Effects of sodium butyrate on expression of members of the IGF-binding protein superfamily in human mammary epithelial cells

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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 cecum 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 NaB on three human mammary epithelial cells and regulation of the IGF axis, specifically, IGF-binding protein-3 (IGFBP-3), a known growth regulator in human mammary cells, and IGFBP-related protein 2 (IGFBP-rP2)/connective tissue growth factor.

NaB inhibited DNA synthesis, as measured by [³H]thymidine incorporation, in estrogen-responsive (MCF-7) and estrogen-non-responsive (Hs578T) breast cancer cells, and normal human mammary epithelial cells (HMEC) to a similar degree (up to 90% inhibition at 1–10 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 NaB 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 HMEC, 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 1–10 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 (i) uniformly suppresses DNA synthesis in both cancerous and non-cancerous mammary cells, and (ii) 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.

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Introduction

Butyric acid 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, Velázquez *et al.* 1997, Gleave *et al.* 1998, 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 (de Haan *et al.* 1986, Archer & Hodin 1999).

It has been reported that butyrate, as well as trichostatin A (TSA), a specific histone deacetylase inhibitor (Yoshida *et al.* 1990), modulates specific genes involved in cell cycle regulation and apoptosis. These include the cyclin-dependent kinase (cdk) inhibitor p21^{Waf1/Cip1} (Archer *et al.* 1998), p16^{INK4} (Schwartz *et al.* 1998), p27^{Kip1} (Litvak *et al.* 1998), retinoblastoma protein (Vaziri *et al.* 1998), cyclin D1 (Lallemand *et al.* 1996, Siavoshian *et al.* 1997), Bcl-2 and Bax (Mandal & Kumar 1996, Hague *et al.* 1997). 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 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 -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 (Oh *et al.* 1995, Gucev *et al.* 1996, Rajah *et al.* 1997), anti-estrogens (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 sodium butyrate (NaB) on members of the IGFBP superfamily in human mammary epithelial cells, using estrogen-responsive (MCF-7) and estrogen-non-responsive (Hs578T) breast cancer cells, and normal human mammary epithelial (HMEC) cells. We report here that NaB upregulates IGFBP-3 and IGFBP-rP2 mRNA and protein in mammary epithelial cells.

Materials and Methods

Materials

NaB, TSA, BSA and 0.4% trypan blue solution were purchased from Sigma Chemical Co. (St Louis, MO, USA). 125 I-labeled IGF-I was kindly provided by Diagnostic Systems Laboratories (Webster, TX, USA). Polyclonal anti-IGFBP-3, anti-IGFBP-rP1, anti-IGFBP-rP2 and anti-HEC1 (specific for both human IGFBP-2 and -3) antisera were generated as previously described (Rosenfeld *et al.* 1990, Oh *et al.* 1993, Wilson *et al.* 1997, Yang *et al.* 1998). Polyclonal anti-IGFBP-5 antibody was purchased from Austral Biologicals (San Ramon, CA, USA). Polyclonal anti-acetyl-lysine, anti-acetylated histone H3 and anti-acetylated histone H4 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal anti-poly(ADP-

ribose)polymerase (PARP) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies, anti-p21^{Waf1/Cip1}, anti-p16^{INK4} and anti-p27^{Kip1} were purchased from Transduction Laboratories (Lexington, KY, USA), PharMingen (San Diego, CA, USA) and Calbiochem (Cambridge, MA, USA) respectively.

