Insulin-like growth factor-II renders LIM 2405 human colon cancer cells resistant to butyrate-induced apoptosis: a potential mechanism for colon cancer cell survival in vivo

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Butyrate has potent anti-tumorigenic effects on many colon cancer cell lines, including inhibition of growth and promotion of apoptosis in vitro. Nevertheless, despite the butyrate concentration in the colonic lumen being sufficient to result in the death of almost all cells in vitro, colon cancers still develop and grow in vivo, suggesting that cancer cells must develop mechanisms by which they escape the effects of butyrate observed in vitro. Insulin-like growth factor-II (IGF-II) is an autocrine growth factor in many colon cancer cells. The aim of this study was to determine whether IGF-II influences butyrate-mediated apoptosis in LIM 2405 human colon cancer cells. Butyrate and trichostatin A, both of which are histone deacetylase inhibitors although the latter is more specific, induced apoptosis as determined by floating cell counting, Hoechst 33258 staining, DNA laddering and a cell death detection ELISA. IGF-II inhibited the effects of both agents. Butyrate but not trichostatin A also induced LIM 2405 cell migration. In contrast to the above results, IGF-II enhanced butyrate-induced cell migration. Levels of IGF binding protein-3 (IGFBP-3), which may induce apoptosis by IGF-dependent or -independent mechanisms, were increased by butyrate and trichostatin A; IGF-II augmented this effect. It is therefore unlikely that IGFBP-3 mediates butyrate-induced apoptosis. We suggest that IGF-II inhibits the pro-apoptotic effect of butyrate downstream of histone deacetylase inhibition. In contrast, IGF-II promotes histone deacetylase-dependent IGFBP-3 expression and histone deacetylase-independent migration. IGF-II may promote tumour growth by mediating the development of resistance to the pro-apoptotic effects of butyrate.

Introduction

Butyrate is a short-chain fatty acid of physiological relevance to the colonic epithelium since it is a major product of bacterial fermentation of luminal carbohydrates (particularly dietary fibre and resistant starch) and is found within the lumen at concentrations of up to 20 mM (1). Butyrate appears to play important physiological roles in the maintenance of colonic mucosal health by, for example, being a major energy source for colonic epithelial cells (2), and promoting barrier function and cell migration following wounding (3, 4). Butyrate also exhibits several potent anti-tumorigenic effects on many colorectal cancer cell lines, including inhibition of growth, induction of differentiation and promotion of apoptosis (5–7). Indeed, butyrate seems to inhibit the development and growth of tumours in vivo (8, 9). An unexplained paradox is that, despite the butyrate concentration in the colonic lumen being sufficient to result in the death of almost all cells in vivo, colon cancers still develop and grow in vivo.

This implies that cancer cells must develop mechanisms by which they escape the powerful effects of butyrate observed in vitro, particularly the induction of apoptosis. Little is known of the phenomenon of resistance to butyrate. We have recently observed that colon cancer cell lines, most particularly Caco-2 cells, develop butyrate resistance in association with their differentiation, and that this at least in part is due to the enhanced ability of the cells to oxidize butyrate and presumably limit intracellular concentrations (10). This mechanism may have more relevance to the ability of normal cells to withstand luminal exposure to high concentrations of butyrate, rather than for less differentiated cancers. It would seem likely, therefore, that colon cancer cells develop other cellular mechanisms for escaping the suppressive and apoptotic effects of butyrate.

Insulin-like growth factors (IGFs) are potent proliferative and anti-apoptotic agents (11) and IGF-II has been implicated as an autocrine growth factor for colon cancer cells (12). Many colon tumours (13) and human colon cancer cell lines express high levels of IGF-II mRNA (14). Simultaneous expression analysis of over 45 000 genes shows that IGF-II expression had the greatest increase in colorectal tumours and cell lines compared with normal colorectal tissues (15). Using a genetic approach, a functional role for IGF-II in colorectal tumour progression has recently been confirmed in mice (16). Incubation of Caco-2 colon cancer cells with an anti-IGF-II antibody significantly retarded cell growth as well as inducing differentiation (17), suggesting that IGF-II is an autocrine growth factor for these cells. The actions of IGF are modulated by a family of high affinity IGF binding proteins (IGFBPs) (18). Some IGFBPs, including IGFBP-3, also have IGF-independent effects on cellular processes including apoptosis (19). The IGFBP-3 response of colon cancer cells to butyrate might also have relevance to butyrate-mediated resistance.

