and -6 and synthesizes the corresponding peptides under serum-free conditions (Zhang *et al.*, 1995; Hoeflich *et al.*, 1996; Park *et al.*, 1996). The secretion of IGF-II (Zarrilli *et al.*, 1994; Zhang *et al.*, 1995; Singh *et al.*, 1996) and the levels of the IGF-I receptor (Zarrilli *et al.*, 1994) are high in proliferating cells and reduced in differentiated cells. A monoclonal antibody against the IGF-I receptor inhibits both basal and IGF-II-stimulated cell proliferation (Zarrilli *et al.*, 1994), suggesting that IGF-II may act as an autocrine growth factor for these cells. In addition to its effect on proliferation, sustained expression of IGF-II inhibits the expression of sucrase-

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isomaltase mRNA, a marker for enterocyte differentiation (Zarrilli *et al.,* 1996). Furthermore, as differentiation of Caco-2 cells proceeds, the secretion of IGFBPs changes, suggesting that IGFBPs may regulate proliferation and differentiation of Caco-2 cells (Oguchi *et al.,* 1994; Heoflich *et al.,* 1996; Park *et al.,* 1996).

IGFBP-3, the major IGFBP in human blood, is synthesized by a variety of cell types and can directly modulate IGF actions (Jones and Clemmons, 1995). IGFBP-3 has a two-fold higher affinity for IGF-II than IGF-I (Oh *et al.,* 1993c) suggesting that it may modulate the effects of locally-produced IGF-II. IGFBP-3 also has IGF-independent effects (Oh *et al.,* 1993a; 1993b Valentinis *et al.,* 1995; Rechler, 1997) and has its own receptor (Leal *et al.,* 1997). Recent evidence indicates that IGFBP-3 may mediate apoptosis induced by a tumor suppressor, p53, in the EBI colon carcinoma cell line (Buckbinder *et al.,* 1995). We have previously shown that IGFBP-3 mRNA accumulation and protein secretion increase as Caco-2 cells differentiate (Park *et al.,* 1996). In the present study, we investigated the role of IGFBP-3 in the proliferation and differentiation of Caco-2 cells by using an anti-sense IGFBP-3 expression construct.

Materials and Methods

Materials Caco-2 cell line was obtained from the American Type Culture Collection (ATCC HTB37, Rockville, USA). Dulbecco's modified Eagle medium/F-12 nutrient mixture (DMEM/F12), fetal bovine serum (FBS), human transferrin, trypsin-EDTA, and penicillin-streptomycin were obtained from GibcoBRL (Gaithersburg, USA). Tissue culture dishes and multi-well plates were purchased from Becton Dickinson (Lincoln Park, USA). Radioimmunoassay-grade bovine serum albumin (BSA) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). A rabbit polyclonal anti-IGFBP-3 antibody, IGFBP-3 g1, was kindly provided by Dr. Youngman Oh (Oregon Health Sciences University, Portland, USA). BA 75 nitrocellulose sheets were purchased from Schleicher and Schuell (Keene, USA) and nylon membranes were purchased from Micron Separation Inc. (Westborough, USA). Totally RNA™ (total RNA isolation kit), BrightStar™ BiotinScript™ (nonisotopic *in vitro* transcription kit), and BrightStar™ BioDetect™ (nonisotopic detection kit) were obtained from Ambion Inc. (Austin, USA).

Preparation of an anti-sense IGFBP-3 cDNA expression construct The full-length, 2591-bp human IGFBP-3 cDNA (Wood *et al.,* 1988) was a gift of Dr. B. Fennie (Genentech, South San Francisco, USA). The cDNA was digested with *Eco*RI and *Bam*HI, and the resultant 1506-bp fragment containing coding sequences was ligated in an anti-sense orientation into pcDNA3 (Invitrogen, San Diego, USA). Frozen competent *E. coli*, MAX Efficiency DH5αF′IQ (GibcoBRL) was transformed with the expression construct, and plasmid DNA was prepared from ampicillin-resistant colonies. Prior to its use in transfection, the plasmid DNA was purified utilizing the Qiagen Plasmid Kid (Qiagen Inc., Chatworth, USA) and then linearized with *Pvu*I.