Cell culture

Hs578T estrogen-non-responsive human breast cancer cells and MCF-7 estrogen-responsive human breast cancer cells were purchased from ATCC (Manassas, VA, USA). Both cell lines were maintained in 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, USA), and maintained in mammary epithelium basal medium (MEBM) with growth supplements (bovine pituitary extract (BPE), human epidermal growth factor, 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% CO₂. At 90% confluence, cells were placed in serum-free media for 12 h, then treated as indicated in the text. After 22 h, 0.1 μ Ci [3 H]thymidine (NEN, Boston, MA, USA) in a volume of 25 μ l PBS was added to each well, and the plate was incubated for 4 h at 37 °C. Cells were washed with cold PBS twice, treated with 10% trichloroacetic acid (TCA) for 10 min at -20 °C, washed with 10% TCA followed by 95% ethanol, and lysed with 400 μ l 0.25 N NaOH per well. Cell lysates (CL) from each well were transferred to scintillant vials, then 10 ml scintillation fluid with 100 μ l 2 N HCl were 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% confluence, they were incubated for 12 h in serum-free DMEM, then treated with various concentrations of NaB as indicated in the text in serum-free media. After a further incubation of 1–4 days, the MTS reagent (Promega Co., Madison, WI, USA) was added in the ratio recommended by the manufacturer. At 15 min intervals, the absorbance of the formazan product at 490 nm was read with a plate reader (Spectra Shell Reader; SLT Lab Instruments GmbH, 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 as to the butyrate-treated wells. After the reading, cells were trypsinized, then the cell number was counted with a

hemacytometer. Using this method, a linear correlation was obtained between direct cell counts from 1×10^3 to 1×10^4 per well and the absorbance ($r=0.85$, $n=70$) (data not shown). The experiment was repeated three times in conditions where the starting untreated control cell number was 8×10^3 per well, and the absorbance at 490 nm read at 45 min 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), which measures cytoplasmic histone-bound DNA fragments produced during apoptotic DNA fragmentation, was used (Mandal & Kumar 1996). Hs578T cells were seeded into a 96-well plate at 80% confluence, they were incubated for 12 h in serum-free DMEM, then treated with various concentrations of NaB in serum-free media. After 72 h, cytoplasmic extracts were made from attached cells by adding 100 μ l lysis buffer to 5×10^3 cells per well. Supernatant (20 μ l from 100 μ l) was analyzed in the ELISA, as directed by the manufacturer's protocol. Briefly, the samples were placed into streptavidin-coated multi-well plates, a mixture of anti-histone-biotin and anti-DNA-peroxidase was added and incubated. The 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 CL

Cells were seeded in 12-well plates. At 95% confluence, they were incubated for 12 h 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. CM samples were collected after 72 h and centrifuged at 1000 *g* for 10 min to remove debris. The harvested CM from duplicate wells within each experiment were pooled and stored at -20°C until assay. Proteins in 40 μ l CM per lane were examined by Western immunoblot or Western ligand blot under non-reducing conditions.

CL samples were harvested at 24 h post-treatment by washing with PBS, and then adding 150 μ l 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) directly to each well. Plates were rocked for 30 min at 4°C , and the lysates were collected and centrifuged at 10 000 *g* for 10 min 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, USA), and 20 μ g 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, USA). Filters were washed in 3% NP-40/ddH₂O for 30 min, blocked with 1% BSA/TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 2 h, and incubated overnight with 2.0×10^6 c.p.m. ¹²⁵I-labeled IGF-I. The membranes were washed, dried and exposed to film (Kodak BioMax MS, Eastman Kodak Co., Rochester, NY, USA) for 12–18 h.

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 electrotransferred onto nitrocellulose, and membranes were blocked with 5% non-fat dry milk/TBS-T for 1 h 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, USA).

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 h, then treated for 18 h 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, USA), and 5 μ g total RNA per sample were separated on a 1% formaldehyde agarose gel and transferred to nylon membranes (GeneScreenPlus; NEN). 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 [³²P]dCTP (Prime-It II; Stratagene, Cedar Creek, TX, USA), washed and autoradiographed.

Densitometric analysis

To quantitate 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).

Figure 1 Effect of NaB (A) and TSA (B) on DNA synthesis in Hs578T, MCF-7 and HMEC cells. Serum-starved cells (85–90% confluent) were incubated in basal medium for 22 h in the absence or presence of various concentrations of NaB or TSA 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.

Results

NaB treatment inhibits DNA synthesis and causes histone hyperacetylation in human mammary epithelial cells

Since it has been demonstrated that treatment of NaB resulted in growth inhibition in a variety of cell systems *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 (Fig. 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 TSA, a specific histone deacetylase inhibitor, and compared its effect on DNA synthesis. As shown in Fig. 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 (Fig. 2). The CL 24 h after treatment with NaB showed an increase of acetylated proteins in a dose-dependent manner, as indicated by the appearance of 11 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 (Fig. 2). Interestingly, only the 11 kDa band was seen at concentrations over 100 nM.