Because of its growth-promoting and anti-apoptotic effects and because of its apparent high level of expression in the majority of colon cancers, we hypothesized that IGF-II may provide one mechanism by which cells become resistant to butyrate. Since apoptosis is an important protective mechanism against tumour development, the aim of this study was to determine if IGF-II induces resistance to butyrate-mediated apoptosis in a poorly differentiated colon cancer cell line, LIM 2405 cells, and whether butyrate and IGF-II influence and/or interact in the production of IGFBP-3 by those cells. The study also aimed to address possible mechanisms for effects

Abbreviations: FCS, fetal calf serum; HDAC, histone deacetylase; IGF, insulin-like growth factor; IGFBP, IGF binding protein; SFM, serum-free medium; TSA, trichostatin A.
observed, particularly with respect to histone deacetylase (HDAC). Butyrate is believed to induce apoptosis by inhibition of HDAC (20,21), while the effect of IGF-II on this enzyme system is not known. To examine this, an additional end-point, the effect on cell migration, was examined, since butyrate promotes cell migration by mechanisms that are independent of HDAC (22).

Materials and methods

Cells
LIM 2405 cells, which derive from a poorly differentiated primary human colon adenocarcinoma (23), were obtained from Dr R.Whitehead (Ludwig Institute, Melbourne, Australia). Cells were maintained in growth medium [RPMI 1640 plus 25 U/l insulin, 10−5 M e-thioglycolic, 1 µg/ml hydrocortisone and 5% fetal calf serum (FCS)] at 37°C in a humidified incubator.

Cell survival assays
LIM 2405 cells (1×10⁵) were grown to confluence in wells of a 24-well plate. Medium was replaced with RPMI 1640 containing thioglycolic and hydrocortisone (SFM) for 16 h. Cells were then incubated with 0–30 mM sodium butyrate (Sigma, St Louis, MO) for 24–72 h. Floating cells collected from conditioned media and adherent cells collected after trypsinization were counted separately in a haemocytometer. Experiments were performed for 24 h after initial time-course experiments. Cells were incubated with 1 µM trichostatin A (TSA; ICSN, Costa Mesa, CA), a specific inhibitor of histone deacetylase, for 24 h prior to counting of attached and floating cells. To determine whether IGF-II modulates the effects of butyrate or TSA, cells were incubated with 100 ng/ml IGF-II for 1 h prior to the addition of these agents for 24 h (i.e. incubation with IGF-II for 25 h).

Floating and adherent cells were analysed for apoptosis by fluorescent DNA staining with 10 µg/ml Hoechst 33258 (Sigma). Apoptotic cleavage of DNA from floating and adherent cells was also assessed by 2% agarose gel electrophoresis (24). Additionally, apoptosis was measured using an ELISA based on detection of histone-associated DNA fragments in the cytoplasm of apoptotic cells according to the manufacturer’s instructions (Roche, Castle Hill, Australia).

To assess which IGF receptor is involved in the IGF-II effect on apoptosis, cells were treated with butyrate with or without 100 ng/ml [Leu27]IGF-II, which binds to the IGF-I receptor with markedly reduced affinity but normally to the IGF-II/mannose 6-phosphate receptor, or 100 ng/ml [Arg4,Arg8]IGF-II, which binds normally to the IGF-I receptor but does not bind to the IGF-II/mannose 6-phosphate receptor (25) (IGF-II mutants kindly provided by Dr K.Sakano, Daiichi Pharmaceuticals, Japan). Floating cells were counted as above in four independent experiments.

Cell migration studies
Circular wounds were created in confluent culture by scratching with a pipette tip and serum-free medium to remove detached cells. Cells were then incubated with serum-free medium with or without 100 ng/ml IGF-II, 2 mM butyrate and 1 µM TSA for 24 h. Diameter of the cell-free area was measured immediately using a calibrated linear eyepiece graticule at low power and the area calculated (π = 0). After 24 h, cell monolayers were fixed in methanol, stained with 0.05% crystal violet and the diameter of the cell-free area measured again. Cell migration was expressed as the difference between cell-free area at t = 0 and 24 h. Within each experiment, four replicate wounds were measured for each treatment. Migration was not affected by the presence of 1 µg/ml mitomycin C (at which proliferation was completely inhibited), indicating that observed effects were not due to proliferation (data not shown).

To determine which IGF receptor was involved in IGF-II-induced migration, the effects of IGF-II, [Leu27]IGF-II and [Arg4,Arg8]IGF-II (100 ng/ml) were compared in two independent experiments.