Transfection and selection of Caco-2 cell clones Caco-2 cells were maintained and subcultured as previously described (Park *et al.,* 1996). The complete medium for cell maintenance consisted of DMEM/F12 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection of Caco-2 cells with the anti-sense IGFBP-3 expression construct or the pcDNA3 vector control was performed using a liposome-mediated method as previously described (Park *et al.,* 1996). The cells between passages 23 and 25 were used for transfection. Forty-eight hours after transfection, the cells were subcultured and switched to medium containing 0.4 mg/ml G418 sulfate (GibcoBRL). After 14 days of selection, single resistant colonies were isolated by means of cloning cylinders prior to expansion. The experiments described herein were performed on a pool of three pcDNA3 clones (clones #1, #2, and #5) and two separate anti-sense IGFBP-3-transfected clones (clone #0 and clone #4).

Cell growth curves To establish the growth curves, 6×10^4 cells per well were plated with the complete medium in 12-well plates. The medium was changed every 2 days. For growth curves in serum-free medium, cells were plated in a similar manner. One day later, the monolayers were rinsed three times with DMEM/F12 supplemented with 5 μg/ml transferrin, 0.5 mg/ml RIA-grade BSA, and 5 ng/ml selenium (serum-free medium) and replaced with 1 ml/well serum-free medium. The medium was subsequently changed every 2 days. The viable cell number was assessed by its ability to reduce MTT to spectrophotometrically detectable (570 nm) formazan (Danizot and Lang, 1986). The standard curve was constructed by comparing MTT assay data to the number of viable cells as determined by counting in a hemacytometer for parallel sets of cell cultures.

IGFBP measurements To obtain serum-free conditioned media, cells were cultured in 12-well plates for 9 days as described above and then the 48-h conditioned media were collected. Ligand blotting was performed using [^{125}I]IGF-II (Amersham, Arlington Heights, USA) as described previously (Park *et al.,* 1992).

Northern blot analysis Total RNA was isolated by the method of Chomczynski and Sacchi (1987) using the Totally RNA™ kit. Briefly, the cell monolayers were collected by scraping in denaturation solution, which contained a guanidine thiocyanate/cationic detergent solution. The RNA was separated from DNA and protein by phenol/chloroform extraction and precipitated by adding isopropanol. Total RNA concentrations were determined by measuring ultraviolet (UV) absorbance at a wavelength of 260 nm. Equivalent aliquots (30 μg) of total RNA were electrophoresed in a 1% agarose formaldehyde gel and capillary-transferred to positively-charged nylon membranes. Equivalence of loading, completeness of transfer, and integrity of the RNA preparation were confirmed for each gel by examining ethidium bromide stained 28S and 18S ribosomal RNA bands both before and after the transfer. An RNA ladder (0.24–9.5 kb) was run in parallel to estimate RNA sizes. The membranes were fixed by UV cross-linker (Stratagene, La Jolla, USA) and hybridized with the appropriate biotin-14-CTP labeled anti-sense cRNA probe. The cRNA probes were labeled with biotin-14-CTP using BrightStar™ BiotinScript™ and detected utilizing BrightStar™ BioDetect™. The relative abundance of each mRNA species was

measured by densitometric scanning of the exposed films using a densitometer (Molecular Dynamics, Sunnyvale, USA). A 475-bp human IGFBP-3 cDNA (pHBP3-502) was a gift from Dr. S. Shimasaki (Wood *et al.,* 1988), and the riboprobe was transcribed using T3 polymerase after linearization with *Hin*dIII. The 2041-bp human sucrase-isomaltase cDNA (Green *et al.,* 1987) was a gift of Dr. Dallas Swallow (London, England). For probe preparation, the insert was digested with *Eco*RI and *Xho*I, and the resulting 790-bp cDNA fragment was ligated into pcDNA3. The riboprobe was prepared using SP6 polymerase after linearization with *Eco*RI.

Results

Analysis of IGFBP-3 and sucrase-isomaltase gene expression during Caco-2 cell growth In order to determine the expression levels of IGFBP-3 and sucrase-isomaltase genes during Caco-2 cell growth, total RNA was isolated from untransfected Caco-2 cells after various times in culture for Northern blot analysis. We have previously shown that the changes in levels of IGFBP-3 and sucrase-isomaltase mRNA corresponded with the changes in IGFBP-3 protein secretion and sucrase-isomaltase activities, respectively (Park *et al.,* 1996). The results are summarized in Fig. 1. The 3.1 kb IGFBP-3 mRNA species increased between 5 and 11 days of culture. As previously observed, a single hybridizing species of 6.5 kb corresponding to sucrase-isomaltase mRNA was barely detectable at 5 days of culture and increased markedly after 9 days of culture.