Effects of NaB on reducing cell number and the induction of apoptosis in human mammary epithelial cells

As NaB inhibits DNA synthesis almost completely by 24 h 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 (Fig. 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: first, nuclear enzyme cleavage, and secondly, DNA fragmentation. The nuclear enzyme 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

Figure 2 Effect of NaB on protein acetylation. Serum-starved cells were treated for 24 h with various concentrations of NaB as indicated. CL harvested from duplicate wells within each experiment were pooled, and 20 µg 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 CL treated with 100 nM TSA are shown on the right. Molecular mass markers are also shown. The results are representative of two independent experiments.

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

Figure 4 Effect of NaB on apoptosis. (A) Immunoblot analysis of PARP in Hs578T and MCF-7 CL obtained during treatment with NaB (day 1 and day 3). The 85 kDa fragment characteristic of apoptosis is shown. The data shown are representative of two independent experiments. (B) Induction of apoptosis in Hs578T cells treated with increasing doses (0–10 mM) of NaB 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 Materials and Methods. Results are expressed as mean absorbance $\pm$ S.D. of two independent experiments performed in duplicate ($n=4$).

analysis of CL. The ~ 85 kDa carboxy-terminal fragment of PARP detected by Western immunoblotting is indicative of apoptosis occurring in the lysates sampled (Kaufmann *et al.* 1993, Lazebnik *et al.* 1994). 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 CL of MCF-7 cells (Fig. 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 CL 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 (Fig. 4B). This sensitive assay demonstrated apoptotic effects initially commencing at 1 mM 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.

NaB 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 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 HMEC 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 Fig. 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 HMEC cells, but not in the cancerous cell lines (Fig. 5B). We did not detect p16^{INK4} in Hs578T and MCF-7 cells, and only a slight induction of this protein was seen in HMEC 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 levels. 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 h after treatment, and with a 2.5-fold increase after treating cells with 5 mM NaB for 24 h, whereas in the non-cancerous HMEC cells, only a slight induction (<1.2 -fold) was observed (Fig. 6B). In MCF-7 cells, IGFBP-3 mRNA was not detected in these analyses.

Figure 5 Effect of NaB 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 NaB as indicated. Total RNA was harvested, and 5 µg per lane were 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. Twenty micrograms total protein from whole CL 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 culture plates. The data shown represent two separate experiments. p16^{INK4} was only detectable in the CL of HMEC cells.

The CM were then examined for changes in IGFBP-3 protein levels after NaB treatment. In order to ascertain a suitable time point to collect CM samples, the time-course induction of media IGFBP-3 protein in Hs578T cells was

studied by Western ligand blot analysis, using ¹²⁵I-labeled IGF-I as the ligand, as described in Materials and Methods. The IGFBP-3 level in the CM was detectably increased at 24 h, even after 1 mM NaB, was further increased at 48 h,

Figure 6 Northern blot analysis of the effect of NaB on the expression of IGFBP-3 mRNA. Total RNA was harvested, and 5 µg per lane were electrophoresed. The membrane was subsequently probed with labeled cDNA fragments of IGFBP-3. (A) Time-course expression of IGFBP-3 mRNA in Hs578T cells. Serum-starved cells were treated for 6, 12 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 detected in 10 µg total RNA.

and peaked at 72 h following NaB treatment (Fig. 7A). Subsequently, CM at 72 h post-treatment were analyzed in further studies. As shown in Fig. 7B, various IGFBPs could be detected by the IGF ligand blot. The identity of

these IGFBPs was confirmed by Western immunoblots using IGFBP-3, -2 and -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 -5, and the 24 kDa band to be IGFBP-4 (data not shown). In HMEC 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 mass 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 (Fig. 7B). Further Western immunoblotting analyses demonstrated no detected 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 HMEC cells, mirroring the mRNA data (shown earlier in Fig. 6B). Levels of IGFBP-2/-5 and -4 showed no significant change up to 10 mM 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 HMEC cells (data not shown).