Western ligand blotting and immunoblotting
LIM 2405 conditioned media were concentrated by ethanol precipitation and resuspended in non-reducing Laemmli sample buffer. Proteins were separated by SDS 12% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Micron Separations Inc, Westborough, USA). The membrane was blocked, washed, incubated overnight with [125I]IGF-II (1–2×10⁶ c.p.m/membrane; specific activity, 90–170 µCi/µg) and exposed to X-ray film (Brilliant Blue F3R, Kodak, Melbourne, Australia) for 1–3 days.

Membranes that had been subjected to Western ligand blotting were stripped, blocked with non-fat skimmed milk, incubated with a polyclonal antiserum against human IGFBP-3 (kindly supplied by Dr J.Martin, University of Sydney, St Leonards, Australia) followed by detection using enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL) and exposure to X-ray film for 1–10 min.

To assess the effects of butyrate and TSA on IGF-II secretion, conditioned media were applied onto a nitrocellulose membrane using a slot-blot apparatus under vacuum. The membrane was dried (10 min, 37°C) and subsequently blocked in non-fat skimmed milk for 1 h at room temperature. The membrane was probed with a monoclonal antibody (1:1000 dilution of 10 µg/ml) against human IGF-II (kindly provided by Prof. Nishikawa, Kanazawa Medical University, Ishikawa, Japan) and signal detected using enhanced chemiluminescence followed by exposure to X-ray film for 5–10 min. This semiquantitative assay is not affected by the presence of IGFBP (27).

Statistics
Results are shown as mean ± SE of 3–10 experiments. The effects of agents were analysed by ANOVA. Prior to analysis of Cell Death Detection ELISA results, data were log-transformed to stabilize variance. Post hoc analyses were performed using Fisher’s protected least significant difference test.

Results
Sodium butyrate inhibits survival of LIM 2405 colon cancer cells
Incubation of LIM 2405 cells with sodium butyrate increased floating cell numbers and decreased adherent cell number in a dose-dependent manner (Figure 1). After 72 h, 10 mM butyrate increased floating cell number to 202 ± 49% of control (P < 0.05) with a concomitant decrease in adherent cell number to 60 ± 11% of control (P < 0.001). Following incubation with butyrate, there was little change in cell number after 2–4 h, but changes in adherent and floating cell numbers were observed after 24–72 h.

To confirm that the floating cells were apoptotic, cellular DNA was stained with Hoechst 33258. After 24 h, about 95% of floating cells had condensed, fragmented nuclei consistent with apoptosis. In contrast, >90% of nuclear staining of adherent cells was uniform (data not shown). Electrophoresis of genomic DNA from floating cells showed laddering, which was more intense in butyrate-treated cells than in untreated cells, consistent with the larger number of floating cells (Figure 2). In contrast, only high molecular weight DNA was present in adherent cells (Figure 2), confirming that floating but not adherent cells were apoptotic.

Inhibition of histone deacetylase promotes apoptosis of LIM 2405 cells
Since butyrate induces apoptosis via inhibition of histone deacetylase in other cell lines, the effect of trichostatin A (TSA), a specific inhibitor of histone deacetylase that is structurally unrelated to butyrate, on LIM 2405 cells was studied. Similarly to 10 mM butyrate, 1 µM TSA decreased the number of attached viable cells to 64 ± 5% (P < 0.01; Figure 3A) and increased the number of floating apoptotic cells to 233 ± 15% of control (P < 0.001; Figure 3B). These results were confirmed using the Cell Death Detection ELISA, which detects cytoplasmic histone-associated DNA fragments. With this assay, the number of apoptotic cells following treatment with butyrate and TSA were 405 ± 77 and 539 ± 146% of control (both P < 0.001; Figure 3C), respectively.

IGF-II inhibits butyrate-induced apoptosis of LIM 2405 cells
LIM 2405 cells were pre-treated with 100 ng/ml IGF-II for 1 h before incubation with 10 mM butyrate or 1 µM TSA + IGF-II for 24 h. Incubation with IGF-II dramatically decreased floating apoptotic cell number due to both butyrate and TSA (both P < 0.001; Figure 3B). Adherent viable cell number increased following IGF-II incubation with butyrate from 69 ± 3 to 95 ± 7% of control (P < 0.01; Figure 3A). Adherent
Sodium butyrate inhibits survival of LIM 2405 colon cancer cells. LIM 2405 cells were incubated with sodium butyrate (0–30 mM) in serum-free medium for 72 h. Floating cells (B) were collected and attached cells (A) then trypsinized and counted separately using a haemocytometer. Results are shown as a percentage of control (mean ± SE) of three experiments. Within each experiment, results were measured in duplicate or triplicate. In control wells, 3.4 ± 0.8×10^5 cells were attached and 1.1 ± 0.2×10^5 cells were floating. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control.

