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PRINCIPAL INVESTIGATOR: Ron G. Rosenfeld, M.D.

CONTRACTING ORGANIZATION: Oregon Health Sciences University
Portland, Oregon 97201-3098

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Ron G. Rosenfeld, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health Sciences University
Portland, Oregon 97201-3098

E-Mail: rosenfer@ohsu.edu

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The insulin-like growth factors (IGFs) are potent mitogens for normal and cancerous prostatic cells. The IGFs are found complexed to IGF binding proteins (IGFBPs) which modulate IGF bioactivity, but also may themselves act in an IGF-independent manner. We have recently characterized a series of IGFBP-related proteins (IGFBP-rPs) which share homology with the IGFBPs in the amino-terminus, bind IGFs with low affinity, and regulate cell growth through both IGF-dependent and IGF-independent actions. This grant is directed at the study of IGFBP-rP2 (also known as connective tissue growth factor) as a regulator of normal and malignant prostatic growth. The specific aims are to: 1) analyze IGFBP-rP2 mRNA and protein expression and distribution in normal and malignant prostatic tissues; 2) determine the transcriptional, translational and post-translational regulation of IGFBP-rP2; and 3) determine the mechanisms by which IGFBP-rP2 regulates prostate growth.

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INTRODUCTION:

The insulin-like growth factors (IGFs) are potent mitogens for normal and cancerous prostatic cells. The IGFs are found complexed to IGF binding proteins (IGFBPs) which modulate IGF bioactivity, but also may themselves act in an IGF-independent manner. We have recently characterized a series of IGFBP-related proteins (IGFBP-rPs) which share homology with the IGFBPs in the amino-terminus, bind IGFs with low affinity, and regulate cell growth through both IGF-dependent and IGF-independent actions. This grant is directed at the study of IGFBP-rP2 (also known as connective tissue growth factor) as a regulator of normal and malignant prostatic growth. The specific aims are to: 1) analyze IGFBP-rP2 mRNA and protein expression and distribution in normal and malignant prostatic tissues; 2) determine the transcriptional, translational and post-translational regulation of IGFBP-rP2; and 3) determine the mechanisms by which IGFBP-rP2 regulates prostate growth.

BODY:

IGFBP-rP2 (also known as connective tissue growth factor) was originally isolated from human umbilical endothelial cells and shown to be mitogenic and chemotactic for fibroblasts (1). IGFBP-rP2 belongs to the CCN (for CYR61, CTGF, and nephroblastoma overexpressed, NOV) family of cysteine-rich proteins involved in a diversity of cellular functions, such as mitogenesis, differentiation, survival, adhesion, migration, and regulation of matrix gene expression (2). IGFBP-rP2 is a major downstream effector of TGF-beta in fibroblasts, where it is regulated through a novel response element.

We have evaluated the expression of IGFBP-rP2 in a wide variety of normal and malignant prostate cell lines (3). In addition to the well-established androgen-dependent (LcaP) and androgen-independent (DU145 and PC-3) cell lines, we studied primary cultures of normal human prostate epithelial cells (HPEC), which were, in turn, transformed with the SV40-T antigen to obtain the low tumorigenic P69 cells and the highly tumorigenic and metastatic M12 cells. IGFBP-rP2 mRNA was detectable in all but one cell line as a single 2.4-kb band, consistent with the molecular size observed in most studies (4). IGFBP-rP2 protein was present in conditioned media and in cell lystaes from these cultures. IGFBP-rP2 was up-regulated with increasing passage numberin HPEC, with concentrations of both mRNA and secreted protein increased more than 5-fold at late, compared with early passage. Cell-cycle inhibitors known to be modified during cellular sensescence, such as $p21^{WAF1}$ and $p16^{INK4a}$, were evaluated, with little change in the former, but marked increases in the latter identified at late passage.

Because TGF-beta, all-trans retinoic acid (atRA) and IGF-I are important regulators of prostate epithelial growth and survival, we investigated their effects on cell proliferation and IGFBP-rP2 expression. Both TGF-beta and atRA caused a dose-dependent inhibition of cellular growth in the normal HPEC, whereas only TGF-beta was inhibitory in PC-3 cells. Paralleling the inhibition of proliferation of HPEC by TGF-beta and atRA, there was a dose-dependent increase in the concentration of both IGFBP-rP2 protein and steady-state mRNA. A maximum protein response equivalent to a 6-fold increase over the non-treated cells was observed at a dose of TGF-beta of 5

ng/ml, whereas 1 uM atRA caused an increase of IGFBP-rP2 mRNA and protein of about 4-fold over basal levels. Importantly, IGFBP-rP2 was also regulated by IGF-I in HPEC, both at the protein and at the mRNA level, with a 90% reduction of both secreted protein and steady-state mRNA upon treatment with IGF-I at a dose of 100 ng/ml. This effect of IGF-I was accompanied by a significant increase in cell proliferation.

Several lines of research support a role for IGFBP-rP2 in tumor suppression. We have shown previously that IGFBP-rP2 is up-regulated by TGF-beta1 in the breast cancer cell line Hs578T (5), and IGFBP-rP2 induces apoptosis in the estrogen receptor-positive breast cancer cell line, MCF-7 (6). It is, thus, noteworthy that IGFBP-rP2 is up-regulated by TGF-beta and atRA, and down-regulated by IGF-I in prostatic epithelial cells. We speculate that, in the human prostate, IGFBP-rP2 is a downstream effector of growth inhibitors and that IGFBP-rP2 expression must be down-regulated by growth factors, to support cellular proliferation. Similarly, IGFBP-rP2 could be a downstream effector in the mechanisms leading to cellular senescence of prostatic epithelial cells.

Since dietary factors are known to play an important role in the development and prevention of human cancers, we studied the effects of sodium butyrate, a major end-product of dietary starch and fiber, produced naturally during digestion by anaerobic bacteria in the caecum and colon (7). Initial studies were performed in two breast cancer cell lines (MCF-7 and Hs578T), and have been extended recently to PC-3 and LNCaP prostate cancer cells. Treatment of cells with sodium butyrate induces histone hyperacetylation and apoptosis, and specifically up-regulates expression of IGFBP-3 and IGFBP-rP2, at both an mRNA and protein level. These studies represent the first demonstration that butyrate regulates the IGFBP system in mammary and prostate cancer cells, and provide an impoirtant potential mechanism of action for this dietary product. Additional studies will be required to extend the evaluation of butyrate to a variety of normal and malignant prostate cell lines, and to evaluate the mechanism by which butyrate transcriptionally regulates IGFBP-rP2 expression.

KEY RESEARCH ACCOMPLISHMENTS:

* First characterization of IGFBP-rP2 mRNA and protein expression in normal and malignant prostatic cells.

* First demonstration of up-regulation of IGFBP-rP2 in the prostate by TGF-beta and all-trans retinoic acid, and down-regulation by IGF-I.

* First demonstration of regulation of IGFBP-rP2 mRNA and protein expression by sodium butyrate.

REPORTABLE OUTCOMES:

1. Lopez-Bermejo A, Buckway CK, Devi GR, Hwa V, Plymate SR, Oh Y, Rosenfeld RG:

Characterization of insulin-like growth factor binding protein-related proteins (IGFBP-rPs) 1, 2, and 3 in human prostate epithelial cells: potential roles for IGFBP-rP1 and 2 in senescence of the prostatic epithelium. Endocrinology 141:4072-4080, 2000.

2. Tsubaki J, Choi W-K, Ingermann AR, Twigg SM, Kim H-S, Rosenfeld RG, Oh Y: Effects of sodium butyrate on expression of members of the insulin-like growth factor binding protein superfamily in human mammary epithelial cells. J Endocrinol (in press).

3. Cohen P, Clemmons DR, Rosenfeld RG: Does the GH-IGF axis play a role in cancer pathogenesis? Growth Horm IGF Res 10:297-305, 2000.

CONCLUSIONS:

These studies have demonstrated that IGFBP-rP2 is widely expressed in prostatic cells, and that regulation of gene expression and protein levels is consistent with its role as a putative tumor suppressor. Thus, TGF-beta, all-trans retinoic acid, and sodium butyrate, all known to act in an anti-proliferative way in prostate cancer, up-regulate IGFBP-rP2 expression, while IGF-I, a potent mitogen for the prostate, dramatically suppresses IGFBP-rP2 expression. Future studies will extend our understanding of IGFBP-rP2 gene expression in the prostate, and evaluate its mechanism of action.

"So what": Prostate carcinogenesis involves the loss of normal growth regulatory machinery, and the IGF system is known to play an important role in the complex system of growth factors and hormones which regulate prostatic proliferation. IGFBP-rP2 regulation by known modulators of prostatic cell growth is consistent with its role as a tumor suppressor in the prostate.

REFERENCES:

1. D.M. Bradham et al., J Cell Biol 114, 1285 (1991)
2. P. Bork, FEBS Lett 327, 125 (1993)
3. A. Lopez-Bermejo et al., Endocrinology 141, 4072 (2000)
4. D.R. Brigstock, Endocr Rev 20, 189 (1999)
5. D-H Yang et al., J Clin Endocrinol Metab 83, 2593 (1998)
6. K. Hishikawa et al., J Biol Chem 274, 37461 (1999)
7. J Tsubaki et al., J Endocrinol (in press)

APPENDICES: Attached.

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Characterization of Insulin-Like Growth Factor-Binding Protein-Related Proteins (IGFBP-rPs) 1, 2, and 3 in Human Prostate Epithelial Cells: Potential Roles for IGFBP-rP1 and 2 in Senescence of the Prostatic Epithelium*

A. LÓPEZ-BERMEJO†, C. K. BUCKWAY, G. R. DEVI, V. HWA, S. R. PLYMATE, Y. OH, AND R. G. ROSENFELD

Department of Pediatrics (A.L.-B., C.K.B., G.R.D., V.H., Y.O., R.G.R.), Oregon Health Sciences University, Portland, Oregon 97201; and Geriatric Research Education and Clinical Center (S.R.P.), Veterans Affairs Health Care System Puget Sound, Seattle/Tacoma, Washington 98493

ABSTRACT

Insulin-like growth factor (IGF)-binding protein (IGFBP)-related proteins (IGFBP-rPs) are newly described cysteine-rich proteins that share significant aminoterminal structural similarity with the conventional IGFBPs and are involved in a diversity of biological functions, including growth regulation. IGFBP-rP1 (MAC25/Angiomodulin/prostacyclin-stimulating factor) is a potential tumor-suppressor gene that is differentially expressed in meningiomas, mammary and prostatic cancers, compared with their malignant counterparts. We have previously shown that IGFBP-rP1 is preferentially produced by primary cultures of human prostate epithelial cells (HPECs) and by poorly tumorigenic P69SV40T cells, compared with the cancerous prostatic LNCaP, DU145, PC-3, and M12 cells. We now show that IGFBP-rP1 increases during senescence of HPEC.

IGFBP-rP2 (also known as connective tissue growth factor), a downstream effector of transforming growth factor (TGF)-β and modulator of growth for both fibroblasts and endothelial cells, was detected in most of the normal and malignant prostatic epithelial cells tested, with a marked up-regulation of IGFBP-rP2 during senescence of HPEC. Moreover, IGFBP-rP2 noticeably increased in response to TGF-β1 and all-*trans* retinoic acid (atRA) in HPEC and PC-3 cells, and it decreased in response to IGF-I in HPEC.

IGFBP-rP3 [nephroblastoma overexpressed (NOV)], the protein product of the NOV protooncogene, was not detected in HPEC but was expressed in the tumorigenic DU145 and PC-3 cells. It was also synthesized by the SV40-T antigen-transformed P69 and malignant M12 cells, where it was down-regulated by atRA.

These observations suggest biological roles of IGFBP-rPs in the human prostate. IGFBP-rP1 and IGFBP-rP2 are likely to negatively regulate growth, because they seem to increase during senescence of the prostate epithelium and in response to growth inhibitors (TGF-β1 and atRA). Although the data collected on IGFBP-rP3 in prostate are modest, its role as a growth stimulator and/or protooncogene is supported by its preferential expression in cancerous cells and its down-regulation by atRA. (*Endocrinology* **141:** 4072–4080, 2000)

THE INSULIN-LIKE growth factor (IGF) system is composed of two ligands (IGF-I and IGF-II), six IGF-binding proteins (IGFBPs -1 to -6), and two receptors (type 1 and type 2 IGF receptors) (1). Recently, the IGFBP family of proteins has been expanded to include additional members that share significant structural similarities, the so-called IGFBP-related proteins (IGFBP-rPs) (2), which not only share the conserved aminoterminal domain of the IGFBPs but also show some degree of affinity for IGFs and insulin.

Abnormalities in the IGF system have been identified in prostate disease, such as prostate hyperplasia and cancer. In this respect, prostate cancer growth seems to be poorly dependent on IGFs, because the type I IGF receptor, which mediates most biological functions of IGF-I and IGF-II, is down-regulated during prostate carcinogenesis (3). Additionally, recent epidemiological studies have shown an increased risk of developing prostatic carcinoma in adult males with high-normal serum concentrations of IGF-I (4).

Despite extensive characterization of the IGFBPs in the human prostate, little is known about their roles in prostate physiology. IGFBP-2 and -4 are known to increase during carcinogenesis of the prostatic epithelium (5, 6), whereas IGFBP-3 is a proteolytic substrate for prostate-specific antigen (7, 8). Recent studies have also shown an increase of several IGFBPs in prostate tissue during involution of the gland in castrated rats and in men taking finasteride (9, 10). These findings suggest that IGFBPs may play a role in apoptosis of prostate cells, either by sequestering IGFs or by direct cellular actions. Our knowledge of the new IGFBP-rPs in the human prostate is only scant at its best.

The IGFBP-rPs are cysteine-rich proteins involved in a diversity of biological functions, including growth regula-

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Address all correspondence and requests for reprints to: Ron G. Rosenfeld, M.D., Department of Pediatrics, School of Medicine, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201-3098. E-mail: rosenfer@ohsu.edu.

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Western immunoblot analyses

Conditioned media and total cell lysates, using RIPA buffer [150 mM NaCl, 20 mM HEPES (pH 7.4), 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Québec, Canada)], from both treated and untreated (control) cells, were normalized for protein concentration, using a DC protein assay (Bio-Rad Laboratories, Inc.). Equal amounts of total protein per sample were dissolved in nondenaturing SDS sample buffer [0.5 M Tris (pH 6.8), 1% SDS, 10% glycerol, and bromphenol blue] and boiled for 5 min. Samples were electrophoresed on 15% SDS-polyacrylamide gels, then electroblotted onto nitrocellulose, and membranes blocked with 4% milk-TBS-T [Tris-buffered saline-Tween-20 (0.1%)] for 1 h at 22 C. Western blots were incubated with IGFBP-rP1, -rP2, or -rP3 antisera at a 1:3000 dilution and with $p16^{INK4a}$ IgG antibody at a dilution of 1:500 (1 μg/ml) in TBS-T overnight at 4 C. Blots were washed with TBS-T and then incubated for 1 h at 22 C with a 1:3000 dilution of horseradish peroxidase-linked antirabbit or antimouse IgG secondary antibodies. Proteins of interest were detected with ECL chemiluminescence reagents, according to the manufacturer's protocol.

RNA analyses

Total cytoplasmic RNA was isolated from cells, by use of RNeasy (QIAGEN, Inc., Chatsworth, CA). Twenty micrograms of each RNA preparation were electrophoresed on a 1.2% agarose-2.2 M formaldehyde gel, transferred overnight onto a nylon membrane (GeneScreen), using 10 × SSC as the transfer solution, and cross-linked to the membrane by UV irradiation in a Stratalinker 1800 (Stratagene). The Northern blots were then probed with an EcoRI/Xho fragment of IGFBP-rP1 (27), or a BamHI/Xho fragment of IGFBP-rP2 (28), which were radiolabeled (1×10^9 dpm/μg) with [α-^{32}P]deoxycycidine triphosphate (NEN Life Science Products-DuPont; SA, 3000 Ci/mmol) using a random priming kit (Prime-a-Gene, Promega Corp.). Northern blots were hybridized overnight at 65 C in hybridization buffer (Rapid-Hyb, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Blots were then washed for 15 min in 2 × SSC/0.1% SDS at 22 C, followed by two more stringent washes in 0.2 × SSC/0.1% SDS at 65 C for 15 min. Blots were exposed to Kodak Biomax film (Eastman Kodak Co., Rochester, NY) for 12 to 48 h at −70 C, using one intensifying screen. Membranes were then reprobed with 18S ribosomal RNA, which acted as a loading control for the RNA samples. An image analyzer (GS-700) equipped with MultiAnalyst version 1.0.2 Software (Bio-Rad Laboratories, Inc.) was used to quantify the resulting bands.

RT-PCR

RT-PCR was performed using 5′-CGCGAATTCGCCATGCAGAGTGTGCAGAGCACG-3′ and 5′-GGGGCTCGAGTTACATTTTCCCTCTGGTAGTC-3′ primers specific for IGFBP-rP3. One microgram of total RNA from each cell line was reversed transcribed in a vol of 20 μl, by use of Reverse Transcription System Kit (Promega Corp.), following the manufacturer's instructions. The reaction was performed at 42 C for 15 min, denatured at 99 C for 5 min, and placed on ice. One microliter of the mixture and 50 pmol of 5′ and 3′ primers were employed in PCR amplification reactions using Advantage GC cDNA PCR Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). Amplification of the cDNA was carried out with 25 cycles of denaturing at 94 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min. One negative and one positive control were included in all reactions.

Statistical analyses

All experiments were performed at least twice. Statistical analyses were performed by a two-tail Student's *t* test, assuming unequal variances, using Excel Data Analysis Software (Microsoft Corp., Redmond, WA). Data are expressed as means ± SE. $P < 0.05$ was considered significant.

Results

Expression of IGFBP-rP1, 2, and 3 in HPECs

In agreement with previous observations (16), IGFBP-rP1 messenger RNA (mRNA) was detected by Northern blot analysis in the P69/M12 lineage and in primary cultures of prostate epithelial cells, with a parallel detection of IGFBP-rP1 in conditioned media from these cultures (Fig. 2). In the malignant LNCaP, DU145, and PC-3 cells, IGFBP-rP1 mRNA was undetectable by Northern blot, and an immunoreactive band was only present in cell lysates but not in conditioned media from these cells.

IGFBP-rP2 mRNA was detectable in all but one cell line as a single 2.4-kb band, consistent with the molecular size observed in most studies (29). IGFBP-rP2 protein was present in conditioned media and in cell lysates from these cultures. In contrast to IGFBP-rP1, both normal and malignant prostate epithelial cells express similar amounts of IGFBP-rP2 (Fig. 2).

IGFBP-rP3 message was evaluated by RT-PCR in only a limited number of cell lines but was identifiable in P69, M12, LNCaP, and PC-3 cells. IGFBP-rP3 protein was undetectable in conditioned media from HPEC, compared with readily detectable levels in the immortalized P69 cell line and malignant M12, DU145, and PC-3 cells (Fig. 2).

Expression of IGFBP-rP1 and 2 during senescence of HPEC

HPEC cells have a limited life span, with no more than 30 population doublings before they enter replicative senescence (corresponding to our 9th culture passage). Thus, early passages of HPEC (<4th) are highly replicative cells (duplication time for 4th passage is approximately 2 days), whereas senescent cells (>9th passage) are unable to replicate, and they die over time (Fig. 3).

Interestingly, both IGFBP-rP1 and 2 were up-regulated on increasing passage of these cells, with concentrations of both mRNA and secreted protein more than 5-fold higher at late passages, compared with early passages (Fig. 4, A and B).