NaB 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 Fig. 8A, a 10-fold induction of IGFBP-rP2 by 5 mM NaB treatment was observed in Hs578T cells, whereas a maximal 2- to 3-fold induction occurred in HMEC cells. IGFBP-rP2 mRNA was not detected in MCF-7 cells.

Western immunoblot analysis against IGFBP-rP2 and -rP1 was then performed. As shown in Fig. 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 h, 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

Figure 7 Effect of NaB on IGFBP production in Hs578T, MCF-7 and HMEC cells analyzed by Western ligand blotting. Serum-starved cells were treated with various concentrations of NaB. CM 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 NaB as indicated and Western ligand blot was performed over 4 days. (B) Representative Western ligand blots using 72 h CM in Hs578T, MCF-7 and HMEC cells. The data shown were derived from at least three independent experiments.

compared with control, especially in Hs578T CM, 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 Fig. 3, where 40–50% of the cells are non-viable by this time of NaB treatment compared with control. IGFBP-rP1 was not detected 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, NaB effects in the human mammary cell system including cancerous and non-cancerous cells, was

Figure 8 Effect of NaB on (A) IGFBP-rP2 mRNA expression, and (B) IGFBP-rP2 and -rP1 protein expression in Hs578T, MCF-7 and HMEC cells. (A) Representative Northern blots. Total RNA was harvested at 18 h post-treatment, and 5 µg per lane were 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 detected in 10 µg total RNA. (B) Representative Western immunoblot analysis. Serum-starved cells were treated with various concentrations of NaB for 72 h. CM 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 detected in the CM of MCF-7 cells.

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 cdk inhibitors studied showed some cellular specificity in their

Figure 9 Schematic diagram showing a summary of sequential effects of NaB 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.

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 Fig. 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 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 (Hassig *et al.* 1997, Archer & Hodin 1999). 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 h after treatment. Also, as shown in Fig. 9, after 24 h of NaB 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 (Hague *et al.* 1993, Carducci *et al.* 1996, Coradini *et al.* 1997). 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 (Siavoshian *et al.* 1997, Schwartz *et al.* 1998), including histone hyperacetylation, especially of H3 and H4 (Archer & Hodin 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 & Pines 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 (Nakano *et al.* 1997, Siavoshian *et al.* 1997, Archer *et al.* 1998, Litvak *et al.* 1998), prostate cancer cells (Huang *et al.* 1999) and breast cancer cells (Lallemand *et al.* 1999). In contrast, Vaziri *et al.* (1998) demonstrated p21^{Waf1/Cip1}-independent G1 cell cycle arrest by butyrate in 3T3 fibroblasts. 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 HMEC cells. Interestingly, butyrate upregulated p27^{Kip1} expression only in HMEC cells. The p16^{INK4} protein was detected only in HMEC 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 (Valentinis *et al.* 1995, Oh 1998). 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 -4 were unaffected. As summarized in Fig. 9, we observed that the mRNA induction had already started at 6 h after treatment and peaked by 24 h after treatment, while the presence of the protein in CM was barely detectable at the 24 h 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 (Zugmaier *et al.* 1989, Oh *et al.* 1995), 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.