Expression of IGFBP-3 mRNA by Caco-2 clones transfected with an anti-sense IGFBP-3 expression construct To determine whether Caco-2 clones transfected with an anti-sense IGFBP-3 construct had an altered steady-state level of IGFBP-3 mRNA, Northern blot analysis was performed on total RNA isolated from cells cultured for 9 days (Fig. 2). The clones transfected with anti-sense IGFBP-3 cDNA had substantially lower levels of the 3.1 kb IGFBP-3 mRNA species compared to the control clones. Quantitative analysis of the bands indicated that the magnitude of the reduction in clone #4 was 55 ± 7% in four separate experiments. There were no statistical differences in the levels of IGFBP-3 mRNA between the two anti-sense clones.

Secretion of IGFBP-3 by clones transfected with an anti-sense IGFBP-3 expression construct To determine whether Caco-2 cell clones transfected with an anti-sense IGFBP-3 construct produce decreased amounts of IGFBP-3, the clones were cultured for 9 days, and the 48 h, serum-free conditioned media were conditioned media were collected for ligand blot analysis. Four classes of IGFBPs were detectable (Fig. 3): a 24,000 M_r band corresponding to IGFBP-4 (Park *et al.,* 1996), a broad 31,000 M_r band that has been identified as IGFBP-6 (Zhang *et al.,* 1995),

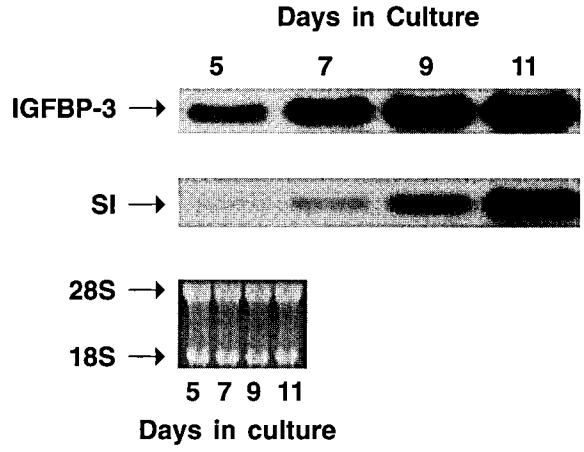

Fig. 1. Expression of IGFBP-3 and sucrase-isomaltase mRNA in Caco-2 cells. Cells were seeded at a density of 6×10^5 cells in 100-mm dishes. On the indicated days, total RNA was isolated for Northern blot analysis. Autoradiographs of representative Northern blots show IGFBP-3 and sucrase-isomaltase (SI) mRNA species with the apparent sizes of 3.1 kb and 6.5 kb, respectively. A photograph of the ethidium bromide-stained membrane after transfer is shown on the bottom to document equal loading and quantitative transfer of RNA in the lanes.

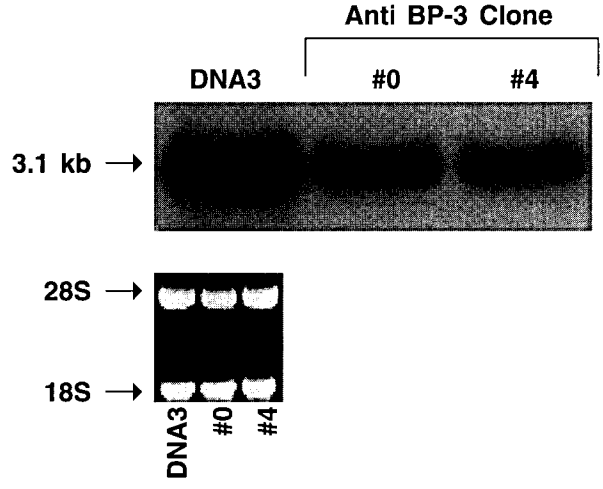

Fig. 2. Expression of IGFBP-3 mRNA in Caco-2 cells stably transfected with pcDNA3 or the anti-sense IGFBP-3 expression construct. Cells were seeded at a density of 6×10^5 cells in 100-mm dishes and cultured for 9 days. Aliquots (30 μg) of total RNA were obtained from a pool of pcDNA3-transfected clones (#1, #2, and #5) (DNA3) or from individual clones transfected with anti-sense IGFBP-3 cDNA (anti BP-3 clones #0 and #4). An autoradiograph of a representative Northern blot shows IGFBP-3 mRNA species with the apparent size of 3.1 kb. A photograph of the ethidium bromide-stained membrane after transfer is shown on the bottom to document equal loading and quantitative transfer of RNA in the lanes.