In support of an increase of these proteins during senescence of HPEC, we also investigated the concentrations of cell-cycle inhibitors that are known to be modified during cellular senescence, such as $p21^{WAF1}$ and $p16^{INK4a}$ (30). In agreement with a previous report in primary cultures of prostate epithelial cells (31), $p21^{WAF1}$ was barely identifiable in our cell lysate preparations, either at early or at late passages (data not shown); whereas a marked up-regulation of $p16^{INK4a}$ at late, low-replicative passages was evidenced (6-fold increase, compared with early passages), paralleling the increases in IGFBP-rP1 and -rP2 (Fig. 4C).

IGFBP-rP2 is responsive to growth regulators in HPECs

Because TGF-β, atRA, and IGF-I are important regulators of prostate epithelial growth and survival (32–34) and because IGFBP-rP2 is tightly regulated by TGF-β in other cellular systems, we wished to investigate the effects of these growth inhibitors (TGF-β and atRA) and growth stimulator (IGF-I) on cell proliferation and IGFBP-rP2 expression in our normal and malignant prostate cells.

Both TGF-β and atRA caused a dose-dependent inhibition

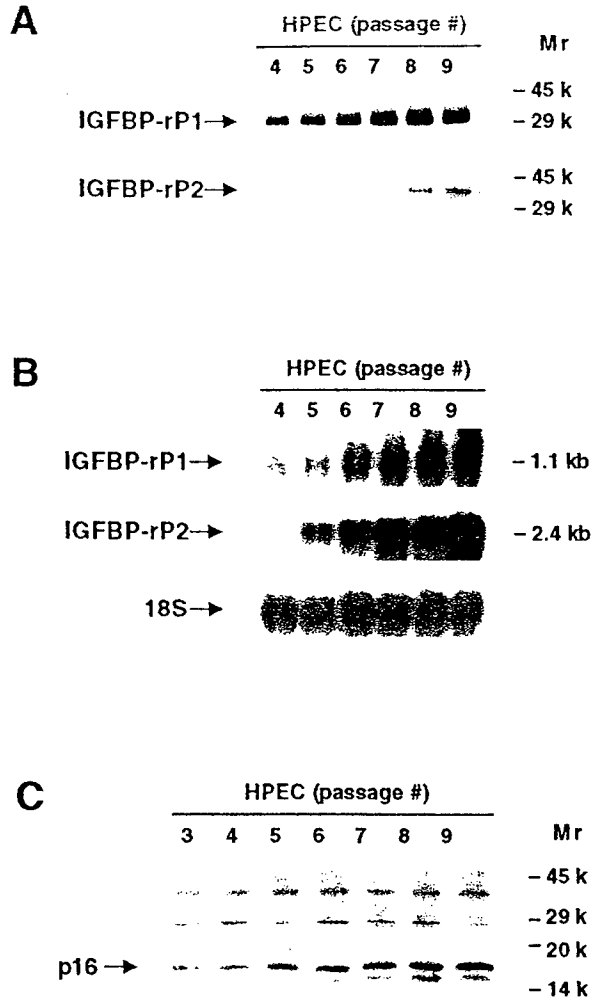

FIG. 4. Expression of IGFBP-rP1 and 2 during serial passages of HPEC. A, Western immunoblot studies of conditioned media from HPEC using polyclonal anti-IGFBP-rP1 and 2 antibodies. Three micrograms of total protein were loaded per lane. Both IGFBP-rP1 and 2 concentrations, in conditioned media, increased approximately 6-fold at late, low-replicative passages, compared with early ones. B, Northern blot analyses of same serial passages of HPEC using [^{32}P] radiolabeled IGFBP-rP1 and 2 probes. Twenty micrograms of total RNA were loaded per lane. Concentrations of 18S ribosomal RNA are shown as an internal control for loading. C, Western immunoblot studies of total cell lysates from HPEC using monoclonal anti-p16^{INK4a} antibody. Ten micrograms of total protein were loaded per lane. Note a marked up-regulation (~6-fold) of p16^{INK4a} concentrations at late, low-replicative passages, compared with early ones, as part of the senescence process of HPEC cells.

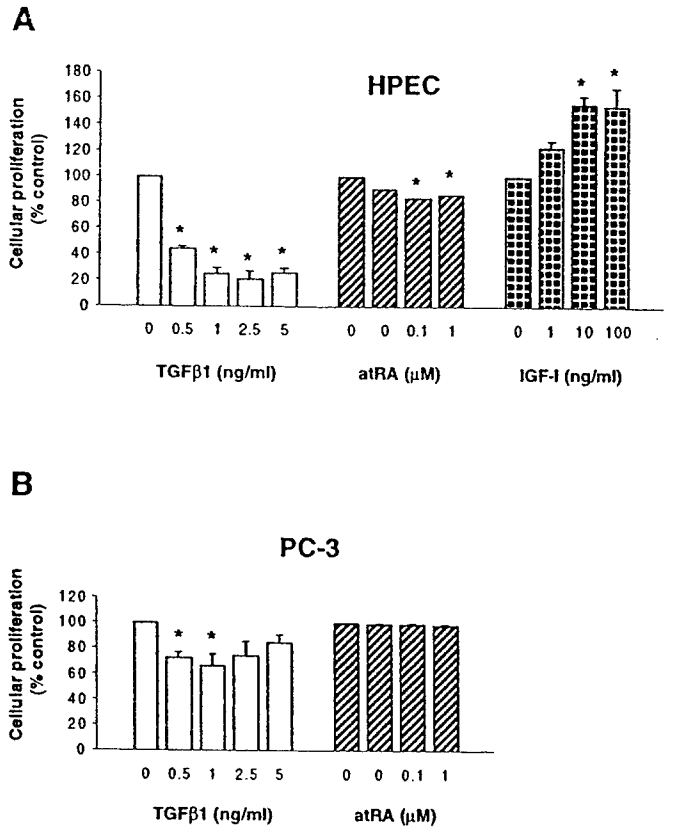

FIG. 5. Effects of growth regulators on cellular proliferation of HPEC and PC-3 cells. A, The growth rates of HPEC cultured with TGF-β, atRA, and IGF-I, for 48 h under serum-free conditions, were investigated by either [^{3}H]thymidine incorporation studies (TGF-β) or by MTS assay (atRA and IGF-I). Both TGF-β and atRA are inhibitory for HPEC, whereas IGF-I is stimulatory for these cells. B, Similarly, the effects of TGF-β and atRA were studied in the malignant PC-3 cells. Only TGF-β was inhibitory for PC-3 cells [atRA did not inhibit the growth of these cells, as judged by either MTS assay or by [^{3}H]thymidine incorporation studies (data not shown)]. Results are expressed as means ± SE of three independent studies for HPEC and two independent studies for PC-3 cells. *Asterisks* indicate statistical significance (compared with control).

rP2 concentrations were studied. TGF-β produced a significant increase (4-fold) in protein levels over nonstimulated values. Similarly, treatment with atRA, under the same experimental conditions, resulted in a 6-fold increase in IGFBP-rP2 concentrations (Fig. 7A). These changes did not, however, follow parallel increases in the concentrations of steady-state mRNA, as the levels of IGFBP-rP2 mRNA did not significantly change on TGF-β treatment and were only increased 2-fold by atRA (Fig. 7B).

In addition, we treated all of the other cell lines with the three above-mentioned growth regulators, but only small responses of IGFBP-rP2 were observed for TGF-β1, atRA, and IGF-I in P69; for TGF-β1 in M12; and for atRA in DU145 cells (data not shown).

IGFBP-rP3 is regulated in HPECs

IGFBP-rP3 has been previously shown to be expressed in prostate epithelial cells, but no regulation has been described, as yet, in these cultures (22). To better understand the role of this protein in the human prostate, we also investigated the effects of TGF-β, atRA, and IGF-I on the expression of IGFBP-rP3 *in vitro*.

IGFBP-rP3 was not produced by normal prostate epithelial cells, nor was it induced by any of the above-mentioned growth regulators in these cells. In the well-established PC-3 (and in DU145) cancer cells, IGFBP-rP3 was readily detected in conditioned media under serum-free incubation, but no significant regulation was observed in these cultures.

In the P69/M12 lineage, both TGF-β and atRA also produced a dose-dependent inhibitory effect on cellular proliferation (Fig. 8). Interestingly, atRA, but not TGF-β, down-

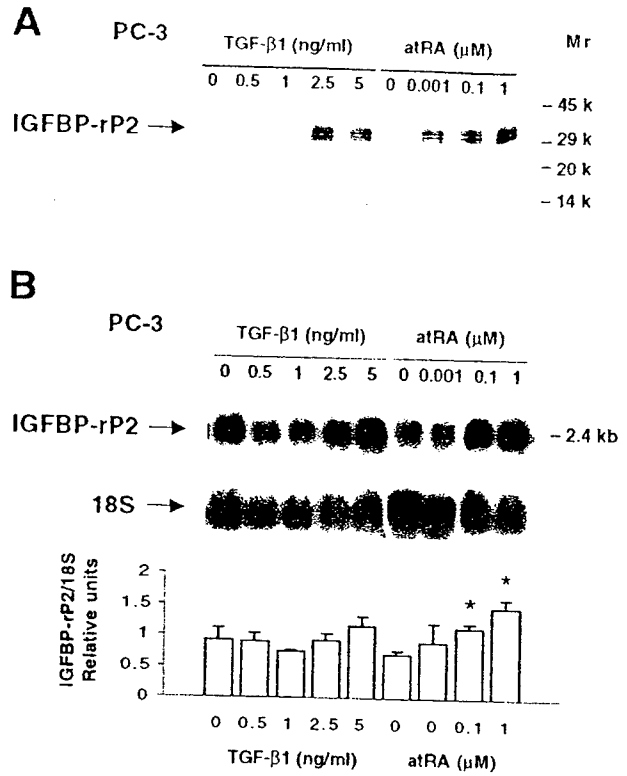

FIG. 7. IGFBP-rP2 is responsive to growth regulators in PC-3. A, Up-regulation of IGFBP-rP2 by the growth inhibitors TGF-β and atRA. Representative Western immunoblot of conditioned media by PC-3 using polyclonal anti-IGFBP-rP2 antibody. Cells were treated with increasing concentrations of TGF-β1 and atRA, for 48 h, in serum-free conditions. A 4-fold increase or greater was seen with both TGF-β1 and atRA in PC-3. B, Northern blot analysis for IGFBP-rP2 indicates only modest increases at the mRNA levels that were statistically different for atRA (graph shows the means ± SE of two independent experiments). *Asterisks* indicate statistical significance (compared with control).

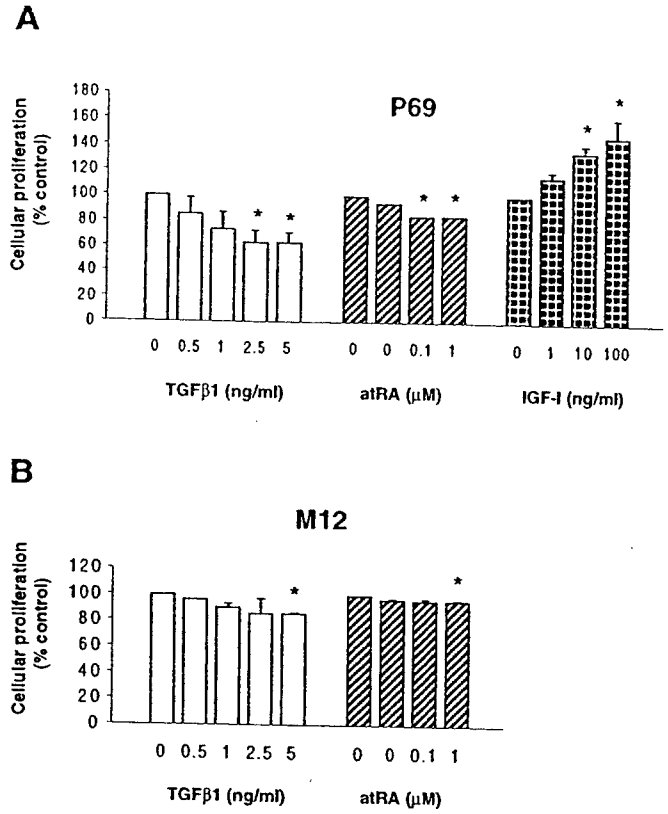

FIG. 8. Effects of growth regulators on cellular proliferation of P69 and M12 cells. A, The growth rates of P69 cultured with TGF-β, atRA, and IGF-I, for 48 h, under serum-free conditions, were investigated by either [^{3}H]thymidine incorporation studies (TGF-β) or by MTS assay (atRA and IGF-I). Both TGF-β and atRA are inhibitory, whereas IGF-I is stimulatory for P69 cells. B, Similarly, the effects of TGF-β and atRA were studied in the malignant M12 cells. Both TGF-β and atRA were inhibitory for M12 cells. Results are expressed as means ± SE of three independent studies for P69 cells and two independent studies for M12 cells. *Asterisks* indicate statistical significance (compared with control).

agreement with observations in human mammary cells, in that the concentrations of both IGFBP-rP1 mRNA and protein were markedly enhanced during replicative senescence of normal prostate epithelial cells. Thus, although the precise role of IGFBP-rP1 in the human prostate has, as yet, to be clarified, our results support the hypothesis that IGFBP-rP1 is a tumor suppressor gene and/or senescence factor.

Several lines of investigation also support roles of IGFBP-rP2 in growth regulation and tumorigenesis. IGFBP-rP2 mediates most of the biological actions of TGF-β in fibroblasts where a novel TGF-β response element in the IGFBP-rP2 promoter has been found (19). Additionally, IGFBP-rP2 is a growth factor for endothelial cells (29, 38). Supporting its role in tumorigenesis, IGFBP-rP2 was expressed by a chondrosarcoma-derived chondrocytic cell line and a fibrosarcoma cell line *in vitro* (29, 39) and has been found in the stromal component of breast and pancreatic cancer and desmoplastic melanomas (40–42). Additionally, it has been shown that IGFBP-rP2 is up-regulated by TGF-β1 in the breast cancer cell line Hs578T (25) and that IGFBP-rP2 induces apoptosis in the estrogen receptor-positive breast cancer cell line MCF-7 (43). These lines of evidence suggest that IGFBP-rP2 plays also a role in modifying the growth of stroma in desmoplastic tumors and in regulating the growth of breast cancer cells.

In this light, our results indicate that IGFBP-rP2 may also regulate the growth of normal and prostate cancer cells, because it is noticeably up-regulated by growth inhibitory factors, such as TGF-β and atRA, in normal and malignant cells, and down-regulated by growth-promoting factors, such as IGF-I, in HPEC. In the prostate, TGF-β is known to limit the proliferation and survival of the normal epithelium, an effect that is lost in malignant cells largely because of a down-regulation of TGF-β receptors during prostate carcinogenesis (44). Retinoids also exert potent inhibitory properties in the normal prostate and have been shown to be present at lower concentration in prostate cancer tissues (33, 45). Thus, both TGF-β and atRA play major roles in controlling the growth and survival of normal prostate cells and probably also in the development of prostate cancer. IGFs regulate also the proliferation of prostate cells (34). Interestingly, the expression of type I IGF-I receptor, which mediates most of the biological activities of IGFs, in markedly down-regulated during carcinogenesis (3). Thus, IGFs seem to play fundamental roles in the regulation of growth and malignant transformation of normal prostate epithelial cells. Because the above-mentioned growth regulators affected the concen-

insulin-like growth factor binding proteins 2, 3, 4, and 5. Endocrinology 139:807–810
10. **Thomas LN, Wright AS, Lazier CB, Cohen P, Rittmaster RS** 2000 Prostatic involution in men taking finasteride is associated with elevated levels of insulin-like growth factor-binding proteins (IGFBPs)-2, -4, and -5. Prostate 15:203–210
11. **Murphy M, Pykett MJ, Harnish P, Zang KD, George DL** 1993 Identification and characterization of genes differentially expressed in meningiomas. Cell Growth Differ 4:715–722
12. **Akaogi K, Okabe Y, Funahashi K, Yoshitake Y, Nishikawa K, Yasumitsu H, Umeda M, Miyazaki H** 1994 Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. Biochem Biophys Res Commun 198:1046–1053
13. **Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K, Miyazaki K** 1996 Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. Proc Natl Acad Sci USA 93:8384–8389
14. **Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H** 1994 Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 303:591–598
15. **Burger A, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M, Papas T, Seth A** 1998 Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. Oncogene 16:2459–2467
16. **Hwa V, Tomasini-Sprenger C, López-Bermejo A, Rosenfeld RG, Plymate SR** 1998 Characterization of insulin-like growth factor binding protein-related protein 1 in prostate cells. J Clin Endocrinol Metab 83:4355–4362
17. **Bradham DM, Igarashi A, Potter RL, Grotendorst GR** 1991 Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 114:1285–1294
18. **Bork P** 1993 The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327:125–130
19. **Grotendorst GR, Okochi H, Hayashi N** 1996 A novel transforming growth factor β response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ 7:469–480
20. **Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, Perbal B** 1992 Proviral rearrangements and overexpression of a new cellular gene (NOV) in myeloblastosis-associated virus type 1-induced nephroblastomas. Mol Cell Biol 12:10–21
21. **Martinerie C, Huff V, Joubert I, Badzioch M, Saunders G, Strong L, Perbal B** 1994 Structural analysis of the human NOV proto-oncogene and expression in Wilms tumor. Oncogene 9:2729–2723
22. **Tatoud R, Padilla A, Sethia K, Gibson I,** The novH proto-oncogen mRNA is differentially expressed in human prostate cell lines: a new marker for tumor progression? Program of the 89th Annual Meeting of The American Association for Cancer Research, New Orleans, LA, 1998, p 209 (Abstract)
23. **Plymate SR, Tennant M, Birnbaum RS, Thrasher JB, Chatta G, Ware JL** 1996 The effect on the insulin-like growth factor system in human prostate epithelial cells of immortalization and transformation by simian virus-40 T antigen. J Clin Endocrinol Metab 81:3709–3716
24. **Wilson EM, Oh Y, Rosenfeld RG** 1997 Generation and characterization of an IGFBP-7 antibody: identification of 31kD IGFBP-7 in human biological fluids and Hs578T human breast cancer conditioned media. J Clin Endocrinol Metab 82:1301–1303
25. **Yang D-H, Kim H-S, Wilson EM, Rosenfeld RG, Oh Y** 1998 Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF-β in Hs578T human breast cancer cells. J Clin Endocrinol Metab 83:2593–2596
26. **Burren CP, Wilson EM, Hwa V, Oh Y, Rosenfeld RG** 1999 Binding properties and distribution of insulin-like growth factor binding protein-related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP superfamily. J Clin Endocrinol Metab 1096–1103
27. **Oh Y, Nagalla SR, Yamanaka Y** 1996 Synthesis and characterization of insulin-like growth factor binding protein-7 (IGFBP-7). J Biol Chem 271:30322–30325
28. **Kim H-S, Nagalla SR, Oh Y** 1997 Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci USA 94:12981–12986
29. **Brigstock DR** 1999 The connective-tissue growth factor/cystein-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20:189–206
30. **Wynford-Thomas D** 1999 Cellular senescence and cancer. J Pathol 187:100–111
31. **Jarrard DF, Sarkar S, Shi Y, Yeager TR, Magrane G, Kinoshita H, Nassif N, Meisner L, Newton MA, Waldman FM, Reznikoff CA** 1999 p16/pRb pathway alterations are required for bypassing senescene in human prostate epithelial cells. Cancer Res 59:2957–2964
32. **Story MT, Hopp KA, Molter M** 1996 Expression of transforming growth factor beta 1 (TGF beta 1), -beta 2, and -beta 3 by cultured human prostate cells. J Cell Physiol 169:97–107
33. **Pasquali D, Rossi V, Prezioso D, Gentile V, Colantuoni V, Lotti T, Bellastella A, Sinisi AA** 1999 Changes in tissue transglutaminase activity and expression during retinoic acid-induced growth arrest and apoptosis in primary cultures of human epithelial prostate cells. J Clin Endocrinol Metab 84:1463–1469
34. **Peehl DM, Cohen P, Rosenfeld RG** 1995 The insulin-like growth factor system in the prostate. World J Urol 13:306–311
35. **Swisshelm K, Ryan K, Tsuchiya K, Sager R** 1995 Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc Natl Acad Sci USA 92:4472–2276
36. **Sprenger CC, Damon SE, Hwa V, Rosenfeld RG, Plymate SR** 1999 Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. Cancer Res 59:2370–2375
37. **Degeorges A, Wang F, Frieson Jr HF, Seth A, Chung LWK, Sikes RA** 1999 Human prostate cancer expresses the low affinity insulin-like growth factor binding protein IGFBP-rP1. Cancer Res 59:2787–2790
38. **Lau LF, Lam SC-T** 1999 Minireview: the CCN family of angiogenic regulators: the integrin connection. Exp Cell Res 248:44–57
39. **Nakanishi T, Kimura Y, Tamura T, Ichikawa H, Yamaai Y-I, Sugimoto T, Takigawa M** 1997 Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF)) mRNA. Biochem Biophys Res Commun 234:206–210
40. **Frazier KS, Grotendorst GR** 1997 Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. Int J Biochem Cell Biol 29:153–161
41. **Kubo M, Kikuchi K, Nashiro K, Kakinuma T, Hayashi N, Nanko H, Tamaki K** 1998 Expression of fibrogenic cytokines in desmoplastic malignant melanoma. Br J Dermatol 139:192–197
42. **Wenger C, Ellenrieder V, Alber B, Lacher U, Menke A, Hameister H, Wilda M, Iwamura T, Beger HG, Adler G, Gress TM** 1999 Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. Oncogene 28:1073–1080
43. **Hishikawa K, Oemar BS, Tanner FC, Nakaki T, Lüscher TF, Fujii T** 1999 Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. J Biol Chem 274:37461–37466
44. **Lee C, Sintich S, Mathews E, Shah A, Kundu S, Perry K, Cho J, Ilio K, Cronauer M, Janulis L, Sensibar J** 1999 Transforming growth factor-beta in benign and malignant prostate. Prostate 39:285–290
45. **Pasquali D, Thaller C, Eichele G** 1996 Abnormal level of retinoic acid in prostate cancer tissues. J Clin Endocrinol Metab 81:2186–2191
46. **Liu C, Liu X-J, Crowe PS, Kelner GS, Fan J, Barry G, Manu F, Ling N, Souza EBD, Maki RA** 1999 Nephroblastoma overexpressed gene (NOV) codes for a growth factor that induces protein tyrosine phosphorylation. Gene 238: 471–478

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REVIEW

Does the GH–IGF axis play a role in cancer pathogenesis?