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References

- Adamo ML, Shao ZM, Lanau F, Chen JC, Clemmons DR, Roberts CT Jr, LeRoith D & Fontana JA 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 SY & Hodin RA 1999 Histone acetylation and cancer. *Current Opinion in Genetics and Development* **9** 171–174.
- Archer SY, Meng S, Shei A & Hodin RA 1998 p21^{WAF1} is required for butyrate-mediated growth inhibition of human colon cancer cells. *PNAS* **95** 6791–6796.
- Baxter RC, Binoux MA, Clemmons DR, Conover CA, Drop SL, Holly JM, Mohan S, Oh Y & Rosenfeld RG 1998 Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. *Journal of Clinical Endocrinology and Metabolism* **83** 3213.
- Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR & Kley N 1995 Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377** 646–649.
- Burger AM, 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.
- Carducci MA, Nelson JB, Chan-Tack KM, Ayyagari SR, Sweatt WH, Campbell PA, Nelson WG & Simons JW 1996 Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clinical Cancer Research* **2** 379–387.
- Colston KW, Perks CM, Xie SP & Holly JM 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. *Journal of Molecular Endocrinology* **20** 157–162.
- Coradini D, Biffi A, Costa A, Pellizzaro C, Pirronello E & Di Fronzo G 1997 Effect of sodium butyrate on human breast cancer cell lines. *Cell Proliferation* **30** 149–159.
- Gleave ME, Sato N, Sadar M, Yago V, Bruchovsky N & Sullivan L 1998 Butyrate analogue, isobutyramide, inhibits tumor growth and time to androgen-independent progression in the human prostate LNCaP tumor model. *Journal of Cellular Biochemistry* **69** 271–281.
- Gucev ZS, Oh Y, Kelley KM & Rosenfeld RG 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor β 2-induced growth inhibition in human breast cancer cells. *Cancer Research* **56** 1545–1550.
- de Haan JB, Gevers W & Parker MI 1986 Effects of sodium butyrate on the synthesis and methylation of DNA in normal cells and their transformed counterparts. *Cancer Research* **46** 713–716.

- Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D & 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. *International Journal of Cancer* **55** 498–505.
- Hague A, Díaz GD, Hicks DJ, Krajewski S, Reed JC & 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. *International Journal of Cancer* **72** 898–905.
- Hassig CA, Tong JK & Schreiber SL 1997 Fiber-derived butyrate and the prevention of colon cancer. *Chemistry and Biology* **4** 783–789.
- 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. *Journal of Biological Chemistry* **274** 37461–37466.
- Huang H, Reed CP, Zhang JS, Shridhar V, Wang L & Smith DI 1999 Carboxypeptidase A3 (CPA3): a novel gene highly induced by histone deacetylase inhibitors during differentiation of prostate epithelial cancer cells. *Cancer Research* **59** 2981–2988.
- Hunter T & Pines J 1994 Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. *Cell* **79** 573–582.
- Huynh H, Yang X & Pollak M 1996 Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. *Journal of Biological Chemistry* **271** 1016–1021.
- Hwa V, Oh Y & Rosenfeld RG 1999 Insulin-like growth factor binding proteins: a proposed superfamily. *Acta Paediatrica. Supplement* **428** 37–45.
- Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE & Poirier GG 1993 Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Research* **53** 3976–3985.
- Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM & Rosenfeld RG 1996 Insulin-like growth factor-binding proteins and their regulatory dynamics. *International Journal of Biochemistry and Cell Biology* **28** 619–637.
- Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R & Dickson RB 1987 Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* **48** 417–428.
- Lallemant F, Courilleau D, Sabbah M, Redeuilh G & Mester J 1996 Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. *Biochemical and Biophysical Research Communications* **229** 163–169.
- Lallemant F, Courilleau D, Buquet-Fagot C, Atfi A, Montagne MN & Mester J 1999 Sodium butyrate induces G2 arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA rereplication. *Experimental Cell Research* **247** 432–440.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG & Earnshaw WC 1994 Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371** 346–347.
- Litvak DA, Evers M, Hwang KO, Hellmich MR, Ko TC & Townsend CM 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 VM 1992 Insulin-like growth factors and cancer. *British Journal of Cancer* **65** 311–320.
- Mandal M & Kumar R 1996 Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. *Cell Growth and Differentiation* **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 & Sakai T 1997 Butyrate activates the *WAF1/Cip1* gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *Journal of Biological Chemistry* **272** 22199–22206.
- Nickerson T, Huynh H & Pollak M 1997 Insulin-like growth factor binding protein-3 induces apoptosis in MCF7 breast cancer cells. *Biochemical and Biophysical Research Communications* **237** 690–693.
- Nishimura A, Fujimoto M, Oguchi S, Fusunyan RD, MacDermott RP & Sanderson IR 1998 Short-chain fatty acids regulate IGF-binding protein secretion by intestinal epithelial cells. *American Journal of Physiology* **275** E55–E63.
- Ogryzko VV, Wong P & Howard BH 1997 WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Molecular and Cellular Biology* **17** 4877–4882.
- Oh Y 1998 IGF-independent regulation of breast cancer growth by IGF binding proteins. *Breast Cancer Research and Treatment* **47** 283–293.
- Oh Y, Müller HL, Lamson G & Rosenfeld RG 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. *Journal of Biological Chemistry* **268** 14964–14971.
- Oh Y, Müller HL, Ng L & Rosenfeld RG 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. *Journal of Biological Chemistry* **270** 13589–13592.
- Pouillart PR 1998 Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies. *Life Sciences* **63** 1739–1760.
- Rajah R, Valentinis B & Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor- β 1 on programmed cell death through a p53- and IGF-independent mechanism. *Journal of Biological Chemistry* **272** 12181–12188.
- Rajaram S, Baylink DJ & Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Reviews* **18** 801–831.
- Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman HL, Kajstura J, Rubin R, Zoltick P & Baserga R 1995 The insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. *Cancer Research* **55** 2463–2469.
- Rosenfeld RG, Lamson G, Pham H, Oh Y, Conover C, De Leon DD, Donovan SM, Ocran I & Giudice L 1990 Insulin-like growth factor binding proteins. *Recent Progress in Hormone Research* **46** 99–159.
- Rozen F, Zhang J & Pollak M 1998 Antiproliferative action of tumor necrosis factor- α on MCF-7 breast cancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. *International Journal of Oncology* **13** 865–869.
- Schwartz B, Avivi-Green C & Polak-Charon S 1998 Sodium butyrate induces retinoblastoma protein dephosphorylation, p16 expression and growth arrest of colon cancer cells. *Molecular and Cellular Biochemistry* **188** 21–30.
- Sheikh MS, Shao ZM, Clemmons DR, LeRoith D, Roberts CT Jr & Fontana JA 1992 Identification of the insulin-like growth factor binding proteins 5 and 6 (IGFBP-5 and 6) in human breast cancer cells. *Biochemical and Biophysical Research Communications* **183** 1003–1010.
- Siavoshian S, Blottiere HM, Cherbut C & Galmiche JP 1997 Butyrate stimulates cyclin D and p21 and inhibits cyclin-dependent kinase 2 expression in HT-29 colonic epithelial cells. *Biochemical and Biophysical Research Communications* **232** 169–172.
- Staiano-Coico L, Khandke L, Krane JF, Sharif S, Gottlieb AB, Krueger JG, Heim L, Rigas B & Higgins PJ 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. *Experimental Cell Research* **191** 286–291.
- Valentinis B, Bhala A, DeAngelis T, Baserga R & 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. *Molecular Endocrinology* **9** 361–367.