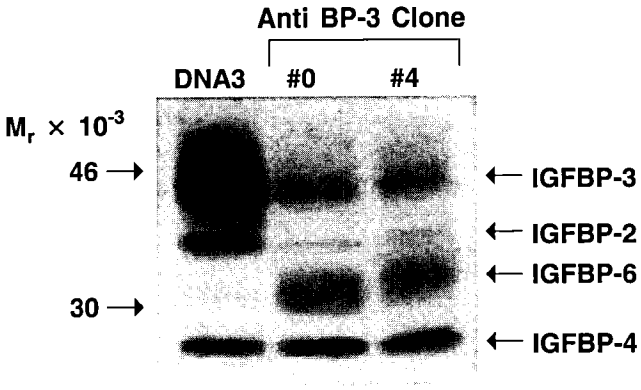

Fig. 3. Ligand blot analysis of media conditioned by Caco-2 cells stably transfected with pcDNA3 or the anti-sense IGFBP-3 expression construct. Forty eight-hour serum-free conditioned media were collected from the cultures of the pool of pcDNA3-transfected clones (#1, #2, and #5) (DNA3) or individual anti-sense IGFBP-3 transfected clones (anti BP-3 clones #0 and 4). The volume of media loaded onto the gel were adjusted for equivalent cell numbers. An autoradiograph of a single blot, which is representative of four independent blots, is shown.

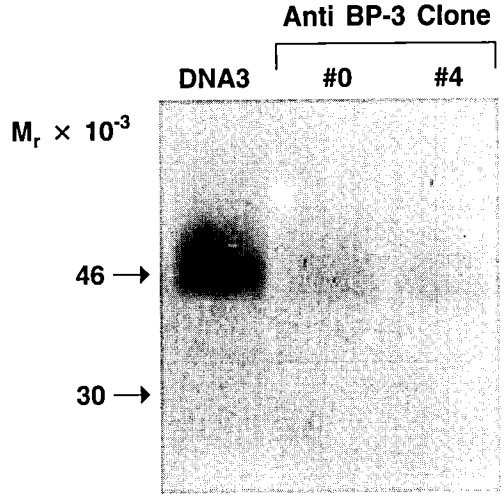

Fig. 4. Immunoblot analysis of media conditioned by Caco-2 cells stably transfected with pcDNA3 or the anti-sense IGFBP-3 expression construct. Conditioned media were collected as described in Fig. 3 and loaded on the gel. After transfer to nitrocellulose paper, the blot was probed with a rabbit polyclonal anti-IGFBP-3 antibody. A photograph of chemiluminescent detection of a representative blot is shown.

a 33,000 M_r band that has been confirmed as IGFBP-2 (Park *et al.*, 1998), and a doublet of bands migrating in the 40,000 to 52,000 M_r range corresponding to IGFBP-3 (Park *et al.*, 1996). The medium conditioned by control cells contained all four classes of IGFBPs, which is in line with our previously published results (Park *et al.*, 1996). In agreement with the data on IGFBP-3 mRNA (Fig. 2), there was a marked decrease in IGFBP-3 concentration in the medium conditioned by anti-sense clones compared to vector controls. The magnitude of decrease in anti-sense clone #0 was 77 ± 7%. There were no statistical differences in IGFBP-3 concentrations between the two anti-sense clones. These anti-sense cells synthesized and secreted more IGFBP-6 and less IGFBP-2 than the vector controls. There were no changes in IGFBP-4 secretion in the anti-sense clones compared to the vector controls. Immunoblot analysis of medium conditioned by clones transfected with the anti-sense IGFBP-3 construct with an anti-IGFBP-3 antibody confirmed that there was a marked decrease in IGFBP-3 secretion by these clones (Fig. 4).