Pinchas Cohen[1], David R. Clemmons[2] and Ron G. Rosenfeld[3]

[1]Department of Pediatrics, UCLA, Los Angeles, CA, [2]Department of Medicine, UNC, Chappel Hill, NC and [3]Department of Pediatrics OHSU, Portland, ON, USA

Summary Recent case-controlled studies have found increases in the serum levels of insulin-like growth factor-I (IGF-I) in subjects who had, or who eventually developed, prostate or premenopausal breast cancers. Since growth hormone (GH) increases IGF-I levels, concern has been raised regarding its potential role as a cancer initiation factor. The epidemiological studies, which indicate an association between serum IGF-I levels and cancer risk, have not established causality. In fact, several alternative explanations for the elevated serum IGF-I levels in cancer patients may be proposed based on human and animal models. First, an effect of IGF-I causing symptomatic benign tissue hyperplasia may result in an ascertainment bias leading to an initiation of procedures resulting in the diagnosis of asymptomatic cancers. Second, elevated serum IGF-I in cancer patients may originate within the tumor (as suggested by some animal studies). Thirdly, serum IGF-I may actually be a surrogate marker of tissue IGF-I levels or of nutritional factors, which are not under GH control and may be involved in cancer initiation. The role of GH in cancer initiation is further negated by the fact that in acromegaly, the incidence of cancer, other than possibly colonic neoplasia does not appear to be significantly increased. Furthermore, GH transgenic mice, with high IGF-I levels, do not develop breast, prostate, or colonic malignancies. It is known that IGFBP-3 can inhibit IGF action on cancer cells *in vitro* and also can induce apoptosis via an IGF-independent mechanism. Importantly, in addition to increasing IGF-I levels, GH also increases the serum levels of IGFBP-3 and serum IGFBP-3 levels have been shown to be negatively correlated with the risk of cancer in the above mentioned epidemiological studies and in a similar study on colon cancer. These studies suggest that cancer risk is increased in individuals in whom both high IGF-I levels and low IGFBP-3 levels are present. In subjects treated with GH, IGF-I and IGFBP-3 levels both rise together and are not within the elevated cancer-risk range, based on published studies. Long-term studies are needed to assess the potential risks, including the long-term cancer risk associated with GH therapy. These should take into account several factors, including the duration of exposure, the risk magnitude associated with the degree of serum IGF-I elevation, and the adjusted risk based on a concomitant increase in IGFBP-3 levels. Since GH treated patients often have sub-normal IGF-I serum levels, which normalize on therapy, one might predict that their cancer risk on GH therapy should not increase above the normal population. Until further research in the area dictates otherwise, on-going cancer surveillance and routine monitoring of serum IGF-I and IGFBP-3 levels in GH-recipients should be the standard of care. At present, the data that are available do not warrant a change in our current management of approved indications for GH therapy.

Key words: growth hormone, insulin-like growth factor-1, cancer.

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Correspondence to: Pinchas Cohen, MD, Professor and Director of Research and Training, Division of Endocrinology, Department of Pediatrics, Mattel Children's Hospital at UCLA, 10833 Le Conte Ave, MDCC 22-315, Los Angeles, CA 90095-1752, USA. Tel: 310-206-5844; Fax: 310-206-5843; E-mail: hassy@mednet.ucla.edu

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EPIDEMIOLOGY OF THE GH–IGF–IGFBP-3 AXIS AND CANCER IN HUMANS

Recently, several epidemiological studies have been published suggesting an association between serum IGF-I levels and the incidence of malignancies. While some of these papers indicated higher IGF-I levels in subjects at

notable that acromegaly is associated with a dramatic increase in the incidence of benign hyperplasia of several organs, including colonic polyps[21], and benign prostatic hyperplasia[22]. In addition, primate studies suggested that treatment with high dose GH resulted in hyperplasia of the mammary gland in aging females[23]. These findings raise the possibility that the GH–IGF axis may lead to symptomatic benign proliferative disease, which could be associated with frequent urination or breast discomfort that would then lead to a potential detection bias. This ascertainment bias for cancer diagnosis needs to be considered as a possible explanation for the published data.

THE IGF AXIS IN *IN VITRO* MODELS OF CANCER

In normal tissues, growth and differentiation are controlled by multiple growth factors including those belonging to the IGF axis[24]. Changes at all levels of the IGF axis have been reported in a variety of cancers[25,26]. As noted previously, similar data are not available for assessing non-IGF-I dependent effects of GH receptor activation. Among the changes in IGF system physiology are the enhanced autocrine production of IGF-I and IGF-II[27-31]. The effects of IGFs on normal and cancer cells are mediated via the type 1 IGF receptor (IGF-1R)[32]. IGF-1R abnormalities may participate in the tumor development process as autocrine stimuli of survival and growth dysregulation. Additional regulators of tissue growth are the IGFBPs and the IGFBP proteases, which modulate IGF action[33]. Local IGFBP-3 levels may be important in the pathogenesis of cancer. IGFBP-3 is reduced in some types of neoplastic cells compared to normal cells by immunostaining[34]. *In vitro* studies of benign and malignant tissues and cell lines have demonstrated that IGFBP-3 has inhibitory effects on cancer cell growth[35]. Recent reports have shown that IGFBP-3 dose-dependently induces apoptosis though an IGF/IGF receptor independent pathway in prostate and breast cancer cells *in vitro*, suggesting a role for reduced IGFBP-3 mediated apoptosis in tumors. IGFPB-3 has been shown to directly induce apoptosis by binding to cell surface associated proteins in addition to blocking IGF interactions with the IGF-1R receptor[36].

IGFBP alterations have also been linked to changes in their proteases[37]. PSA is an IGFBP-3 protease, capable of acting as a co-mitogen with IGFs in the presence of IGFBP-3 *in vitro*[38]. IGFBP-3 proteolysis by PSA[39] and cathepsin D[40], within the breast and the prostate or their metastatic foci, could potentially contribute to the local propagation of neoplasia or metastasis. Elevation in serum PSA level has been correlated with decreased intact IGFBP-3 as well as to the stage of prostate cancer[41,42].

Of note is the fact that GH-independent regulation of IGF system components has been well characterized. In the prostate, the expression of the tissue IGF-related molecules is under potent control of androgens, which induce IGF-I and IGF receptors and suppress IGFBPs[43]. In the breast, estrogen regulates local IGF system components[44].

Thus, when cancer cells are studied *in vitro*, it appears that IGF, IGFBP and IGFBP protease perturbations play an important role in tumor cell propagation. GH is not thought to be an active participant in these cellular events, *in vitro*, and its non-IGF dependent effects *in vivo* have not been determined.

THE GH–IGF SYSTEM IN ANIMAL MODELS OF CANCER

Several rodent models have shed light on the GH–IGF-cancer issue. Key among these are the GH transgenic mice[45]. A central component of this model is the effect of various forms of GH on the prolactin receptor. An early publication using human growth hormone (hGH) transgenic mice demonstrated the development of mammary tumors[46]. A later publication using bovine GH as the transgene and contrasting it with prolactin transgenesis, demonstrated that prolactin is capable of inducing breast cancer[47]. Human growth hormone can activate the murine prolactin receptor and can thus cause mammary tumors in mice. The bovine GH transgenic mouse model showed that activation of the GH receptor alone, does not result in breast tumors, even though IGF-I levels were dramatically elevated and the mice displayed features of acromegaly over their lifetime. Animal models of tumor bearing animals, in which the GH–IGF axis has been manipulated, suggest that this system does affect the growth of established tumors. In GH deficient mice, breast cancer xenografts grow more slowly [48]. Similarly a GHRH antagonist or somatostatin analogues can inhibit cancer xenograft progression in SCID mice [49,50]. IGF-I infusion can promote tumor growth *in vivo*[51]. Other studies, however, did not find an effect of IGF-I infusion on rhabdomyosarcoma xenograft models[52] and one study showed that tumor-bearing animals actually benefited from IGF-I therapy through preservation of host lean tissue mass and reduction in cancer cachexia[53]. Conversely, IGFBP-3 infusion inhibits the growth of cancer xenografts in nude mice[54]. These models indicate that existing active tumors should constitute a contraindication to GH therapy as is currently stated in GH product labels. These studies, however, do not suggest a role of GH in *de novo* carcinogenesis in humans. On the other hand, treatment of cancer xenograft-bearing animals serving as models of tumor cachexia indicate that GH therapy might be favorable to the host survival and anabolism, while having no growth promoting effects on the tumor[55,56]. In fact some models reported a shrinking

in a 70% decrease in plasma IGF-I[74]. This decrease is dependent upon both protein and total energy intake, and at energy intakes below 20 kcal/day or protein intakes below 0.6 kg/day substantial decreases in IGF-I occur[75]. Catabolic conditions result in major decreases in IGF-I, but it is difficult in these states, such as hepatic failure, inflammatory bowel disease and renal failure, to discern the portion of change that occurs as a consequence of changes in nutritional intake, compared to the underlying inflammatory process itself, and concomitant tissue breakdown. Poorly controlled diabetes mellitus is associated with low plasma IGF-I concentrations that rise into the normal range with appropriate insulin substitution therapy[76].

Serum concentrations of IGF-I are also genetically determined and are related to polymorphisms of the IGF-I gene. Such a polymorphism, located near the P2 promotor is associated with varying serum IGF-I levels and with a different risk of developing osteoporosis. Analysis of this microsatellite repeat, which is approximately 1 kb upstream from the IGF-I gene transcription start site, shows a higher prevalence of the 192/192 genotype of this polymorphism among men with idiopathic osteoporosis compared to controls[77] and also predicted certain other bony conditions[78]. This polymorphism was also related to obesity and fat mass[79], two independent predictors of cancer; however, it was not related to serum GH and the differences in serum IGF-I among men with different genotypes and different serum IGF-I were not associated with differences in plasma GH. A study of cancer incidence in twins indicated that prostate, colon and breast malignancies, the three cancers linked to serum IGF-I have a strong genetic component, which was not seen in other types of cancer [80].

Thus, elevations of serum IGF-I, which are associated with cancer, may be related to non-GH sources and could represent a genetically determined marker of other cancer risk factors.

PHARMACO-VIGILANCE DATA ON GH RECIPIENTS AND CANCER

In a number of reported studies, no increased incidence of cancer was found in GH recipients among adults who were treated for GH deficiency [81]. Furthermore, careful follow-up of pediatric patients indicated no increased risk of solid tumor recurrence or development of leukemia[82]. Clearly, these reports represent imperfect, uncontrolled studies, but the experience gained through them, particularly in the pediatric population is extremely vast and demonstrates that, in the absence of other risk factors, GH therapy is not associated with tumor recurrence, leukemia, or other *de novo* tumors[83–86]. These findings indicate that GH therapy in GH deficient individuals does not increase the incidence of cancer, even though the IGF-I levels are normalized.

HYPOTHESES REGARDING GH–IGF-I AND CANCER

Table 2 summarizes the potential explanations for the observed association between IGF-I and cancer seen in some studies. It is possible that there is no true association and that an ascertainment bias or a methodological error contributed to the observation. It is possible that other cancer-controlling factors, such as nutrition, modulate IGF-I and thus IGF-I is actually a marker, rather than a cause of cancer. It is also possible that tumors secrete IGF-I, which then serves as a "tumor marker". It is also possible that autocrine IGF-I is involved in the pathogenesis of some tumors, but the opposing role of IGFBP-3 also needs to be considered. Finally it is possible that the GH-IGF axis is involved in the growth of some established cancers. Given the evidence available at this time it is prudent to continue to monitor patients receiving GH for tumor development.

The results could have arisen through diagnostic bias, in that IGF-1 might have caused benign symptoms, which led to diagnosis of malignancy, or have caused growth of preclinical cancers, leading to their more rapid diagnosis. This is specifically a concern for prostatic cancer because of the high prevalence of latent, asymptomatic prostatic cancers in elderly men[87], such that more intensive scrutiny can often detect tumors that might have remained undiagnosed or been diagnosed later without deliberate investigation. This scrutiny and detection of asymptomatic cancers could occur if raised IGF-1 causes benign prostatic hypertrophy, because the latter will lead to PSA measurements, prostate biopsies and prostatectomy and pathological examination of the removed tissue.

Raised IGF-I levels might be caused by the cancer (for instance, because the cancer may secrete IGF-I) rather than being the cause of it. This is supported by some

Table 2 Hypotheses regarding IGF-I and cancer

The "null (laboratory or statistical errors)" hypothesis—relating to the possibility of chance or error
The "ascertainment bias" hypothesis
The "IGF-I as a marker" hypothesis
The "nutrition" hypothesis—relating to the possibility of confounding effects
The "tumor as a source" hypothesis—relating to the possibility of reverse causation
The "IGF-I causes, and IGFBP-3 prevents, cancer (and GH is neutral)" hypothesis
The "GH causes cancer through IGF-I" hypothesis

levels in women with and without breast cancer: the Rancho Bernardo Study. J Women's Health Gend Based Med 1999; 8: 1265–1272.
9. Rosenfeld RG, Gargosky SE. Assays for insulin-like growth factors and their binding proteins: practicalities and pitfalls. J Pediatr 1996; 128: S52–S57.
10. Petridou E, Dessypris N, Spanos E, *et al.* Insulin-like growth factor-I and binding protein-3 in relation to childhood leukaemia. Int J Cancer 1999; 80: 494–496
11. Ma J, Pollak MN, Giovannucci E, *et al.* Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J Natl Cancer Inst 1999; 91: 620–625.
12. Ron E, Gridley G, Hrubec Z, Page W, Arora S, Fraumeni JF Jr. Acromegaly and gastrointestinal cancer. Cancer 1991; 68: 1673–1667.
13. Popovic V, Damjanovic S, Micic D, *et al.* Increased incidence of neoplasia in patients with pituitary adenomas. The Pituitary Study Group. Clin Endocrinol (Oxf) 1998; 49: 441–445.
14. Bengtsson BA, Eden S, Ernest I, Oden A, Sjogren B. Epidemiology and long-term survival in acromegaly. A study of 166 cases diagnosed between 1955 and 1984. Acta Med Scand 1988; 223: 327–335.
15. Delhougne B, Deneux C, Abs R, *et al.* The prevalence of colonic polyps in acromegaly: a colonoscopic and pathological study in 103 patients. J Clin Endocrinol Metab 1995; 80: 3223–3226.
16. Ladas SD, Thalassinos NC, Ioannides G, Raptis SA. Does acromegaly really predispose to an increased prevalence of gastrointestinal tumours? Clin Endocrinol (Oxf) 1994; 41: 597–601.
17. Orme SM, McNally RJ, Cartwright RA, Belchetz PE. Mortality and cancer incidence in acromegaly: a retrospective cohort study. United Kingdom Acromegaly Study Group. J Clin Endocrinol Metab 1998; 83: 2730–2734.
18. Sonksen PH, Jacobs H, Orme S, Belchetz P. Acromegaly and colonic cancer. Clin Endocrinol 1997; 47: 647–648.
19. Renehan AG, O'dwyer St, Shalet SM. Colorectal neoplasia in acromegaly: the reported increased prevalence is overestimated. Gut 2000; 46: 440–441.
20. Maison P, Balkau B, Simon D, Chanson P, Rosselin G, Eschwege E. Growth hormone as a risk for premature mortality in healthy subjects: data from the Paris prospective study. BMJ 1998; 316: 1132–1133.
21. Colao A, Balzano A, Ferone D, *et al.* Increased prevalence of colonic polyps and altered lymphocyte subset pattern in the colonic lamina propria in acromegaly. Clin Endocrinol (Oxf) 1997; 47: 23–28.
22. Colao A, Marzullo P, Ferone D, *et al.* Prostatic hyperplasia: an unknown feature of acromegaly. J Clin Endocrinol Metab 1998; 83: 775–779.
23. Ng ST, Zhou J, Adesanya OO, Wang J, LeRoith D, Bondy CA. Growth hormone treatment induces mammary gland hyperplasia in aging primates. Nat Med 1997; 3: 1141–1144.
24. Butt AJ, Firth SM, Baxter RC. The IGF axis and programmed cell death. Immunol Cell Biol 1999; 77: 256–262.
25. Grimberg A, Cohen P: Role of IGFs in growth control and carcinogenesis. J Cell Physiol 2000; 183: 1–9.
26. LeRoith D, Butler AA. Insulin-like growth factors in pediatric health and disease. J Clin Endocrinol Metab 1999; 84: 4355–4361.
27. Angelloz-Nicoud P, Binoux M. Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostate carcinoma cell line (PC-3). Endocrinol 1995; 136: 5485–5492.
28. Figueroa JA, Lee AV, Jackson JG, Yee D. Proliferation of cultured human prostate cancer cells is inhibited by insulin-like growth factor (IGF) binding protein-I: evidence for an IGF-II autocrine growth loop. J Clin Endocrinol Metab 1995; 80: 3476–3482.
29. Plymate SR, Tennant M, Birnbaum RS, Thrasher JB, Chatta G, Ware JL. The effect on the insulin-like growth factor system in human prostate epithelial cells of immortalization and transformation by simian virus-40 T antigen. J Clin Endocrinol Metab 1996; 81: 3709–3716.
30. Tennant MK, Thrasher JB, Twomey PA, Drivdahl RH, Birnbaum RS, Plymate SR. Protein and messenger ribonucleic acid (mRNA) for the type I insulin-like growth factor (IGF) receptor is decreased and IGFII mRNA is increased in human prostate carcinoma compared to benign prostate epithelium. J Clin Endocrinol Metab 1996; 81: 3774–3782.
31. Wang YZ, Wong YC. Sex hormone-induced prostatic carcinogenesis in the Noble rat: the role of insulin-like growth factor-I (IGF-I) and vascular endothelial growth factor (VEGF) in the development of prostate cancer. Prostate 1998; 35: 165–177.
32. Baserga R. The IGF-I receptor in cancer research. Exp Cell Res 1999; 253: 1–6.
33. Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. IGFBP proteases – functional regulators of cell growth. Progress in Growth Factors Research 1996; 6: 273–284.
34. Hampel OZ, Kattan MW, Yang G, *et al.* Quantitative immunohistochemical analysis of insulin-like growth factor binding protein-3 in human prostatic adenocarcinoma: a prognostic study. J Urol 1998; 159: 2220–2225.
35. Cohen P, Peehl DM, Rosenfeld RG. The IGF axis in the prostate. Horm Metab Res 1994; 26: 81–84.
36. Rajah R, Valentinis B, Cohen P. Insulin-like growth factor (IGF)-binding protein-3 induces apotosis and mediates the effects of transforming growth factor-beta 1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 1997; 272: 12181–12188.
37. Nunn SE, Gibson TB, Rajah R, Cohen P. Regulation of prostate cell growth by the insulin-like growth factor binding proteins and their proteases. Endocrine 1997; 7: 115–118.
38. Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab 1992; 75: 1046–1053.
39. Cohen P, Peehl DM, Graves HCB, Rosenfeld RG. Biological effects of prostate specific antigen (PSA) as an IGFBP-3 protease. J Endocrinol 1994; 142: 407–411.
40. Nunn SE, Peehl DM, Cohen P. Acid-activated insulin-like growth factor binding protein protease activity of cathepsin D in normal and malignant prostatic epithelial cells and seminal plasma. J Cell Physiol 1997; 171: 196–200.
41. Kanety H, Madjar Y, Dagan Y, *et al.* Serum insulin-like growth factor binding protein-2 is increased and IGFBP3 is decreased in patients with prostate cancer: correlation with serum prostate-specific antigen. J Clin Endocrinol Metab 1993; 77: 229–233.
42. Stamey TA, Kabalin JN, McNeil JE, *et al.* Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. J Urol 1989; 141: 1076.
43. Nickerson T, Pollak M, Huynh H. Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2, 3, 4, and 5. Endocrinol 1998; 139: 807–831.
44. Westley BR, Clayton SJ, Daws MR, Molloy CA, May FE. Interactions between the oestrogen and insulin-like growth factor signalling pathways in the control of breast

cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 2000; 343: 78–85.
81. Abs R, Bengtsson BA, Hernberg-Stahl E, *et al.* GH replacement in 1034 growth hormone deficient hypopituitary adults: demographic and clinical characteristics, dosing and safety. Clin Endocrinol (Oxf) 1999; 50: 703–713.
82. Nishi Y, Tanaka T, Takano K, *et al* and the GH Treatment Study Committee of the Foundation for Growth Science, Japan. Recent status in the occurrence of leukemia in growth hormone-treated patients in Japan. J Clin Endocrinol Metab 1999; 84: 1961–1965.
83. Graves DA. Utility of the National Cooperative Growth Study database for safety reporting. J Pediatr 1996; 128: S1–3.
84. Price DA, Wilton P, Jonsson P, *et al.* Efficacy and safety of growth hormone treatment in children with prior craniopharyngioma: an analysis of the Pharmacia and Upjohn International Growth Database (KIGS) from 1988 to 1996. Horm Res 1998; 49: 91–97.
85. Tuffi GA, Johanson A, Rundle AC, Allen DB. Lack of increased risk for extracranial, nonleukemic neoplasms in recipients of recombinant deoxyribonucleic acid growth hormone. J Clin Endocrinol Metab 1995; 80: 1416–1422.
86. Allen DB, Rundle AC, Graves DA, Blethen SL. Risk of leukemia in children treated with human growth hormone: review and reanalysis. J Pediatr 1997; 131: S32–36.
87. Breslow N, Chan CW, Dhom G, *et al.* Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France. Int J Cancer 1977; 20: 680–688.
88. Shim M, Cohen P. Growth hormone, IGFs IGFBPs and cancer. Horm Res 1999; 51(supp 3): 42–51.
89. Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency. J Clin Endocrinol Metab 1998; 83: 379–381

Effects of Sodium Butyrate on Expression of Members of the Insulin-like Growth Factor Binding Protein Superfamily in Human Mammary Epithelial Cells

Junko Tsubaki, Won-Kyu Choi, Angela R. Ingermann, Stephen M. Twigg, Ho-Seong Kim, Ron G. Rosenfeld and Youngman Oh

Department of Pediatrics, Oregon Health Sciences University, Portland, OR 97201

*Corresponding address: Youngman Oh, Department of Pediatrics, School of Medicine, NRC 5, Oregon Health Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97201-3042. Telephone: (503)494-1931. Fax: (503)494-0428. E-mail: ohy@ohsu.edu

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Abstract

Dietary factors play an important role in both the development and prevention of human cancers, including breast carcinoma. One dietary micronutrient, sodium butyrate (NaB), is a major end product of dietary starch and fiber, produced naturally during digestion by anaerobic bacteria in the caecum and colon. NaB is a potent growth inhibitor and initiates cell differentiation for many cell types *in vitro*. In this study, we investigated the effects of butyrate on three human mammary epithelial cells and regulation of the insulin-like growth factor (IGF) axis, specifically, insulin-like growth factor binding protein-3 (IGFBP-3), a known growth regulator in human mammary cells, and IGFBP-related protein 2 (IGFBP-rP2)/connective tissue growth factor (CTGF).

NaB inhibited DNA synthesis, as measured by [^{3}H]thymidine incorporation, in estrogen-responsive (MCF-7) and estrogen-nonresponsive (Hs578T) breast cancer cells, and normal human mammary epithelial cells (HMEC) to a similar degree (up to 90% inhibition at 1 to 10 mM concentrations). Treatment of cells with NaB induced histone hyperacetylation, suggesting that NaB exerts its biological effects, at least in part, as a histone deacetylase inhibitor in mammary epithelial cells. Treatment of Hs578T cells with butyrate caused an induction of apoptotic cell death. NaB treatment resulted in increased levels of $p21^{Waf1/Cip1}$ mRNA and protein in Hs578T cells and distinct

upregulation of $p27^{Kip1}$ in HME cells, suggesting that NaB activates different genes involved in cell cycle arrest, depending upon the cell type. In the same context, among the IGFBP superfamily members tested, NaB specifically upregulated the expression of IGFBP-3 and IGFBP-rP2. These two proteins are known to be involved in inhibition of mammary epithelial cell replication. Northern blot analysis showed that NaB treatment at 1 to 10 mM concentrations caused a dose-dependent stimulation of IGFBP-3 mRNA expression in cancerous cells and IGFBP-rP2 mRNA expression in both cancerous and non-cancerous cells. Protein data from Western ligand blot and immunoblot analyses demonstrated parallel results.

In summary, we have demonstrated that NaB 1) uniformly suppresses DNA synthesis in both cancerous and non-cancerous mammary cells, and 2) upregulates IGFBP-3 and IGFBP-rP2 mRNA and protein levels in cancerous and non-cancerous mammary cells. These results provide the first demonstration that butyrate regulates the IGFBP system in the human mammary system.

Introduction

Butyrate is a 4-carbon fatty acid which is the major product from microbial fermentation of dietary fibers in the large intestine (Velázquez *et al.* 1997). It is a potent growth inhibitor and initiates cell differentiation in several cell types, including breast cancer cells *in vitro* (Coradini *et al.* 1997, Gleave *et al.* 1998, Velázquez *et al.* 1997, Yamamoto *et al.* 1998). Although the molecular mechanisms by which butyrate exerts its effects are still unclear, it is known to induce a number of alterations within the nucleus, including histone hyperacetylation (Archer *et al.* 1999, de Haan *et al.* 1986).

It has been reported that butyrate, as well as trichostatin A (TSA), a specific histone deacetylase inhibitor (Yoshida *et al.* 1990), modulate specific genes involved in cell cycle regulation and apoptosis. These include the cyclin-cdk inhibitor $p21^{Waf1/Cip1}$ (Archer *et al.* 1998), $p16^{INK4}$ (Schwartz *et al.* 1998), $p27^{Kip1}$ (Litvak *et al.* 1998), retinoblastoma protein (Rb) (Vaziri *et al.* 1998), cyclinD1 (Lallemand *et al.* 1996, Siavoshian *et al.* 1997), Bcl-2 and Bax (Hague *et al.* 1997, Mandal *et al.* 1996). Furthermore, butyrate has also been reported to upregulate the expression of transforming growth factor-β (TGF-β) (Staiano-Coico *et al.* 1990), which is known to be involved in growth suppression of various cancer cells, including breast cancer cells (Knabbe *et al.* 1987).

Insulin-like growth factors (IGFs) are potent mitogens for several cell types (Macaulay *et al.* 1992, Resnicoff *et al.* 1995). The IGF system consists of IGF-I and IGF-II ligands, the transmembrane type I and type II IGF receptors, the IGF binding proteins (IGFBPs) and IGFBP proteases (Hwa *et al.* 1999). Recently, the concept of the IGFBP superfamily has been proposed (Baxter *et al.* 1998); it consists of high affinity IGF binders (IGFBP-1 to IGFBP-6) and low affinity IGF binders (IGFBP-related proteins, IGFBP-rPs). The IGFBPs modulate IGF bioactivity, and bind with differential affinities to IGFs in serum and various biological fluids (Kelley *et al.* 1996, Rajaram *et al.* 1997). In addition, recent evidence suggests that some IGFBPs may have direct receptor-mediated effects independent of IGFs (Oh *et al.* 1993). IGFBP-3, for example, has been demonstrated to be an important mediator of other growth inhibitory agents, such as retinoic acid (Gucev *et al.* 1996), vitamin D (Colston *et al.* 1998), TGF-β (Gucev *et al.* 1996, Oh *et al.* 1995, Rajah *et al.* 1997), antiestrogens (Huynh *et al.* 1996), tumor necrosis factor-α (Rozen *et al.* 1998), and p53 (Buckbinder *et al.* 1995), independently of the IGF signaling system. Furthermore, the importance of IGF-independent biological effects of the IGFBP superfamily, such as IGFBP-3 (Oh *et al.* 1993), IGFBP-rP1 (Burger *et al.* 1998) and –rP2 (Hishikawa *et al.* 1999) on cell replication has been demonstrated in human breast cancer cell systems.

In this study, we have investigated the effects of NaB on members of the IGFBP superfamily in human mammary epithelial cells, using estrogen-responsive (MCF-7) and estrogen-nonresponsive (Hs578T) breast cancer cells, and normal human mammary epithelial cells (HMEC). We report here that NaB upregulates IGFBP-3 and IGFBP-rP2 mRNA and protein in mammary epithelial cells.

Materials and Methods

Materials

Sodium butyrate, trichostatin A, bovine serum albumin (BSA) and 0.4% trypan blue solution were purchased from Sigma Chemical Co (St. Louis, MO). [^{125}I]-labeled IGF-I was kindly provided by Diagnostic Systems Laboratories (Webster, TX). Polyclonal α-IGFBP-3, α-IGFBP-rP1, α-IGFBP-rP2 and α-HEC1 antisera were generated as previously described (Oh *et al.* 1993) (Wilson *et al.* 1997) (Yang *et al.* 1998) (Rosenfeld *et al.* 1990). Polyclonal α-IGFBP-5 antibody was purchased from Austral Biologicals (San Ramon, CA). Polyclonal α-acetyl-lysine, α-acetylated histone H3 and α-acetylated histone H4 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal α-PARP (H-250) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies, α-p21$^{Waf1/Cip1}$, α-p16^{INK4}, α-p27^{Kip1} were purchased from Transducion Laboratories (Lexington, KY), PharMingen (San Diego, CA), Calbiochem (Cambridge, MA), respectively.

Cell Culture

Hs578T estrogen-nonresponsive human breast cancer cells and MCF-7 estrogen-responsive human breast cancer cells were purchased from ATCC (Manassas, VA). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 10% fetal bovine serum. HMEC normal human mammary epithelial cells were purchased from Clonetics (San Diego, CA), and maintained in mammary epithelium basal medium (MEBM) with growth supplements (bovine pituitary extract: BPE, hEGF, insulin, hydrocortisone, gentamicin and amphotericin-B) as directed by the manufacturer.

[^{3}H] Thymidine Incorporation Assay

Cells were seeded into 24-well dishes at 37°C in 5% CO_2. At 90% confluency, cells were placed in serum-free media for 12 hours, then treated as indicated in the text. After 22 hours, 0.1 µCi of [^{3}H]thymidine (NEN, Boston, MA) in a volume of 25 µl phosphate-buffered saline (PBS) was added to each well, and the plate was incubated for 4 hours at 37°C. Cells were washed with cold PBS twice, treated with 10% trichloroacetic acid (TCA) for 10 minutes at –20°C, washed with 10% TCA followed by 95% ethanol, and lysed with 400 µl of 0.25N NaOH per well. Cell lysates from each well were transfered to scintillant vials, then10 ml of scintillation fluid with 100 µl of 2N HCl was added to the vials, and the radioactivity was measured in a scintillation counter.

MTS assay

Hs578T cells were seeded into 96-well plates. At 80% confluency, they were incubated for 12 hours in serum-free DMEM, then treated with various concentrations of sodium butyrate as indicated in the text in serum-free media. After a further incubation of 1 day to 4 days, the MTS reagent (Promega Co., Madison, WI) was added in the ratio as recommended by the manufacturer. At 15 minutes intervals, the absorbance of the formazan product at 490 nm was read by plate reader (Spectra Shell Reader; SLT Labinstruments Ges.m.b.H., Austria). On the same plate, cells were dispensed at the differential confluency. These cells were left untreated in serum-free media, and then the MTS reagent was added at the same time of the butyrate-treated wells. After the reading, cells were trypsinized, then the cell number was counted by hemacytometer. Using this method, a linear correlation was obtained between direct cell counts from 1 x 10^3 to 1 x 10^4 per well and the absorbance (r=0.85, n=70) (data not shown). The experiment was repeated three times in condition where the starting untreated control cell number was 8 x 10^3 per well, and the A 490 nm reading at 45 minutes after adding the MTS reagent to the wells was ~ 0.8.

Quantitation of Apoptosis

To quantitate apoptotic cell death, the "Cell Death Detection ELISA^{PLUS}" kit (Roche Molecular Biochemicals, Mannheim, Germany) that measures cytoplasmic histone-bound DNA fragments produced during apoptotic DNA fragmentation, was used (Mandal *et al.* 1996). Hs578T cells were seeded into a 96-well plate at 80% confluency, they were incubated for 12 hours in serum-free DMEM, then treated with various concentrations of sodium butyrate in serum-free media. After 72 hours, cytoplasmic extracts were made from attached cells by adding 100 µl of lysis buffer to 5 x 10^3 cells per well. Supernatant (20 µl from 100 µl) was analyzed in the ELISA, as directed by manufacturer's protocol. Briefly, the samples were placed into a streptavidin-coated multi-well plates, a mixture of anti-histone-biotin and anti-DNA-peroxidase were added and incubated. ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured against 490 nm as a reference wavelength. The experiment was performed from duplicate samples for each data point generated, and was repeated twice independently.

Preparation of Conditioned Media (CM) and Cell Lysates (CL)

Cells were seeded in 12-well plates. At 95% confluency, they were incubated for 12 hours in serum-free DMEM (Hs578T and MCF-7) or supplement-free MEBM with added BPE (HMEC), then treated as indicated in the text in serum- or supplement-free

media. Conditioned media samples were collected after 72 hours and centrifuged at 1000 x g for 10 minutes to remove debris. The harvested CM from duplicate wells within each experiment were pooled and stored at –20°C until assay. Proteins in 40 µl of CM per lane were examined by Western immunoblot or Western ligand blot under non-reducing conditions.

Cell lysate samples were harvested at 24 hours post-treatment by washing with PBS, and then adding 150 µl of cold RIPA lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1% SDS) plus protease inhibitors cocktail (Roche Molecular Biochemicals, Mannheim, Germany) directly to each well. Plates were rocked for 30 minutes at 4°C, and the lysates were collected and centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatants from duplicate wells within each experiment were pooled and stored at -20°C until assay. Total protein concentration was determined for each sample using DC Protein Assay Reagent (Bio-Rad, Hercules, CA), and 20 µg of total protein per sample were examined by Western immunoblot under reducing conditions.

Western Ligand Blot Analysis

Proteins from CM samples were size-fractionated by 12% SDS-PAGE under non-reducing conditions and electroblotted onto nitrocellulose filters (Hybond; Amersham

Pharmacia Biotech, Piscataway, NJ). Filters were washed in 3% NP-40 / ddH_2O for 30 minutes, blocked with 1% BSA / TBS-T (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 2 hours, and incubated overnight with $2.0x10^6$ cpm of [^{125}I]-labeled IGF-I. The membranes were washed, dried and exposed to film (Kodak BioMax MS, Eastman Kodak Co., Rochester, NY) for 12-18 hours.

Western Immunoblot Analysis

For IGFBP-3, IGFBP-rP1 and IGFBP-rP2 detection, CM samples were separated on non-reducing 12% or 15% SDS-PAGE. For $p21^{Waf1/Cip1}$ detection, CL samples were separated on reducing 15% SDS-PAGE. Proteins were electro-transferred onto nitrocellulose, and membranes were blocked with 5% non-fat dry milk / TBS-T for 1 hour at room temperature, then incubated in 1:3000 dilution of primary antibody at 4°C overnight. Immunoreactive proteins were detected using enhanced chemiluminescence (NEN, Boston, MA).

Total RNA Isolation and Northern Blot Analysis

Cells were grown in 6-well plates until 95% confluent. Cells were then incubated in serum-free media for 12 hours, then treated for 18 hours in serum- or supplement-free media as indicated in the text. Total RNA was isolated from duplicate wells, using the

RNeasy Kit (Qiagen, Valencia, CA), and 5 μg of total RNA per sample were separated on a 1% formaldehyde agarose gel and transferred to nylon membranes (GeneScreenPlus; NEN, Boston, MA). Membranes were stained with 0.02% methylene blue in 0.3 M NaOAc, pH 5.5, and 18S and 28S rRNA bands were used as internal controls to adjust for sample loading. The blot was then hybridized at 65°C with full length cDNA probes random-labeled with [^{32}P]dCTP (Prime-It II; Stratagene, Cedar Creek, TX), washed and autoradiographed.

Densitometric Analysis

To quantify the relative induction after Western blot analyses or Northern blot analyses, densitometric measurement was performed by using a GS-700 Imaging Densitometer with Multi-Analyst software (Bio-Rad, Hercules, CA).

Results

Butyrate (NaB) treatment inhibits DNA synthesis and causes histone hyperaceylation in human mammary epithelial cells.