- Vaziri C, Stice L & Faller DV 1998 Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. *Cell Growth and Differentiation* **9** 465–474.
- Velázquez OC, Lederer HM & Rombeau JL 1997 Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implication. *Advances in Experimental Medicine and Biology* **427** 123–134.
- Wilson EM, Oh Y & Rosenfeld RG 1997 Generation and characterization of IGFBP-7 antibody: identification of 31 kD IGFBP-7 in human biological fluids and Hs578T human breast cancer conditioned media. *Journal of Clinical Endocrinology and Metabolism* **82** 1301–1303.
- Yamamoto H, Fujimoto J, Okamoto E, Furuyama J, Tamaoki T & Hashimoto-Tamaoki T 1998 Suppression of growth of hepatocellular carcinoma by sodium butyrate *in vitro* and *in vivo*. *International Journal of Cancer* **76** 897–902.
- Yang DH, Kim HS, 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. *Journal of Clinical Endocrinology and Metabolism* **83** 2593–2596.
- Yoshida M, Kijima M, Akita M & Beppu T 1990 Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *Journal of Biological Chemistry* **265** 17174–17179.
- Zugmaier G, Ennis BW, Deschauer B, Katz D, Knabbe C, Wilding G, Daly P, Lippman ME & Dickson RB 1989 Transforming growth factors type β 1 and β 2 are equipotent growth inhibitors of human breast cancer cell lines. *Journal of Cellular Physiology* **141** 353–361.

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