Growth curves of Caco-2 cell clones transfected with an anti-sense IGFBP-3 expression construct As shown in Fig. 5A, when anti-sense IGFBP-3 clones and the pooled vector control were cultured in serum-free medium, both of them grew at the same rate until the eighth day of culture. Between days 8 and 10, the anti-sense IGFBP-3 clones grew faster than the pooled control clones. The pooled vector controls and anti-sense clone #4 did not grow between days 10 and 12, but anti-sense clone #0 grew substantially during this period. Consequently, the cell number of two anti-sense IGFBP-3 clones was much higher than that of the pooled control clones at day 12 of culture. Basically, the same results were obtained when these clones were cultured in medium containing 10% FBS (Fig. 5B). Clone #4 proliferated at the same rate as the control until the eighth day of culture and then grew faster than the pooled control clones. Again, anti-sense IGFBP-3 clone #4 reached higher final cell densities compared to the pcDNA3 control. There were no differences in the rate of cell proliferation between clone #0 and clone #4 in the medium containing FBS (data not shown).

Expression of sucrase-isomaltase mRNA by Caco-2 cell clones transfected with an anti-sense IGFBP-3 expression construct Sucrase-isomaltase mRNA was determined as a marker of Caco-2 cell differentiation (Pinto *et al.*, 1983). Northern blot analysis was performed on total RNA isolated from Caco-2 cell clones cultured for 9 days. The levels of sucrase-isomaltase mRNA were 50% lower in clones transfected with an anti-sense IGFBP-3 construct compared to the pcDNA3 controls (Fig. 6)

Discussion

The results presented herein suggest that IGFBP-3 inhibits the proliferation and stimulates the differentiation of Caco-2 cells. Anti-sense IGFBP-3 clone #4 grew at a rate similar to that of control cells until the eighth day of culture, and then growth was arrested at a higher density. In addition, anti-sense IGFBP-3 clone #0 did not reach the growth-arresting density at 10 days. This is in agreement with the

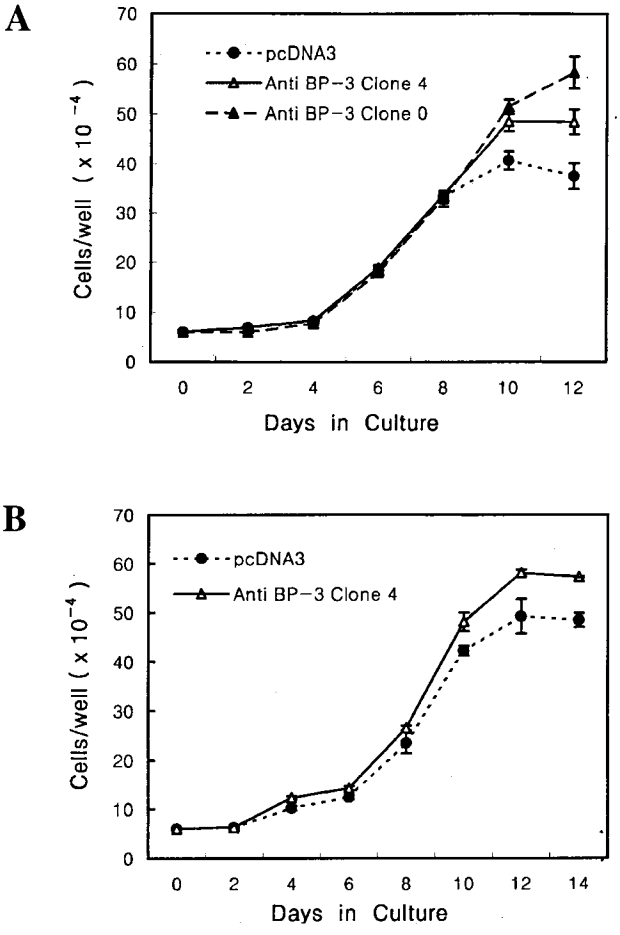

Fig. 5. Growth curves of Caco-2 cells stably transfected with pcDNA3 or the anti-sense IGFBP-3 expression construct. Cells were plated in 12-well plates at 6×10^4 cells/well in DMEM/F12 supplemented with 10% FBS. A. One day later, the monolayers were rinsed three times with serum-free DMEM/F12 supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. B. Cells were grown continuously in DMEM/F12 supplemented with 10% FBS. In both A and B, the medium was replaced every two days. Cell numbers were estimated by MTT assay, and represent mean ± SEM, $n = 4$.