Since it has been demonstrated that treatment of NaB resulted in growth inhibition in a variety of cell system *in vitro*, we first examined the effect of NaB on DNA synthesis in normal (HMEC) and cancerous (Hs578T, MCF-7) human mammary epithelial cells, using the [^{3}H]thymidine incorporation assay. NaB suppressed DNA synthesis in both cancerous and non-cancerous human mammary epithelial cells in a dose-dependent manner, with 90-100% inhibition at NaB concentrations of 5 mM (Figure 1A). One major function of NaB is inhibition of histone deacetylase activity, resulting in histone hyperacetylation. In order to determine whether NaB-induced inhibition of DNA synthesis might be due to histone hyperacetylation, we treated the cells with trichostatin A (TSA), a specific histone deacetylase inhibitor, and compared its effect on DNA synthesis. As shown in Figure 1B, TSA also suppressed DNA synthesis, with 90-100% inhibition at TSA concentrations of 100 nM in these cells. This suggests that butyrate-induced suppression of DNA synthesis in these human mammary cells may involve histone hyperacetylation, as is shown by Western immunoblot with an anti-acetyl-lysine antibody (Figure 2). The cell lysates 24 hours after treatment with NaB showed an

increase of acetylated proteins in a dose-dependent manner, as indicated by the appearance of 11 kDa and 16 kDa bands, which were identified as H4 and H3 histones respectively by Western immunoblot with specific antibodies (data not shown). Histone hyperacetylation was similarly demonstrated by treatment with TSA (Figure 2). Interestingly, only the 11 kDa band was seen at concentrations over 100 nM.

Effects of sodium butyrate on reducing cell number and the induction of apoptosis in human mammary epithelial cells.

As NaB inhibits DNA synthesis almost completely by 24 hours' treatment, cell viability over 4 days after NaB treatment in Hs578T cells was then studied. The MTS assay was used, as described in Materials and Methods, as a marker of relative viable cell number. A progressive reduction in cell number by MTS assay was observed from day 2 onwards using 10 mM NaB, and from day 3 onwards after 5 mM NaB treatment (Figure 3). In parallel wells, when cell numbers were counted after trypsinizing by direct visualization using a hemacytometer, the attached cell numbers were reduced over the same time course and concentrations of NaB, as was detected in the MTS assay above (data not shown). Using trypan blue exclusion during hemacytometer counting, when counted at the same time point, the same number of attached cells were shown to take up

trypan blue in the NaB treated wells compared with the control wells, throughout the full 4 days of the study (data not shown). This trypan blue staining pattern indicates that cell plasma membrane integrity was maintained in the attached cells after NaB treatment compared with the control.

As a reduction in viable cell number by NaB was occurring over time, the possibility that apoptosis was being induced by NaB in the mammary epithelial cells was then addressed. Two independent methods of analysis were used to detect apoptosis: firstly, nuclear enzyme cleavage, and secondly, DNA fragmentation. The nuclear enzyme poly(ADP-ribose)polymerase, or (PARP), is proteolytically cleaved during apoptosis *in vitro* in many cell types, including breast cancer cells (Kaufmann *et al.* 1993). Figure 4A shows Western immunoblots with an anti-PARP antibody after treatment of Hs578T and MCF-7 cells with NaB followed by analysis of cell lysates. The ~85 kDa carboxy terminal fragment of PARP detected by Western immunoblotting, is indicative of apoptosis occurring in the lysates sampled (Lazebnik *et al.* 1994, Kaufmann *et al.* 1993). In Hs578T cells, some induction of the fragment was already observed by day 1 at concentrations of 5 mM NaB and above, and was more marked in the day 3 lysates, particularly after 10 mM NaB. Detectable increases in the PARP fragment were also seen in the day 3 cell lysates of MCF-7 cells (Figure 4A).

To verify and more accurately quantitate the apoptosis induced by NaB, an ELISA kit that detects histone-associated DNA fragments (mono- and oligo-nucleosomes) in the cytoplasmic fraction of cell lysates was used, as described in Materials and Methods. In the Hs578T cytoplasmic extracts from attached cells studied at 3 days post-treatment, NaB induced apoptosis in a dose-dependent manner (Figure 4B). This sensitive assay demonstrated apoptotic effects initially commencing at 1 mM of NaB. Taken together, the two methods of measuring apoptosis show that NaB induces apoptosis in the cells studied, in a more delayed time course compared with the earlier effects on [^{3}H]thymidine incorporation. The apoptosis induced by NaB would be expected to reduce the viable cell number, which was observed, as described earlier.

Sodium butyrate upregulates expression of p21$^{Waf1/Cip1}$ mRNA and protein levels in human mammary epithelial cells.

As the major mechanism for butyrate-induced growth inhibition in various cell systems is known to be through upregulation of cyclin-dependent kinase (cdk) inhibitors, in particular, p21$^{Waf1/Cip1}$, the induction of p21$^{Waf1/Cip1}$ in mammary epithelial cells was next investigated. Figure 5A is a Northern blot of p21$^{Waf1/Cip1}$ from Hs578T, MCF-7 and HME cells treated with or without NaB. p21$^{Waf1/Cip1}$ mRNA expression was upregulated in all

three cell lines, and was most marked in Hs578T cells. As shown in Figure 5B, an upregulation of $p21^{Waf1/Cip1}$ protein levels occurred in these cells, with the greatest increase in $p21^{Waf1/Cip1}$ observed in Hs578T cells, which parallels the mRNA data. As the degree of induction was different between Hs578T cells and the other two cell lines, the NaB effect on cdk inhibitors $p27^{Kip1}$ and $p16^{INK4}$ was further investigated. A distinct upregulation of $p27^{Kip1}$ by NaB treatment (a 2.5 fold increase at 5 mM treatment) was reproducibly seen in HME cells, but not in the cancerous cell lines (Figure 5B). We did not detect $p16^{INK4}$ in Hs578T and MCF-7 cells, and only a slight induction of this protein was seen in HME cells. Taken together, these data suggest that differential cdk inhibitors are induced by NaB treatment, in a cell-type dependent manner.

Butyrate upregulates IGFBP-3 mRNA and protein levels in cancerous, but not in non-cancerous mammary cells.

To investigate any correlation between the effect of NaB and the regulation of IGFBP system, we first examined IGFBP-3, a known growth suppressor in human mammary cells. Northern blotting was firstly performed to measure steady-state mRNA level. Figure 6A shows the time-course effect of NaB treatment on steady-state levels of IGFBP-3 mRNA in Hs578T cells. NaB induced the expression of IGFBP-3 mRNA in a

time-dependent manner, with increases first detectable at 6 hours after treatment, and with a 2.5 fold increase after treating cells with 5 mM NaB for 24 hours, whereas in the non-cancerous HME cells, only a slight induction (< 1.2 fold) was observed (Figure 6B). In MCF-7 cells, IGFBP-3 mRNA was not detectable in these analyses.

The conditioned media was then examined for changes in IGFBP-3 protein levels after NaB treatment. In order to ascertain a suitable time point to collect conditioned media samples, the time-course induction of media IGFBP-3 protein in Hs578T cells was studied by Western ligand blot analysis, using [^{125}I]-labeled IGF-I as the ligand, as described in Materials and Methods. The IGFBP-3 level in the conditioned media was detectably increased at 24 hours, even after 1 mM NaB, was further increased at 48 hours, and peaked at 72 hours following NaB treatment (Figure 7A). Subsequently, conditioned media at 72 hours post-treatment was analyzed in further studies. As shown in Figure 7B, various IGFBPs could be detected by the IGF ligand blot. The identity of these IGFBPs was confirmed by Western immunoblots using IGFBP-3, IGFBP-2 and IGFBP-5 specific antibodies, showing the 42 – 46 kDa doublet bands to be IGFBP-3, the broad 29 – 36 kDa bands to contain IGFBP-2 and IGFBP-5, and the 24 kDa band to be IGFBP-4 (data not shown). In HME cells, the Western ligand blot did not reveal an IGFBP-5 band, whereas the western immunoblot with IGFBP-5 antibody revealed a low intensity band of the predicted molecular weight for IGFBP-5 (data not shown) (Adamo

et al. 1992, Sheikh *et al.* 1992). IGFBP-3 protein levels were upregulated in both Hs578T (2.1 fold over the control) and MCF-7 (12.6 fold over the control) cells, each after 5 mM NaB treatment (Figure 7B). Further Western immunoblotting analyses demonstrated no detectable IGFBP-3 fragments in all samples tested in these cells (data not shown). As the basal level of IGFBP-3 in MCF-7 cells was nearly undetectable, the induction of IGFBP-3 protein by NaB was more conspicuous in this cell line. In contrast, only slight upregulation of IGFBP-3 (< 1.5 fold) was observed in HME cells, mirroring the mRNA data (shown earlier in Figure 6B). Levels of IGFBP-2/-5 and IGFBP-4 showed no significant change up to 10 mM of NaB treatment, after accounting for effects of NaB on cell number (not shown). The effect of TSA treatment on IGFBP-3 protein levels by Western ligand blot and immunoblot was also studied. TSA treatment of both Hs578T and MCF-7 cells caused a dose-dependent increase in IGFBP-3 protein levels (not shown), suggesting that NaB-induced upregulation of IGFBP-3 is, at least in part, through histone hyperacetylation. This effect was not seen in the HME cells (data not shown).

Sodium butyrate upregulates IGFBP-rP2 mRNA and protein expression in both cancerous and non-cancerous mammary cells.

The induction of the low affinity IGF binders, especially IGFBP-rP2, was then investigated, as this protein also has recently been shown to have a growth suppressive effect in human mammary cells (Hishikawa *et al.* 1999). The effect of NaB treatment on IGFBP-rP2 mRNA expression was potent, particularly in Hs578T cells, where effects occurred with 0.5 mM NaB. As seen in Figure 8A, a 10 fold induction of IGFBP-rP2 by 5 mM NaB treatment was observed in Hs578T cells, whereas a maximal 2-3 fold induction occurred in HME cells. IGFBP-rP2 mRNA was not detectable in MCF-7 cells.

Western immunoblot analysis against IGFBP-rP2 and –rP1 was then performed. As shown in Figure 8B, NaB highly upregulated IGFBP-rP2 protein levels in all three cell lines in a dose-dependent manner (>10 fold at 10 mM NaB over the control in all three cell lines). Increases in media IGFBP-rP2 were initially detectable within 24 hours, even after only 1 mM NaB (data not shown). In contrast to the effects on IGFBP-rP2, the IGFBP-rP1 band intensity was not increased by NaB treatment. The apparent reduction in IGFBP-rP1 by NaB treatment compared with control, especially in Hs578T conditioned media, was 52% on average using densitometric analysis at day 3 after 10 mM NaB. This reduction in IGFBP-rP1 could be fully accounted for by considering the effects of NaB on cell number, as shown earlier in Figure 3, where 40-50% of the cells are non-viable by this time of NaB treatment compared with control. IGFBP-rP1 was not detectable in

MCF-7 cells. These results show that IGFBP-rP2 mRNA and protein are specifically induced by NaB in both cancerous and non-cancerous breast epithelial cells.

Discussion

In this study, sodium butyrate effects in the human mammary cell system including cancerous and non-cancerous cells, was investigated, in order to obtain a greater understanding of the cellular mechanism of action of NaB in this cell type. NaB was found to cause an initial inhibition in new DNA synthesis, followed by apoptotic changes, and a reduction in viable cell number. Subsequently, the cyclin-dependent kinase (cdk) inhibitors studied showed some cellular specificity in their upregulation by NaB. IGFBP superfamily members known to induce growth inhibition and apoptosis in breast epithelial cells were also upregulated by NaB.

A schematic summary of our observations in Hs578T cells is shown in Figure 9. We first studied the general effects of NaB on cell growth regulation. Butyrate induces diverse and reversible biological effects on cell differentiation, apoptosis and cell growth *in vitro* (Pouillart *et al.* 1998). In colonic epithelial cells, butyrate is known to exert paradoxical effects, with induction of proliferation in normal cells and growth inhibition in neoplastic phenotypes (Archer *et al.* 1999, Hassig *et al.* 1997). In the mammary system studied here, butyrate inhibits DNA synthesis in both normal and cancerous cells as measured by [^{3}H]thymidine incorporation at 24 hours after treatment. Also as shown in Figure 9, after 24 hours of butyrate treatment at concentrations over 1 mM, cell numbers

became reduced in a dose- and time-dependent manner. This reduction in cell number was explained, at least in part, by NaB-induced apoptosis. The observed induction of apoptosis is consistent with previous reports of NaB induced apoptosis in other cell systems (Carducci *et al.* 1996, Coradini *et al.* 1997, Hague *et al.* 1993). In summary, NaB was found to induce both inhibition of DNA synthesis and programmed cell death, in Hs578T cells.

It is known that butyrate induces a variety of changes within the nucleus (Schwartz *et al.* 1998, Siavoshian *et al.* 1997), including histone hyperacetylation, especially of H3 and H4 (Archer *et al.* 1999), and DNA methylation (de Haan *et al.* 1986). Previous studies indicate that the effect of butyrate and other histone deacetylase inhibitors on cells closely corresponds to the effects of $p21^{Waf1/Cip1}$ expression in the regulation of G1 (Hunter *et al.* 1994), S (Ogryzko *et al.* 1997) and G2 (Coradini *et al.* 1997, Lallemand *et al.* 1999) phases of the cell cycle (Hassig *et al.* 1997). The major mechanism for butyrate-induced cell cycle arrest is reported to be through upregulation of $p21^{Waf1/Cip1}$ in various cell systems, including hepatocellular carcinoma cells (Yamamoto *et al.* 1998), colon cancer cells (Archer *et al.* 1998, Litvak *et al.* 1998, Nakano *et al.* 1997, Siavoshian *et al.* 1997), prostate cancer cells (Huang *et al.* 1999) and breast cancer cells (Lallemand *et al.* 1999). In contrast, Vaziri et al. demonstrated $p21^{Waf1/Cip1}$-independent G1 cell cycle arrest by butyrate in 3T3 fibroblasts (Vaziri *et al.* 1998). In our present studies, the prominent

induction of $p21^{Waf1/Cip1}$ was observed in estrogen non-responsive Hs578T cancer cells, whereas modest induction was observed in estrogen-responsive MCF-7 cancer cells and non-cancerous HME cells. Interestingly, butyrate upregulated $p27^{Kip1}$ expression only in HME cells. The $p16^{INK4}$ protein was detected only in HME cells and no further regulation was observed after butyrate treatment. Taken together, these data imply that the biological function of butyrate is mediated through more than one mechanism, even in butyrate-induced cell cycle arrest, suggesting that butyrate possesses multifunctional mechanisms of action.

This is the first demonstration that butyrate upregulates members of the IGFBP superfamily in human mammary cells. One previous report showed that only IGFBP-2 was upregulated by butyrate in colon cancer cells (Nishimura et al. 1998). In contrast, our studies demonstrate that among the six IGFBPs and IGFBP-rP-1 and –2, only IGFBP-3 and IGFBP-rP2 are upregulated by butyrate in cancerous and non-cancerous mammary epithelial cells. Recent evidence has suggested that, in addition to modulating the access of IGFs to their receptors, IGFBP-3 has the ability to suppress proliferation in various cell systems, including human breast cancer cells, working through IGF-independent mechanisms (Oh 1998, Valentinis *et al.* 1995). Further studies have demonstrated that IGFBP-3 directly induces apoptosis through an IGF-independent pathway in PC-3 human prostatic adenocarcinoma cells (Rajah *et al.* 1997) as well as in other cell systems

(Nickerson *et al.* 1997). We, therefore, hypothesized that IGFBP-3 may be a major downstream effector of growth inhibitory and apoptosis-inducing agents.

Indeed, NaB treatment significantly upregulated IGFBP-3 steady-state mRNA and protein levels in a time- and dose-dependent manner in Hs578T and MCF-7 human breast cancer cells, while levels of IGFBP-2 and IGFBP-4 were unaffected. As summarized in Figure 9, we observed that the mRNA induction had already started at 6 hours after treatment and peaked by 24 hours after treatment, while the presence of the protein in the conditioned media was barely detectable at the 24 hour time-point and gradually increased up to day 3. Notably, both the mRNA and protein induction by NaB were detectable earlier than the reduction in cell number and apoptosis, consistent with a specific regulation of these IGFBP superfamily members by NaB rather than effects on these IGFBPs occurring secondary to cell death. In contrast to effects seen in these cancer cell lines, the NaB regulation of IGFBP-3 in normal human mammary epithelial cells was less marked. Additionally, treatment with TSA gave similar results, indicating that histone hyperacetylation may be involved. The differential regulation of IGFBP-3 by NaB in cancerous versus non-cancerous cells may point to an important mechanism for growth inhibition by IGFBP-3 in cancer.

It has been demonstrated that TGF-β, which is a potent growth suppressing factor in human breast cancer cells (Oh *et al.* 1995, Zugmaier *et al.* 1989), induces expression

of IGFBP-rP2 mRNA and protein levels (Yang *et al.* 1998). In addition, recent studies have demonstrated that IGFBP-rP2 has a direct apoptotic effect in MCF-7 cells (Hishikawa *et al.* 1999). Our present data show that IGFBP-rP2 was significantly upregulated by NaB treatment in a time- and dose-dependent manner in both cancerous and non-cancerous human mammary epithelial cell lines, suggesting that IGFBP-rP2 may also play a role in the bioactivity of butyrate on cells. In Hs578T cells, the induction of IGFBP-rP2 mRNA was already marked at 0.5 mM, while the protein levels were significantly increased at 5 mM. One potential explanation for this dose discrepancy is that some post-transcriptional modification by NaB of IGFBP-rP2 may also be occurring.

On the basis of this work, it can be speculated that the IGFBP superfamily members, IGFBP-3 and IGFBP-rP2, participate in butyrate-induced sequential cell growth inhibition, particularly in the later event of apoptosis. Detailed studies involving regulation of IGFBP-3 and IGFBP-rP2 bioactivity following butyrate treatment in breast epithelial cells are now required to formally address this issue. It is hoped that exploration of butyrate-induced biological effects subsequent to the cell cycle arrest in this cell system and the investigation of the interaction between butyrate and the IGF axis will lead to a more complete understanding of the complex mechanisms of cell growth control, and to the development of better therapeutic reagents.

Figure Legends

Figure 1. Effect of sodium butyrate (A) and TSA (B) on DNA synthesis in Hs578T, MCF-7 and HME cells. Serum-starved Hs578T, MCF-7 and HME cells (85-90% confluent) were incubated in basal medium for 22 h in the absence or presence of various concentrations of sodium butyrate (A) or TSA (B) as indicated. [^{3}H]Thymidine was added, and the incubation was continued for another 4 h. The incorporation of [^{3}H]thymidine was determined relative to control cells incubated without the addition of these reagents. Results represent the average of two independent experiments each performed in triplicate.

Figure 2. Effect of sodium butyrate on protein acetylation. Serum-starved cells were treated for 24 h with various concentrations of sodium butyrate as indicated. Cell lysates harvested from duplicate wells within each experiment were pooled, and 20 μg of protein per lane were loaded onto 15% SDS-PAGE under reducing conditions. Gels were immunoblotted with an anti-acetyl-lysine antibody as described in Materials and Methods. The immunoblots of cell lysates treated with 100 nM TSA are shown on the right. Molecular weight markers are also shown. The results are representative of two independent experiments.

Figure 3. Growth inhibition by sodium butyrate. Hs578T cells were seeded into 96-well plates, then serum-starved cells were treated with various concentrations of sodium butyrate as indicated. On subsequent days, cell proliferation was measured using the MTS assay. A dose-response effect with added sodium butyrate is seen over 4 days. Results are expressed in absorbance readings at 490 nm as a % of the untreated control ± SE (n=16).