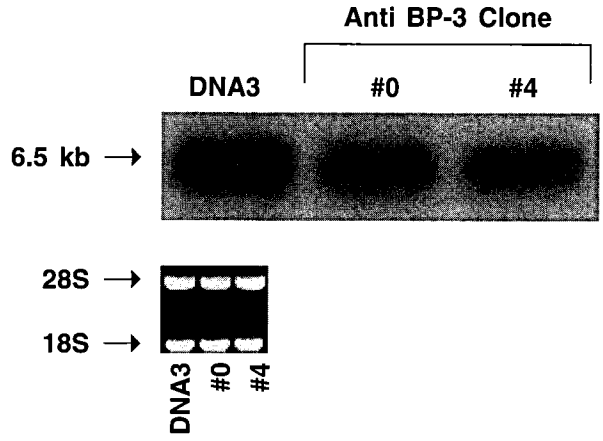

Fig. 6. Sucrase-isomaltase mRNA levels in Caco-2 cells stably transfected with pcDNA3 (DNA3) or the anti-sense IGFBP-3 expression construct (anti BP-3 clones #0 and #4). Cells were seeded at a density of 6×10^5 cells in 100-mm dishes and cultured for 9 days. Total RNA was isolated for Northern blot analysis of sucrase-isomaltase mRNA utilizing methods as described in the legend to Fig. 2

results of Cohen *et al.* (1993) that Balb/c 3T3 fibroblasts overexpressing IGFBP-3 growth-arrested at a lower density than controls. The steady-state levels of sucrase-isomaltase mRNA as a marker of Caco-2 cell differentiation were lower in clones with reduced secretion of IGFBP-3.

The mechanisms that regulate this aspect of Caco-2 cell growth were not investigated in the present study. IGFBP-3 can regulate Caco-2 cell proliferation and differentiation by IGF-dependent or IGF-independent mechanisms (Rechler, 1998). In serum-free medium, endogenously-produced IGFBP-3 inhibits the autocrine mitogenic action of endogenously-produced IGF-II by a sequestering mechanism that reduces IGF-II availability for binding to the IGF-I receptor. In the present study, more IGF-II may have been available for binding to the IGF-I receptor in the medium with the anti-sense clones where there were decreased levels of IGFBP-3 to bind endogenously-produced IGF-II. In serum-containing medium, the balance among serum-derived and endogenously-produced IGFs and IGFBPs may have been disturbed by decreased concentrations of IGFBP-3 in conditioned medium, so that IGFs arising from both endogenous and exogenous sources were more available for binding to the IGF-I receptor. Zarrilli *et al.* (1994) have shown previously that IGF-II operates through binding to the IGF-I receptor and that IGF-II is an autocrine growth factor for Caco-2 cells. The relationship between endogenous IGF-II expression and induction of differentiation in Caco-2 cells has been studied by several investigators (Zarrilli *et al.*, 1994; 1996; Zhang *et al.*, 1995; Park *et al.*, 1996). Accumulation of IGF-II mRNA and IGF-II peptide decreases as expression of differentiation markers increases, leading to the hypothesis that down-regulation of the autocrine IGF-II-IGF-I receptor growth stimulatory loop is necessary for differentiation. This hypothesis has been supported by recent findings that treatment of preconfluent Caco-2 cells with the IGF-I receptor-blocking antibody αIR-3 decreased autonomous cell proliferation by 40% and induced differentiation, as assessed by an increase in sucrase-isomaltase activity (Zarrilli *et al.*, 1996). Zarrilli *et al.* (1996) also observed that sustained expression of IGF-II resulted in inhibition of apolipoprotein A-I and sucrase-isomaltase mRNA expression. A stimulatory role for IGFBP-3 in Caco-2 cell differentiation is suggested by the inverse correlation between Caco-2 cell growth and the

rise in IGFBP-3 secretion by cells during the differentiation program (Oguchi *et al.,* 1994; Hoeflich *et al.,* 1996; Park *et al.,* 1996). As shown in Fig. 1, the increase in IGFBP-3 mRNA preceded the increase in sucrase-isomaltase mRNA, a marker for Caco-2 cell differentiation. Thus, one possible interpretation of our data in the present study is that the decrease in sucrase-isomaltase mRNA in the anti-sense clones was caused by the availability of more IGFs to the IGF-I receptor due to decreased IGFBP-3. Therefore, our working hypothesis is that IGFBP-3 works by the IGF-dependent mechanism to inhibit proliferation and stimulate differentiation of these cells. We speculate that increasing concentrations of IGFBP-3 in the medium of control cells reached sufficient levels to sequester substantial amounts of IGF-II after day 9 of culture, at which time the proliferative rate of those cells declined. At that time, the decrease in IGFBP-3 by transfection with an anti-sense IGFBP-3 expression construct may have prevented the sequestration of IGF-II, resulting in higher cell numbers in the anti-sense clones.

One can speculate that IGFBP-3 may bind to the putative IGFBP-3 receptor (Leal *et al.,* 1997) which induces Caco-2 cell differentiation, and differentiated cells lose their ability to proliferate. Compared to pcDNA3 cells, the anti-sense IGFBP-3 clones produced less IGFBP-2 and more IGFBP-6, which are characteristics of less-differentiated Caco-2 cells (Oguchi *et al.,* 1994; Park *et al.,* 1996). Future studies are needed to ascertain whether Caco-2 cells have the IGFBP-3 receptor and whether antibody against this receptor can prevent IGFBP-3 effects on Caco-2 cell proliferation and differentiation.

In summary, we have demonstrated that clones stably transfected with an anti-sense IGFBP-3 expression construct grew normally but reached cell densities higher than control cells. These anti-sense IGFBP-3 clones expressed less sucrase-isomaltase mRNA compared to the vector controls. The results indicate that IGFBP-3 inhibits proliferation and stimulates differentiation of Caco-2 cells.

Acknowledgments This work was supported by a grant from the Korea Science and Engineering Foundation (#961-0713-086-2). The author thanks Dr. Youngman Oh for the donation of IGFBP-3 antibody and Drs. B. Fennie, D. Swallow, and S. Shimasaki for their donations of cDNA.

References

Aaronson, S. A. (1991) Growth factors and cancer. *Science* **254**, 1146–1153.

Baxter, R. C., Binoux, M. A., Clemmons, D. R., Conover C. A., Drop, L. S., Holly, J. M. P., Mohan, S., Oh, Y. and Rosenfeld, R. G. (1998) Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. *Endocrinology* **139**, 4036.

Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, F. R. and Kley, N. (1995) Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377**, 656–649.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Cohen, P., Lamson, G., Okajima, T. and Rosenfeld, R. G. (1993) Transfection of the human insulin-like growth factor binding protein-3 gene into Balb/c fibroblasts inhibits cell growth. *Mol. Endocrinol.* **7**, 380–386.

Cohick, W. S. and Clemmons, D. R. (1993) The insulin-like growth factors. *Annu. Rev. Physiol.* **55**, 131–153.

Conover, C. A. (1992) Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: Studies of underlying mechanism. *Endocrinology* **130**, 3191–3199.

Daughaday, W. H. and Rotwein, P. (1987) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocrine Rev.* **10**, 68–91.

Denizot, F. and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival: modification of the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.* **89**, 271–277.

Green, F., Edwards, Y., Hauri, H.-P., Povey, S., Ho, M. W., Pinto, M. and Swallow, D. (1987) Isolation of a cDNA probe for a human jejunal brush-border hydrolase, sucrase-isomaltase, and assignment of the gene to chromosome 3. *Gene* **57**, 101–110.

Guo, Y.-S., Narayan, S., Yallampalli, C. and Singh, P. (1992) Characterization of insulin-like growth factor I receptors in human colon cancer. *Gastroenterology* **102**, 1102–1108.

Guo, Y.-S., Jin, G.-F., Townsend, C. M. Jr., Zhang, T., Sheng, H.-M., Beauchamp, R. D. and Thompson, J. C. (1995) Insulin-like growth factor-II expression in carcinoma in colon cell lines: implications for autocrine actions. *J. Am. Coll. Surg.* **181**, 145–154.

Hoeflich, A., Yang, Y., Huber, S., Rascher, W., Koepf, G., Blum, W. F., Heinz-Erian, P., Kolb. H. J. and Kiess, W. (1996) Expression of IGFBP-2, -3, and -4 mRNA during differentiation of Caco-2 colon epithelial cells. *Am. J. Physiol.* **271**, E922–E931.

Jones, J. I. and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Rev.* **16**, 3–34.

Lambert, S., Vivario, J., Boniver, J. and Gol-Winkler, R. (1990) Abnormal expression and structural modification of the insulin-like growth factor II gene in human colorectal tumors. *Int. J. Cancer* **46**, 405–410.

Lamonerie, T., Lavialle, C., Haddada, H. and Brison, O. (1995) IGF-2 autocrine stimulation in tumorigenic clones of a human colon-carcinoma cell line. *Int. J. Cancer* **61**, 587–592.