Figure 4. Effect of sodium butyrate on apoptosis. (A) Immunoblot analysis of PARP in Hs578T and MCF-7 cell lysates obtained during treatment with sodium butyrate (Day1 and Day3). The 85 kDa fragment characteristic of apoptosis is shown. The data shown is representative of two independent experiments. (B) Induction of apoptosis in Hs578T cells treated with increasing doses (0-10 mM) of sodium butyrate for 72 h. Cytoplasmic extracts were prepared from attached cells, and apoptotic cell death was quantitated by ELISA measuring cytoplasmic histone-bound DNA complexes characteristic of apoptosis, as described in the "Materials and Methods." Results are expressed as mean absorbance ± SD of two independent experiments performed in duplicate (n=4).

Figure 5. Effect of sodium butyrate on expression of $p21^{Waf1/Cip1}$ mRNA (A), and protein of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p16^{INK4}$ (B). (A) Representative Northern blots. Serum-starved cells were treated for 18 h with various concentrations of sodium butyrate as indicated. Total RNA was harvested, and 5 μg per lane was electrophoresed. The membrane was probed with labeled cDNA fragments for $p21^{Waf1/Cip1}$. The 28S rRNA is presented as an indicator of loading. The data shown are representative of at least three separate experiments. (B) Representative Western immunoblots. 20 μg of total protein from whole cell lysates obtained at 24 h post-treatment per lane were loaded onto 15% SDS-PAGE under reducing conditions, and immunoblotted with anti-$p21^{Waf1/Cip1}$, anti-$p27^{Kip1}$ and anti-$p16^{INK4}$, as indicated in Materials and Methods. Two sets of untreated controls were derived from different wells in the same cultured plates. The data shown represent two separate experiments. $p16^{INK4}$ was only detectable in the cell lysates of HME cells.

Figure 6. Northern blot analysis of the effect of sodium butyrate on the expression of IGFBP-3 mRNA. Total RNA was harvested, and 5 μg per lane was electrophoresed. The membrane was subsequently probed with labelled cDNA fragments of IGFBP-3. (A) Time-course expression of IGFBP-3 mRNA in Hs578T cells. Serum-starved cells were treated for 6 h, 12 h and 24 h, with or without NaB as indicated. (B) Representative

Northern blots for IGFBP-3 mRNA expression at 18 h (HMEC) and 24 h (Hs578T) post-treatment. 28S rRNA methylene blue membrane staining is presented as an indicator of equal loading. Densitometric analysis adjusted for 18S and 28S rRNA is also shown. Each result represents at least three independent experiments. In MCF-7 cells, IGFBP-3 mRNA was not detectable in 10 μ g of total RNA.

Figure 7. Effect of sodium butyrate on IGFBP production in Hs578T, MCF-7 and HME cells analyzed by Western ligand blotting. Serum-starved cells were treated with various concentrations of sodium butyrate. Conditioned media harvested from duplicate wells within each experiment were pooled, size-fractionated using 12% SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes, and treated with [^{125}I]-labeled IGF-I, as indicated in Materials and Methods. (A) Time-course expression of IGFBP-3 protein in Hs578T cells. Serum-starved cells were treated with or without sodium butyrate as indicated and Western ligand blot was performed over 4 days. (B) Representative Western ligand blots using 72 h conditioned media in Hs578T, MCF-7 and HME cells. The data shown were derived from at least three independent experiments.

Figure 8. Effect of sodium butyrate on (A) IGFBP-rP2 mRNA expression, and (B) IGFBP-rP2 and –rP1 protein expression in Hs578T, MCF-7 and HME cells. (A) Representative Northern blots . Total RNA was harvested at 18 h post-treatment, and 5 μg per lane was electrophoresed. The membrane was subsequently probed with labeled cDNA fragments of IGFBP-rP2. 28S rRNA methylene blue membrane staining is presented as an indicator of equal loading. Densitometric analysis adjusted for 18S and 28S rRNA is also shown. Each result represents two independent experiments. In MCF-7 cells, IGFBP-rP2 mRNA was not detectable in 10 μ g of total RNA. (B) Representative Western immunoblot analysis. Serum-starved cells were treated with various concentrations of sodium butyrate for 72 h. Conditioned media harvested from duplicate wells within each experiment were pooled, size-fractionated using 12% (for IGFBP-rP2) or 15% (for IGFBP-rP1) SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes, and treated with appropriate antibodies, as indicated in Materials and Methods. IGFBP-rP1 was not detectable in the conditioned media of MCF-7 cells.

Figure 9. Schematic diagram showing a summary of sequential effects of sodium butyrate in Hs578T cells observed in this study. The upregulation of IGFBP-3 and IGFBP-rP2 is shown above the time line and other effects of NaB including cell growth

inhibition and cell death are shown below. For further description refer to the discussion in the text.

References

Adamo M L, Shao Z M, Lanau F, Chen J C, Clemmons D R, Roberts C T, Jr., LeRoith D, and Fontana J A 1992 Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs):IGFBP-2, -3, and –4 gene expression and protein secretion in a breast cancer cell line. Endocrinology 131: 1858-1866.

Archer S Y, and Hodin R A 1999 Histone acetylation and cancer. Curr Opin Genet Dev 9: 171-174

Archer S Y, Meng S, Shei A, and Hodin R A 1998 $p21^{WAF1}$ is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci USA 95: 6791-6796.

Baxter R C, Binoux M A, Clemmons D R, Conover C A, Drop S L, Holly J M, Mohan S, Oh Y, and Rosenfeld R G 1998 Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. J Clin Endocrinol Metab 83: 3213.

Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger B R, and Kley N 1995 Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 377: 646-649.

Burger A M, Zhang X, Li H, Ostrowski J L, Beatty B, Venanzoni M, Papas T, and Seth A 1998 Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. Oncogene 16: 2459-2467.

Carducci M A, Nelson J B, Chan-Tack K M, Ayyagari S R, Sweatt W H, Campbell P A, Nelson W G, and Simons J W 1996 Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. Clin Cancer Res 2: 379-387.

Colston K W, Perks C M, Xie S P, and Holly J M 1998 Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3. J Mol Endocrinol 20: 157-162.

Coradini D, Biffi A, Costa A, Pellizzaro C, Pirronello E, and Di Fronzo G 1997 Effect of sodium butyrate on human breast cancer cell lines. Cell Prolif 30: 149-159.

de Haan J B, Gevers W, and Parker M I 1986 Effects of sodium butyrate on the synthesis and methylation of DNA in normal cells and their transformed counterparts. Cancer Res 46: 713-716.

Gleave M E, Sato N, Sadar M, Yago V, Bruchovsky N, and Sullivan L 1998 Butyrate analogue, isobutyramide, inhibits tumor growth and time to androgen-independent progression in the human prostate LNCaP tumor model. J Cell Biochem 69: 271-281.

Gucev A S, Oh Y, Kelley K M, and Rosenfeld R G 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor β2-induced growth inhibition in human breast cancer cells. Cancer Res 56: 1545-1550.

Hague A, Díaz G D, Hicks D J, Krajewski S, Reed J C, and Paraskeva C 1997 bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however overexpression of bcl-2 does not protect against bak-mediated apoptosis. Int J Cancer 72: 898-905.

Hague A, Manning A M, Hanlon K A, Huschtscha L I, Hart D, and Paraskeva C 1993 Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. Int J Cancer 55: 498-505.

Hassig C A, Tong J K, and Schreiber S L 1997 Fiber-derived butyrate and the prevention of colon cancer. Chem Biol 4: 783-789.

Hishikawa K, Oemar B S, Tanner F C, Nakaki T, Lüscher T F, and Fujii T 1999 Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. J Biol Chem 274: 37461-37466.

Huang H, Reed C P, Zhang J S, Shridhar V, Wang L, and Smith D I 1999 Carboxypeptidase A3 (CPA3): a novel gene highly induced by histone deacetylase

inhibitors during differentiation of prostate epithelial cancer cells. Cancer Res 59: 2981-2988.

Hunter T, and Pines J 1994 Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell 79: 573-582.

Huynh H, Yang X, and Pollak M 1996 Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. J Biol Chem 271: 1016-1021.

Hwa V, Oh Y, and Rosenfeld R G 1999 Insulin-like growth factor binding proteins: a proposed superfamily. Acta Paediatr Suppl 428: 37-45.

Kaufmann S H, Desnoyers S, Ottaviano Y, Davidson N E, and Poirier G G 1993 Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 53: 3976-3985.

Kelley K M, Oh Y, Gargosky S E, Gucev Z, Matsumoto T, Hwa V, NG L, Simpson D M, and Rosenfeld R G 1996 Insulin-like growth factor-binding proteins and their regulatory dynamics. Int J Biochem Cell Biol 28: 619-637.

Knabbe C, Lippman M E, Wakefield L M, Flanders K C, Kasid A, Derynck R, and Dickson R B 1987 Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417-428.

Lallemand F, Courilleau D, Buquet-Fagot C, Atfi A, Montagne M N, and Mester J 1999 Sodium butyrate induces G2 arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA rereplication. Exp Cell Res 247: 432-440.

Lallemand F, Courilleau D, Sabbah M, Redeuilh G, and Mester J 1996 Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. Biochem biophys Res Commun 229: 163-169.

Lazebnik Y A, Kaufmann S H, Desnoyers S, Poirier G G, and Earnshaw W C 1994 Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371: 346-347.

Litvak D A, Evers M, Hwang K O, Hellmich M R, Ko T C, and Townsend C M Jr 1998 Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Surgery 124: 161-170.

Macaulay V M 1992 Insulin-like growth factors and cancer. Br J Cancer 65: 311-320.

Mandal M, and Kumar R 1996 Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth Differ 7: 311-318.

Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, Fujita T, Ohtani-Fujita N, Matsukawa Y, Tokino T, Yamagishi H, Oka T, Nomura H, and Sakai T 1997 Butyrate activates the *WAF1/Cip1* gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 272: 22199-22206.

Nickerson T, Huynh H, and Pollak M 1997 Insulin-like growth factor binding protein-3 induces apoptosis in MCF7 breast cancer cells. Biochem Biophys Res Commun 237: 690-693.

Nishimura A, Fujimoto M, Oguchi S, Fusunyan R D, MacDermott R P, and Sanderson I R 1998 Short-chain fatty acids regulate IGF-binding protein secretion by intestinal epithelial cells. Am J Physiol 275: E55-E63.

Ogryzko V V, Wong P, and Howard B H 1997 WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. Mol Cell Biol 17: 4877-4882.

Oh Y 1998 IGF-independent regulation of breast cancer growth by IGF binding proteins. Breast Cancer Res Treat 47: 283-293.

Oh Y, Müller H L, Lamson G, and Rosenfeld R G 1993 Insulin-like growth factor (IGF)- independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. Cell surface binding and growth inhibition. J Biol Chem 268: 14964-14971.

Oh Y, Müller H L, NG L, and Rosenfeld R G 1995 Transforming growth factor-β-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. J Biol Chem 270: 13589-13592.

Pouillart P R 1998 Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies. Life Sci 63: 1739-1760.

Rajah R, Valentinis B, and Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-β1 on programmed cell death through a p53-and IGF-independent mechanism. J Biol Chem 272: 12181-12188.

Rajaram S, Baylink D J, and Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18: 801-831.

Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman H L, Kajstura J, Rubin R, Zoltick P, and Baserga R 1995 The insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. Cancer Res 55: 2463-2469.

Rosenfeld R G, Lamson G, Pham H, Oh Y, Conover C, De Leon D D, Donovan S M, Ocrant I, and Giudice L 1990 Insulin-like growth factor binding proteins. Recent Prog Horm Res 46: 99-159.

Rozen F, Zhang J, and Pollak M 1998 Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breast cancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. Int J Oncol 13: 865-869.

Schwartz B, Avivi-Green C, and Polak-Charcon S 1998 Sodium butyrate induces retinoblastoma protein dephosphorylation, p16 expression and growth arrest of colon cancer cells. Mol Cell Biochem 188: 21-30.

Sheikh M S, Shao Z M, Clemmons D R, LeRoith D, Roberts C T Jr, and Fontana J A 1992 Identification of the insulin-like growth factor binding proteins 5 and 6 (IGFBP-5 and 6) in human breast cancer cells. Biochem Biophys Res Commun 183: 1003-1010.

Siavoshian S, Blottiere H M, Cherbut C, and Galmiche J P 1997 Butyrate stimulates cyclin D and p21 and inhibits cyclin-dependent kinase 2 expression in HT-29 colonic epithelial cells. Biochem Biophys Res Commun 232: 169-172.

Staiano-Coico L, Khandke L, Krane J F, Sharif S, Gottlieb A B, Krueger J G, Heim L, Rigas B, and Higgins P J 1990 TGF-α and TGF-β expression during sodium-n-butyrate-induced differentiation of human keratinocytes: evidence for subpopulation-specific up-regulation of TGF-β mRNA in suprabasal cells. Exp Cell Res 191: 286-291.

Valentinis B, Bhala A, DeAngelis T, Baserga R, and Cohen P 1995 The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol Encocrinol 9: 361-367.

Vaziri C, Stice L, and Faller D V 1998 Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. Cell Growth Differ 9: 465-474.

Velázquez O C, Lederer H M, and Rombeau J L 1997 Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implication. Adv Exp Med Biol 427: 123-134.

Wilson E M, Oh Y, and Rosenfeld R G 1997 Generation and characterization of IGFBP-7 antibody: identification of 31kD IGFBP-7 in human biological fluids and Hs578T human breast cancer conditioned media. J Clin Endocrinol Metab 82: 1301-1303.

Yamamoto H, Fujimoto J, Okamoto E, Furuyama J, Tamaoki T, and Hashimoto-Tamaoki T 1998 Suppression of growth of hepatocellular carcinoma by sodium butyrate *in vitro* and *in vivo*. Int J Cancer 76: 897-902.

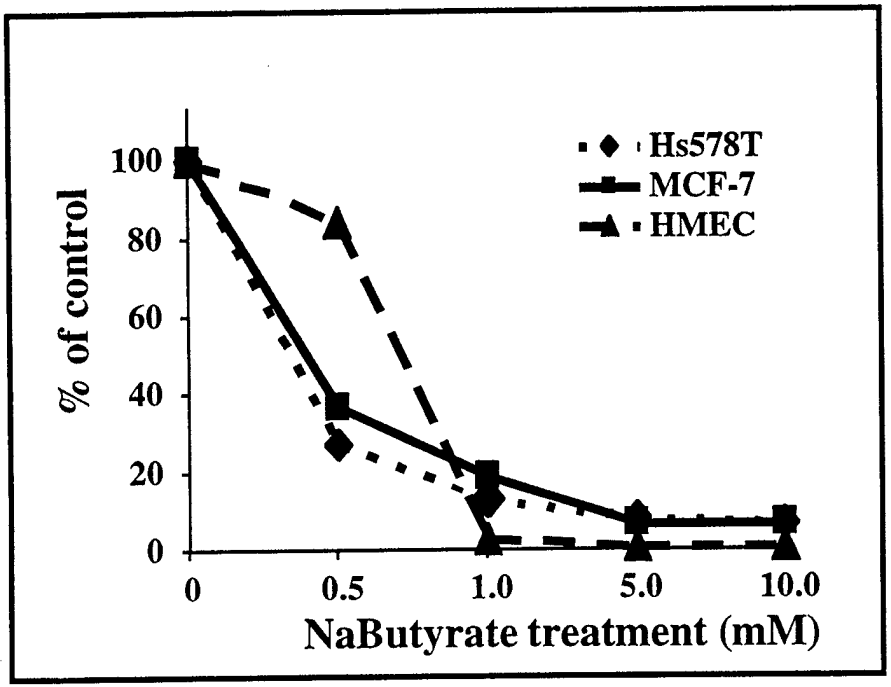
Yang D H, Kim H S, Wilson E M, Rosenfeld R G, and Oh Y 1998 Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF-β in Hs578T human breast cancer cells. J Clin Endocrinol Metab 83: 2593-2596.

Yoshida M, Kijima M, Akita M, and Beppu T 1990 Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem 265: 17174-17179.

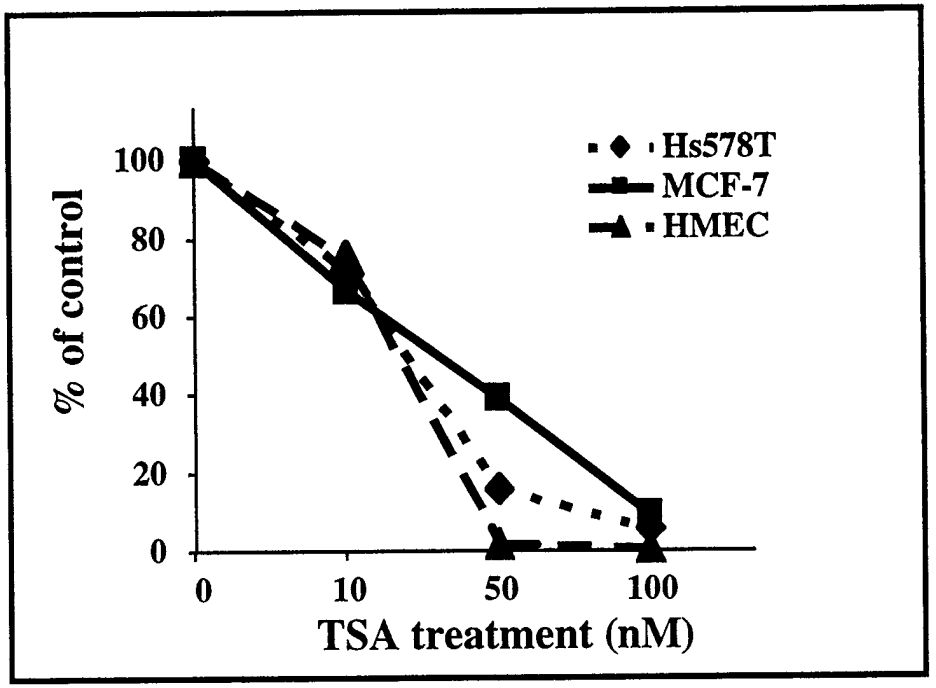
Zugmaier G, Ennis B W, Deschauer B, Katz D, Knabbe C, Wilding G, Daly P, Lippman M E, and Dickson R B 1989 Transforming growth factors type β1 and β2 are equipotent growth inhibitors of human breast cancer cell lines. J Cell Physiol 141: 353-361.

Figure 1.

(A)


% of control
100
80
60
40
20
0
0
0.5
1.0
5.0
10.0
NaButyrate treatment (mM)
Hs578T
MCF-7
HMEC

(B)


% of control
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0
10
50
100
TSA treatment (nM)
Hs578T
MCF-7
HMEC

Figure 2.