Leal, S. M., Liu, Q., Huang, S. S. and Huang, J. S. (1997) The type V transforming growth factor β receptor is the putative insulin-like growth factor-binding protein 3 receptor. *J. Biol. Chem.* **272**, 20572–20576.

Macauley, V. M. (1994) Insulin-like growth factors and cancer. *Br. J. Cancer* **65**, 311–320.

Oguchi, S., Walker, W. A. and Sanderson, I. R. (1994) Profile of IGF-binding proteins secreted by intestinal epithelial cells changes with differentiation. *Am. J. Physiol.* **267**, G843–G850.

Oh, Y., Muller, H. L., Lamson, G. and Rosenfeld, R. G. (1993a)

Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. *J. Biol. Chem.* **268**, 14964–14971.

Oh, Y., Muller, H. L., Pham, H. and Rosenfeld, R. G. (1993b) Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. *J. Biol. Chem.* **268**, 26045–26048.

Oh, Y., Muller, H. L., Lee, D. Y., Fielder, P. J. and Rosenfeld, R. G. (1993c) Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. *Endocrinology* **132**, 1337–1344.

Park, J. H. Y., McCusker, R. H., Vanderhoof, J. A., Mohammadpour, H., Harty, R. F. and MacDonald, R. G. (1992) Secretion of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 by intestinal epithelial (IEC-6) cells: implication for autocrine growth regulation. *Endocrinology* **131**, 1359–1368.

Park, J. H. Y., Corkins, M. R., Vanderhoof, J. A., Caruso, N. M., Hrbek, M. H., Schaffer, B. S., Slentz, D. H., McCusker, R. H. and MacDonald, R. G. (1996) Expression of insulin-like growth factor-II and insulin-like growth factor binding proteins during Caco-2 cell proliferation and differentiation. *J. Cell. Physiol.* **166**, 396–406.

Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simmon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Enterocyte differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47**, 323–330.

Rechler, M. M. (1993) Insulin-like growth factor binding proteins. *Vitam. Horm.* **47**, 1–114.

Rechler, M. M. (1997) Editorial: growth inhibition by insulin-like growth factor (IGF) binding protein-3 — what's IGF got to do with it? *Endocrinology* **138**, 2645–2647.

Singh, P., Dai, B., Yallampalli, U., Lu, X. and Schroy, P. C. (1996) Proliferation and differentiation of a human colon cancer cell line (Caco2) is associated with significant changes in the expression of insulin-like growth factor (IGF) IGF-II and IGF binding protein-4: role of IGF-II. *Endocrinology* **137**, 1764–1774.

Tricoli, J. V., Rall, L. B., Karakousis, C. P., Herrera, L., Petrelli, N. J., Bell, G. I. and Shows, T. B. (1986) Enhanced levels of insulin-like growth factor messenger RNA in human colon carcinomas and liposarcomas. *Cancer Res.* **46**, 6169–6173.

Velentinis, B., Bhala, A., DeAngelis, T., Baserga, R. and Cohen, P. (1995) The human insulin-like factor (IGF) binding protein-3 inhibits the mitogenic action of fibroblast growth factor on mouse fibroblasts with a targeted disruption of the type I IGF receptor gene. *Mol. Endocrinol.* **9**, 361–367.

Westley, B. R. and May, F. E. B. (1995) Insulin-like growth factors: the unrecognised oncogenes. *Br. J. Cancer* **72**, 1065–1066.

Wood, W. I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spencer, S. A., Hellmiss, R., Martin, J. L. and Baxter, R. C. (1988) Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. *Mol. Endocrinol.* **2**, 1176–1185.

Zarrilli, R., Pignata, S., Romano, M., Gravina, A., Casola, S., Bruni, C. B. and Acquaviva, A. M. (1994) Expression of insulin-like growth factor (IGF)-II and IGF-I receptor during proliferation and differentiation of Caco-2 human colon carcinoma cells. *Cell Growth Differ.* **5**, 1085–1091.

Zarrilli, R., Romano, M., Pignata, S., Casola, S., Bruni, C. B. and Acquaviva, A. M. (1996) Constitutive insulin-like growth factor-II expression interferes with the enterocyte-like differentiation of Caco-2 cells. *J. Biol. Chem.* **271**, 8108–8114.

Zhang, Y., Wick, D. A., Seetharam, B. and Dahm, N. M. (1995) Expression of IGF-II and IGF binding proteins in differentiating human intestinal Caco-2 cells. *Am. J. Physiol.* **269**, E804–E813.