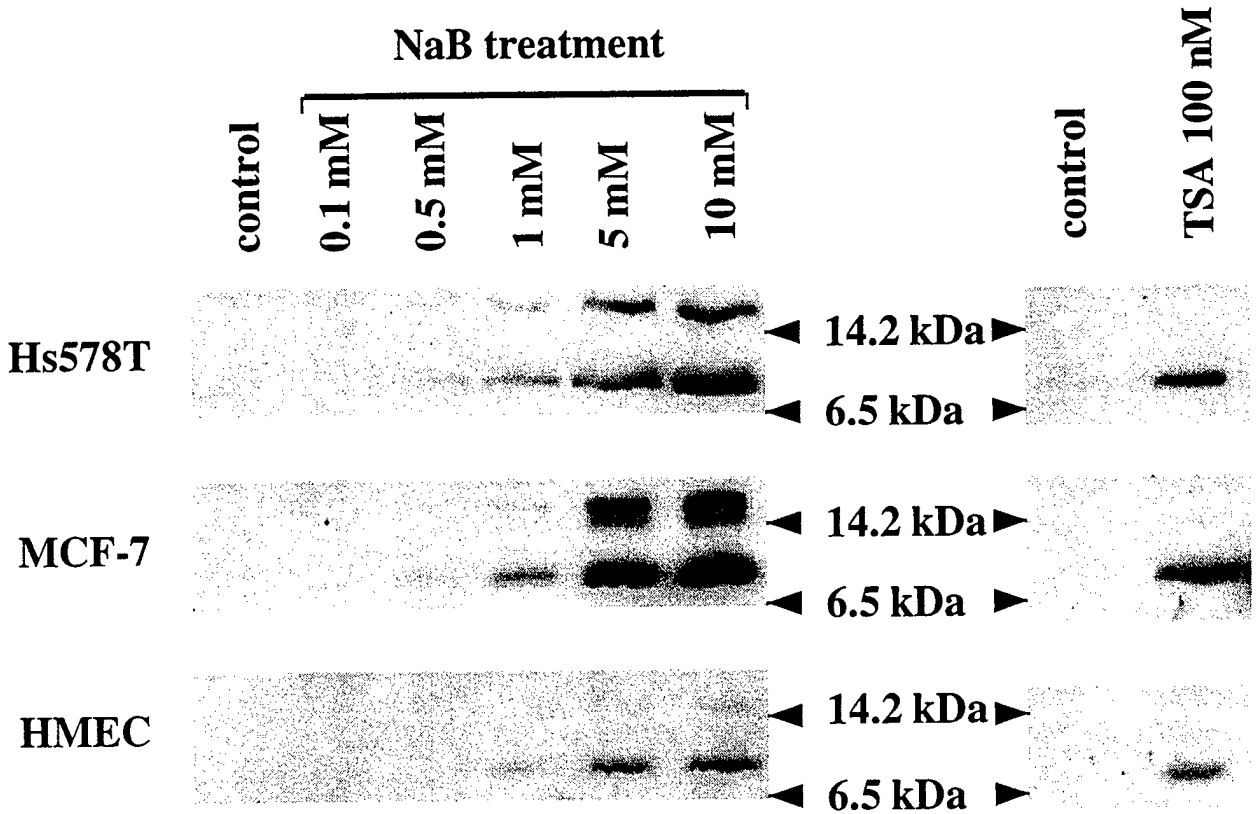
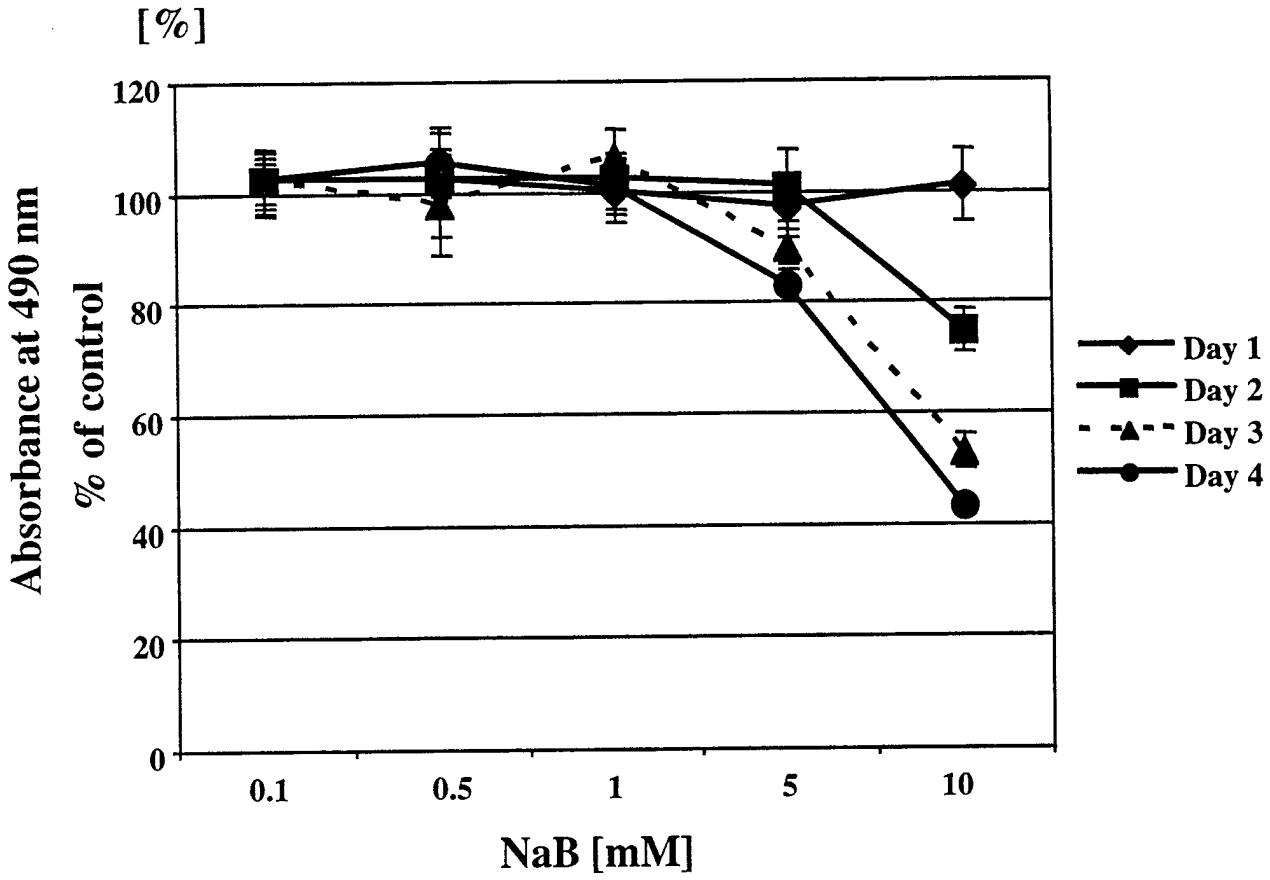

NaB treatment
control
0.1 mM
0.5 mM
1 mM
5 mM
10 mM
control
TSA 100 nM
Hs578T
MCF-7
HMEC
14.2 kDa
6.5 kDa
14.2 kDa
6.5 kDa
14.2 kDa
6.5 kDa

Figure 3.


[%]
120
100
80
60
40
20
0
Absorbance at 490 nm
% of control
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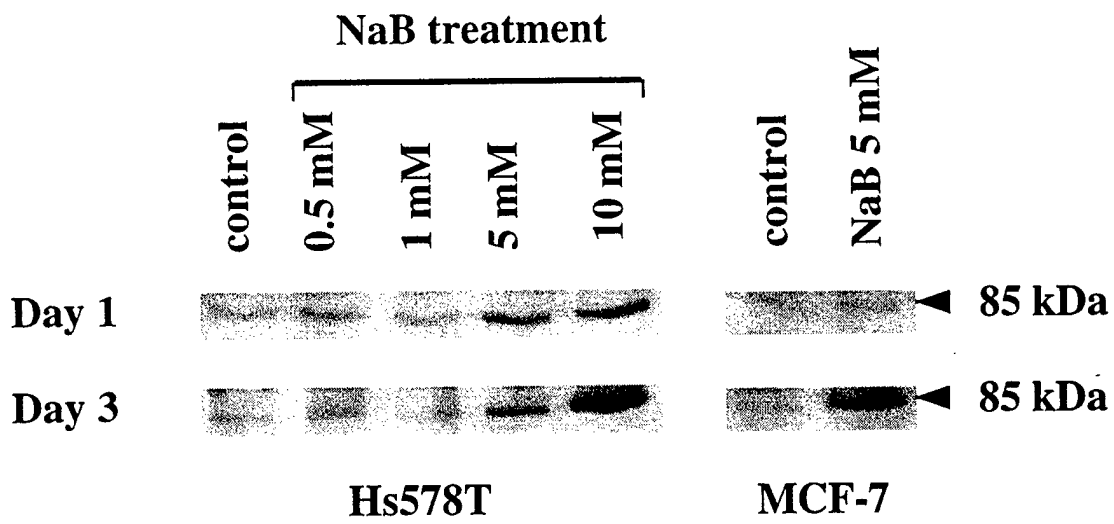
0.5
1
5
10
NaB [mM]
Day 1
Day 2
Day 3
Day 4

Figure 4. (A)


NaB treatment
control
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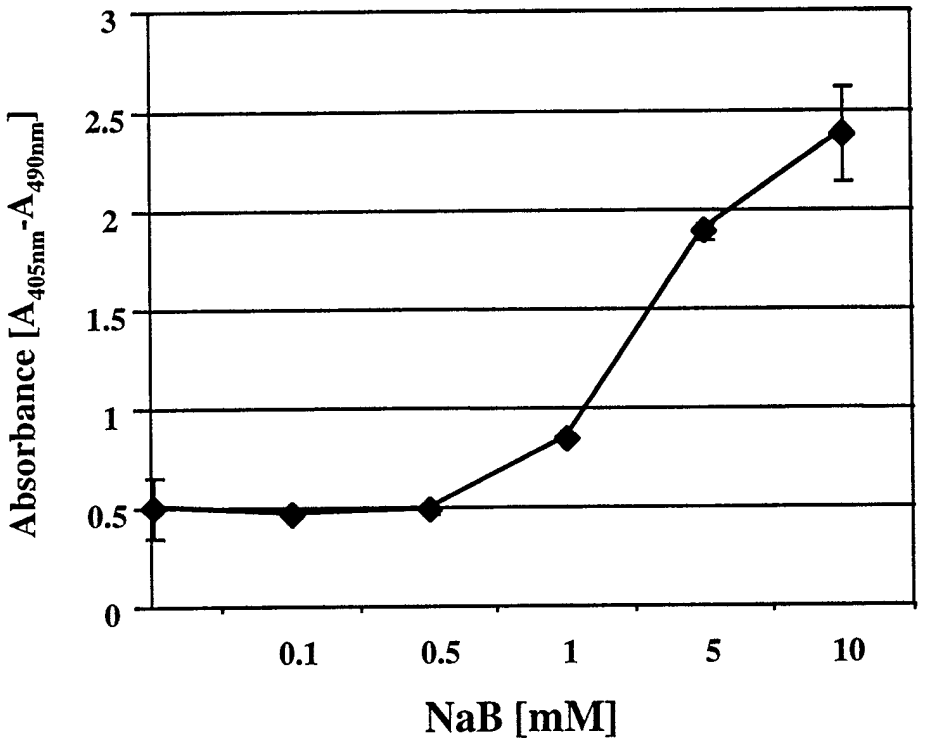
1 mM
5 mM
10 mM
control
NaB 5 mM
Day 1
Day 3
85 kDa
85 kDa
Hs578T
MCF-7

Figure 4. (B)



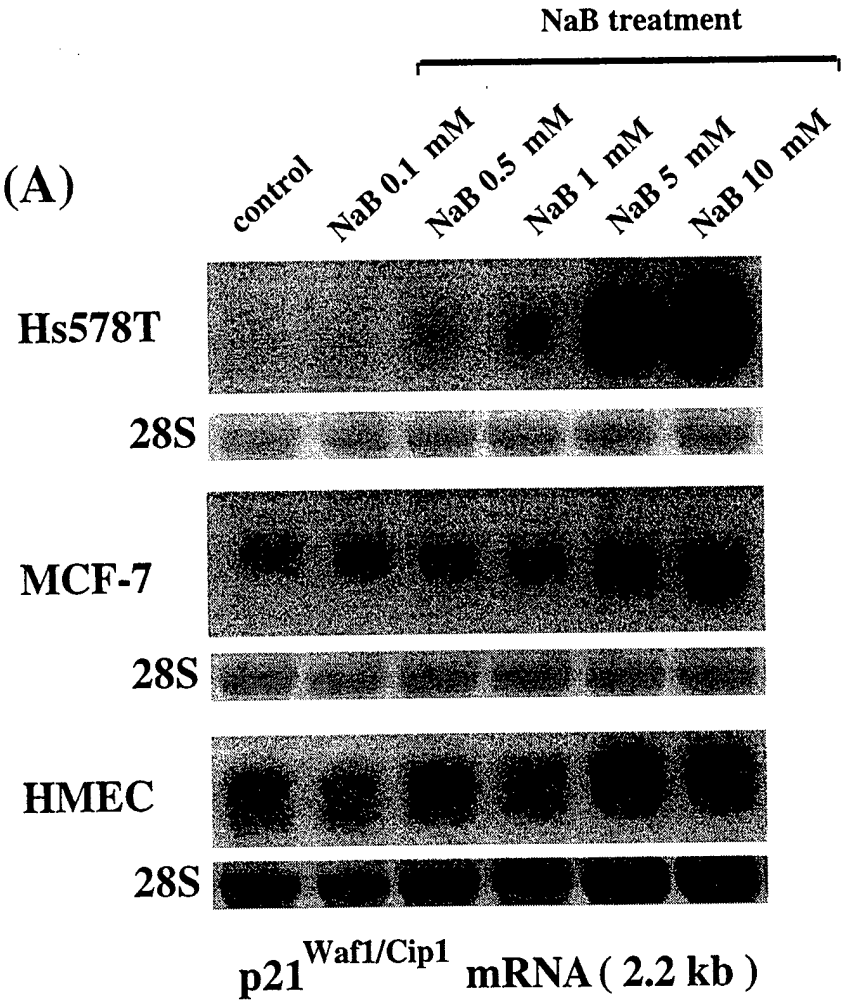

NaB treatment
(A)
control
NaB 0.1 mM
NaB 0.5 mM
NaB 1 mM
NaB 5 mM
NaB 10 mM
Hs578T
28S
MCF-7
28S
HMEC
28S
p21Waf1/Cip1 mRNA (2.2 kb)

Figure 5.

Figure 5. **(B)**

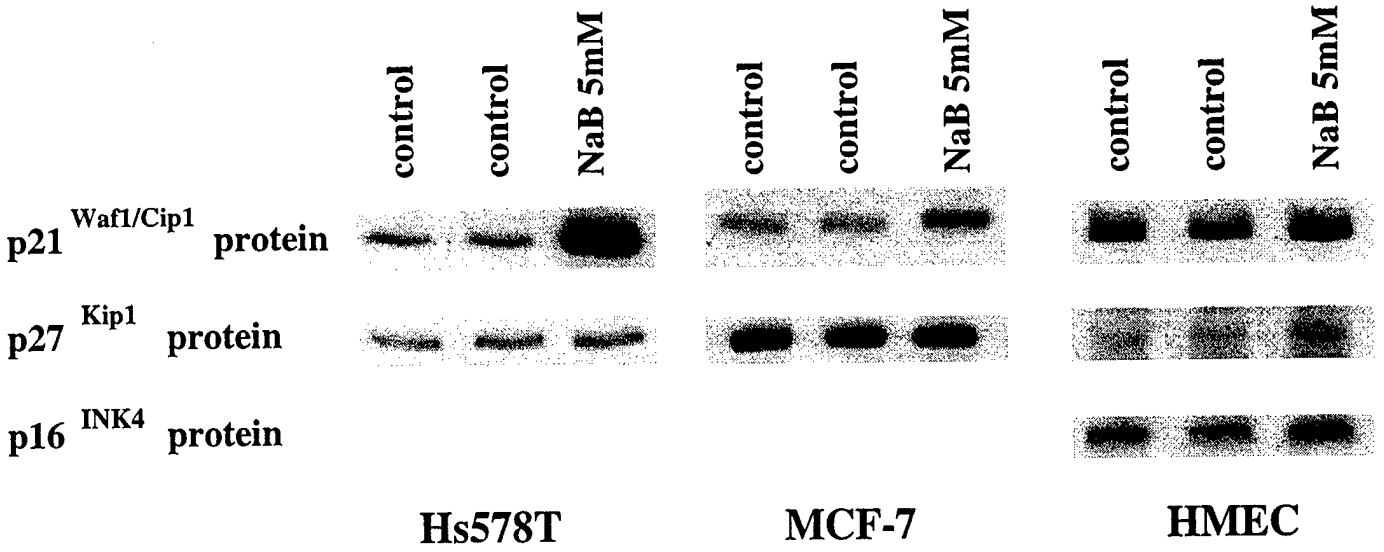

control
control
NaB 5mM
control
control
NaB 5mM
control
control
NaB 5mM
p21 Waf1/Cip1 protein
p27 Kip1 protein
p16 INK4 protein
Hs578T
MCF-7
HMEC

Figure 6.

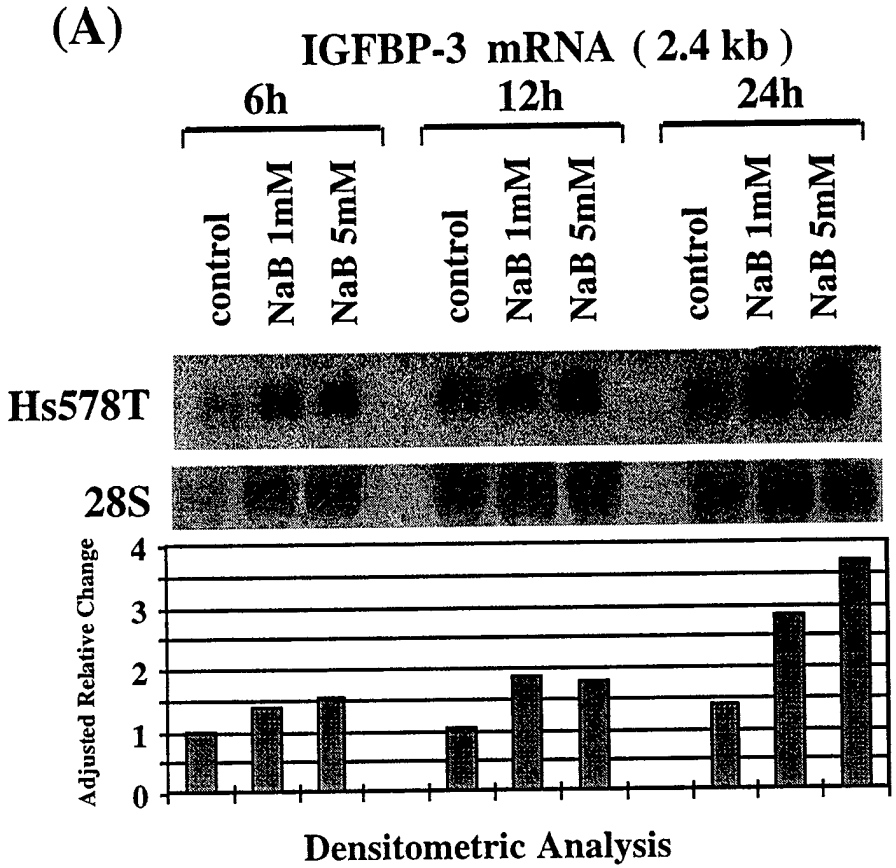

(A)
IGFBP-3 mRNA (2.4 kb)
6h
12h
24h
control
NaB 1mM
NaB 5mM
control
NaB 1mM
NaB 5mM
control
NaB 1mM
NaB 5mM
Hs578T
28S
Adjusted Relative Change
0
1
2
3
4
Densitometric Analysis

Figure 6.

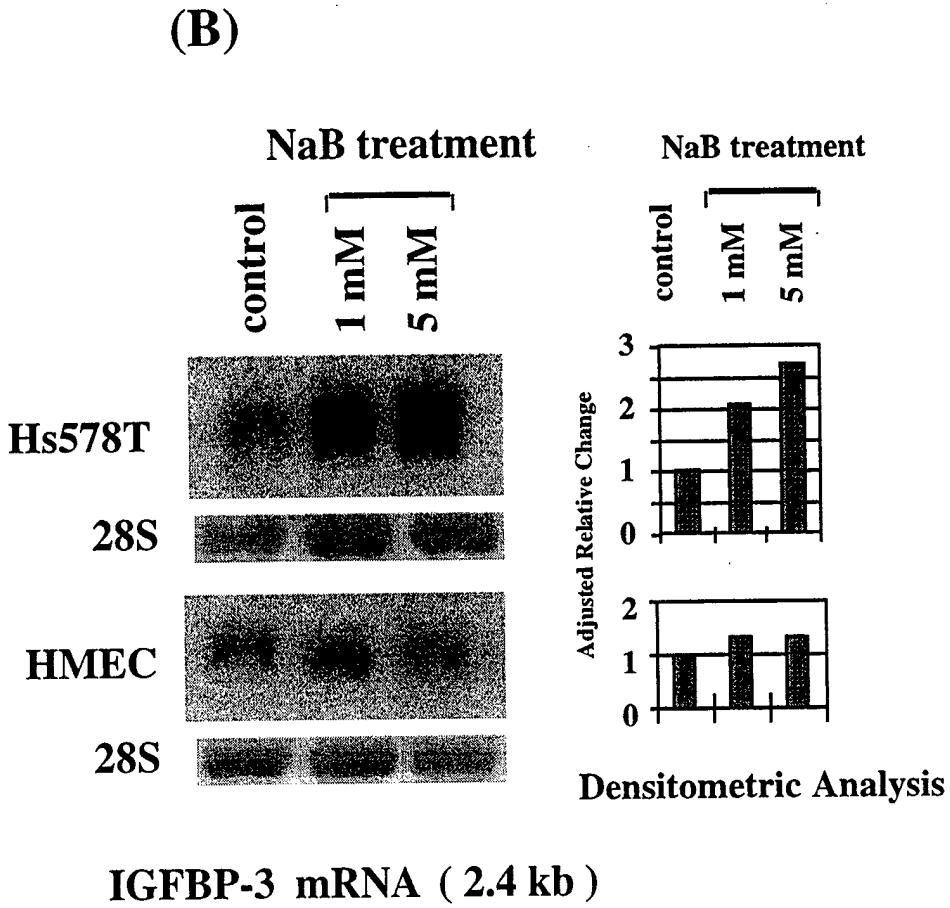

(B)
NaB treatment
control
1 mM
5 mM
Hs578T
28S
HMEC
28S
NaB treatment
control
1 mM
5 mM
Adjusted Relative Change
3
2
1
0
2
1
0
Densitometric Analysis
IGFBP-3 mRNA (2.4 kb)

Figure 7. **(A)**

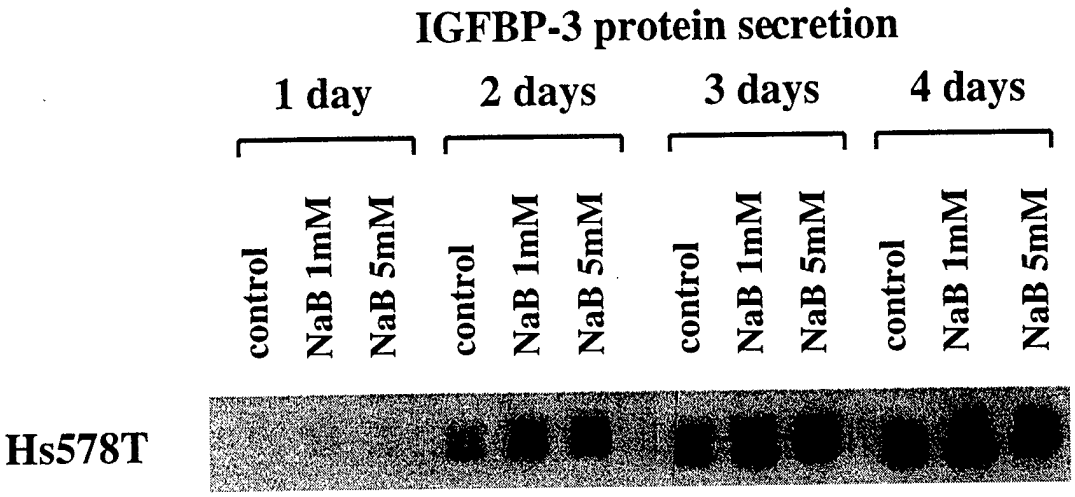

IGFBP-3 protein secretion
1 day
2 days
3 days
4 days
control
NaB 1mM
NaB 5mM
control
NaB 1mM
NaB 5mM
control
NaB 1mM
NaB 5mM
control
NaB 1mM
NaB 5mM
Hs578T

Figure 7. **(B)**

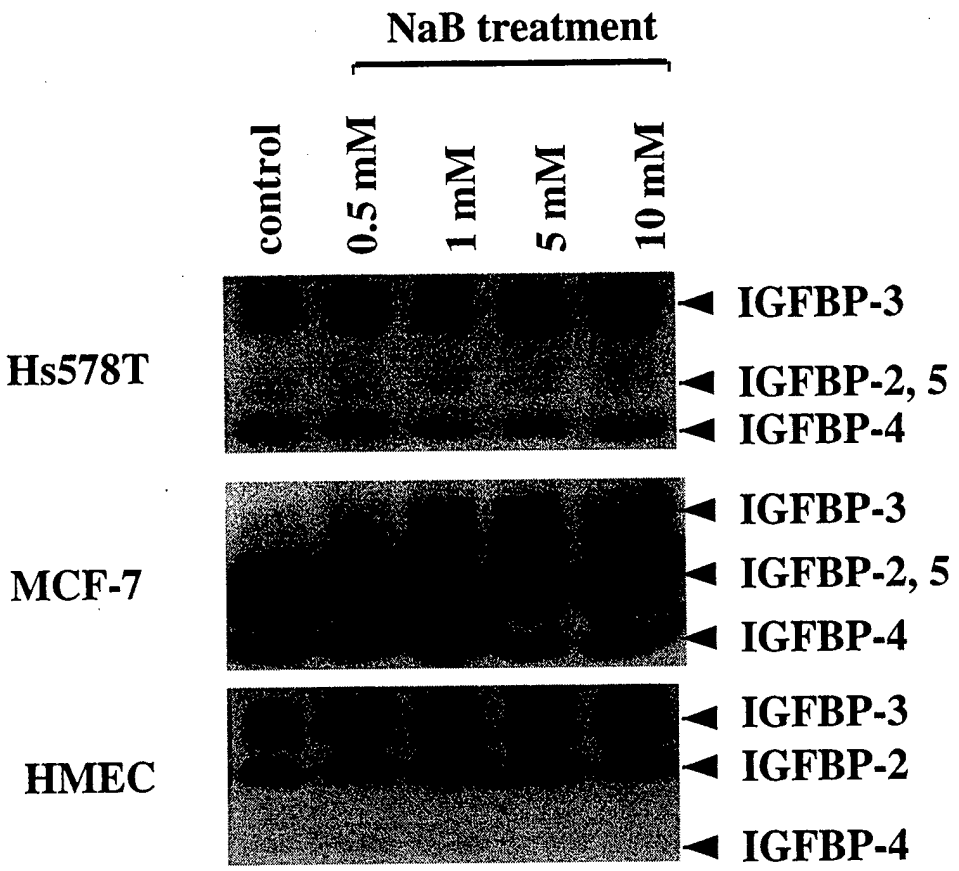

NaB treatment
control
0.5 mM
1 mM
5 mM
10 mM
Hs578T
IGFBP-3
IGFBP-2, 5
IGFBP-4
MCF-7
IGFBP-3
IGFBP-2, 5
IGFBP-4
HMEC
IGFBP-3
IGFBP-2
IGFBP-4

Figure 8.

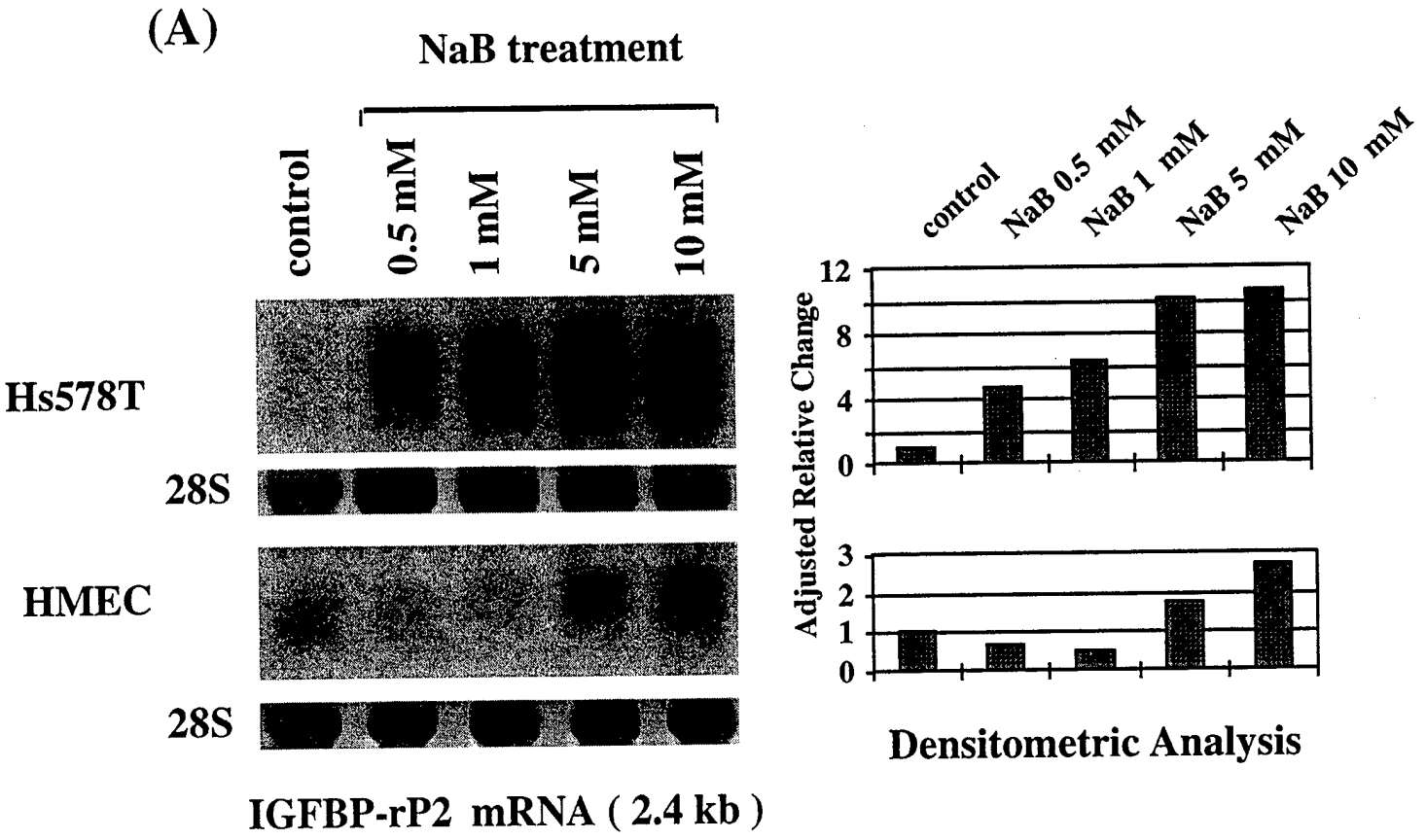

(A)
NaB treatment
control
0.5 mM
1 mM
5 mM
10 mM
Hs578T
28S
HMEC
28S
IGFBP-rP2 mRNA (2.4 kb)
control
NaB 0.5 mM
NaB 1 mM
NaB 5 mM
NaB 10 mM
Adjusted Relative Change
12
8
4
0
3
2
1
0
Densitometric Analysis

Figure 8. **(B)**

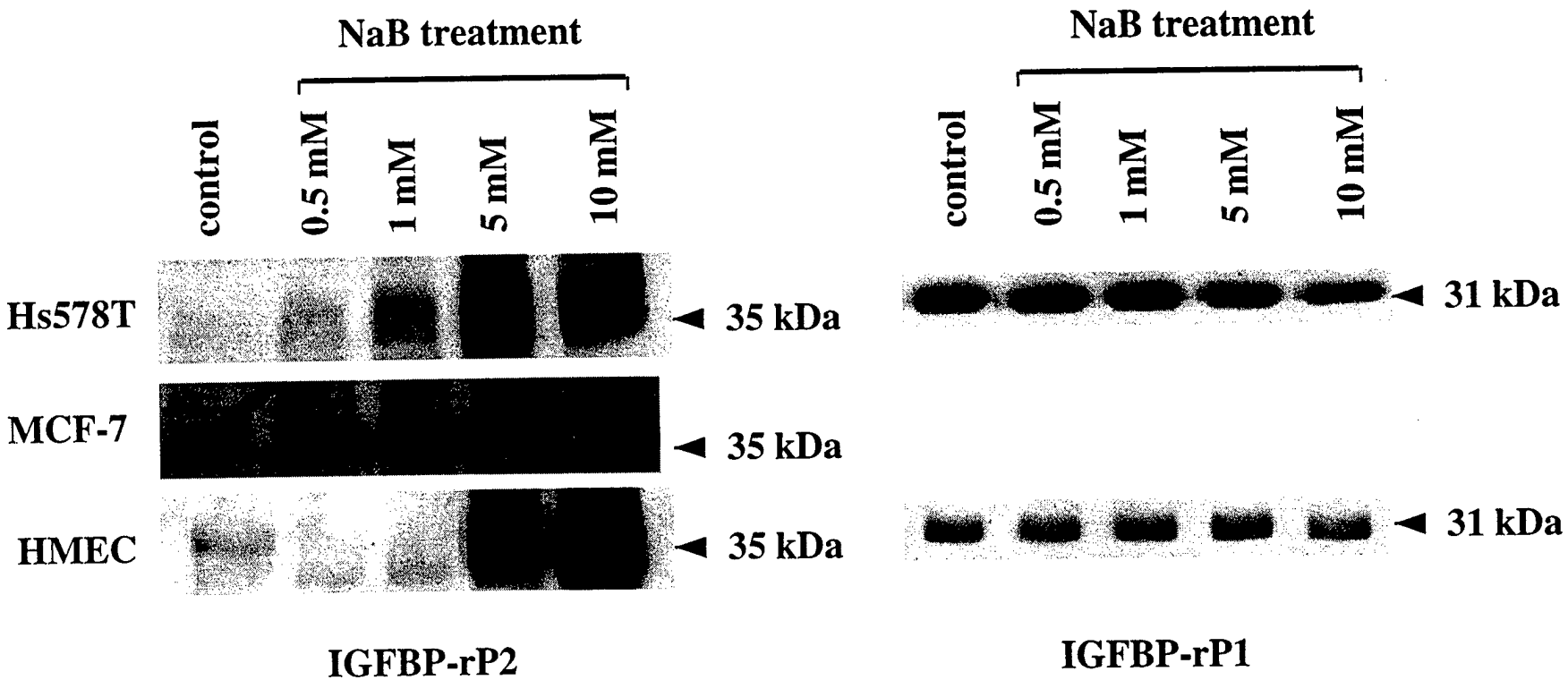
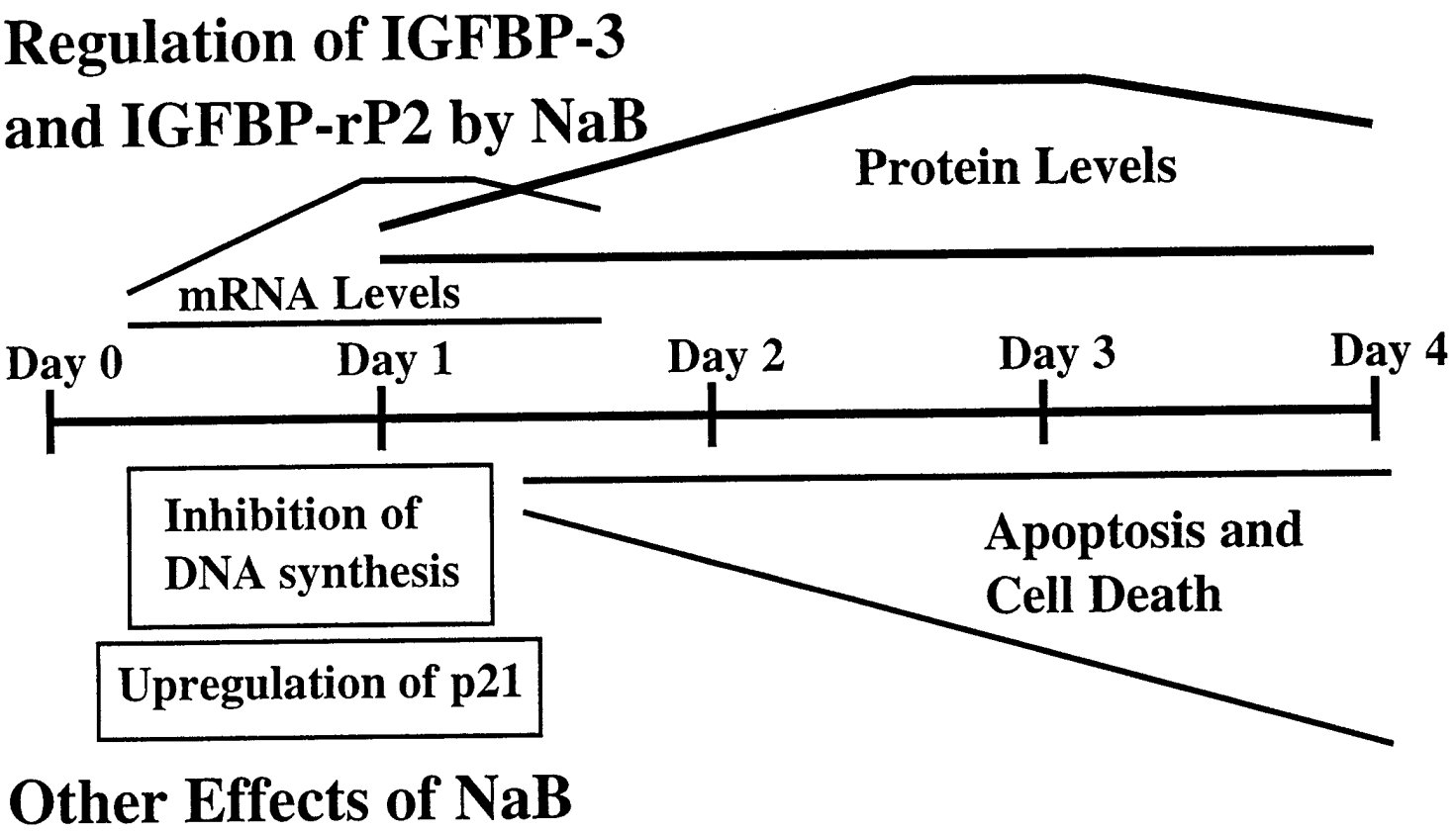

NaB treatment
control
0.5 mM
1 mM
5 mM
10 mM
Hs578T
MCF-7
HMEC
35 kDa
35 kDa
35 kDa
IGFBP-rP2
NaB treatment
control
0.5 mM
1 mM
5 mM
10 mM
31 kDa
31 kDa
IGFBP-rP1

Figure 9.


Regulation of IGFBP-3
and IGFBP-rP2 by NaB
Protein Levels
mRNA Levels
Day 0
Day 1
Day 2
Day 3
Day 4
Inhibition of
DNA synthesis
Upregulation of p21
Apoptosis and
Cell Death
Other Effects of NaB