Cell number following IGF-II incubation with TSA increased from 64 ± 5 to 73 ± 6% but this difference was not statistically significant (Figure 3A). The anti-apoptotic effect of IGF-II was confirmed by decreased intensity of DNA ladders following incubation with IGF-II and butyrate (Figure 2) and by Cell Death Detection ELISA, which showed that IGF-II reduced the number of apoptotic cells to 45 ± 13% of control (P < 0.05; Figure 3C).

[Leu^{77}]IGF-II, which binds to the IGF-I receptor with markedly reduced affinity but normally to the IGF-II/mannose 6-phosphate receptor (25), had no significant effect on butyrate-induced apoptosis as determined by counting floating cell number ([Leu^{77}]IGF-II + butyrate, 382 ± 46% versus butyrate, 440 ± 69% of control). In contrast, [Arg^{54},Arg^{55}]IGF-II, which binds normally to the IGF-I receptor does not bind to the IGF-II/mannose 6-phosphate receptor (25), significantly reduced butyrate-induced apoptosis ([Arg^{54},Arg^{55}]IGF-II + butyrate, 258 ± 51% versus butyrate, 440 ± 69% of control, P < 0.002) to a similar extent to IGF-II (IGF-II + butyrate, 289 ± 75% of control). These results indicate that the inhibitory effect of IGF-II on butyrate-induced apoptosis is mediated by the IGF-I receptor.

IGF-II inhibits butyrate-induced apoptosis

Butyrate has been previously shown to increase migration of colon cancer cells, including LIM 2405 cells (4) and, at least in other cell lines, acts via mechanisms independent of HDAC. The interaction of IGF-II and butyrate in the migration of LIM 2405 cells was therefore examined. Migration was increased by 73 ± 22 and 141 ± 23, respectively, following incubation with either 100 ng/ml IGF-II (P < 0.05) or 2 mM butyrate (P < 0.001) (Figure 4). In contrast to the effect on apoptosis, incubation of IGF-II together with butyrate had an additive effect on migration (263 ± 36%, P < 0.001 versus butyrate; Figure 4). TSA (1 µM) did not increase migration (101 ± 8% of control; data not shown), suggesting that this effect of butyrate is not dependent on inhibition of histone deacetylase.
To determine which IGF receptor was involved in IGF-II-induced migration, the effects of IGF-II, [Leu27]IGF-II and [Arg54,Arg55]IGF-II (100 ng/ml) were compared. In the absence of butyrate, [Arg54,Arg55]IGF-II significantly increased migration to 270 ± 61% of control (P < 0.01), which was similar to the effect of IGF-II (253 ± 25% of control, P < 0.01 versus control), whereas [Leu27]IGF-II had no effect on migration (124 ± 21% of control). Similarly, IGF-II and [Arg54,Arg55]IGF-II but not [Leu27]IGF-II significantly increased butyrate-induced migration (butyrate, 143 ± 11% of control, versus butyrate + IGF-II, 327 ± 8% versus butyrate + [Arg54,Arg55]IGF-II, 341 ± 21% versus butyrate + [Leu27]IGF-II, 173 ± 35%; P < 0.01, butyrate + IGF-II, butyrate + [Arg54,Arg55]IGF-II versus butyrate). These results indicate that the effect of IGF-II on migration is mediated by the IGF-I receptor.

**Butyrate increases levels of IGFBP-3 in conditioned media from LIM 2405 cells**

Conditioned media from LIM 2405 cells were analysed by Western ligand blotting (Figure 5A). In the absence of butyrate, bands sized 35 and 24 kDa and a fainter band sized 30 kDa were observed. Following incubation with butyrate, a 40–43 kDa doublet appeared and levels of the other bands also increased. These changes were dose-dependent. Immunoblotting confirmed the identity of the 40–43 and 30 kDa bands as IGFBP-3 (Figure 5B).

IGFBP-3 was first seen 24–48 h after addition of butyrate and was maximal after 72 h (data not shown). After 24 h, incubation with butyrate, TSA or IGF-II alone increased IGFBP-3 levels (Figure 6). Incubation of IGF-II together with butyrate or TSA resulted in a marked increase in IGFBP-3 levels (Figure 6).

**Discussion**

In the present study, butyrate induced apoptosis in the poorly differentiated LIM 2405 cells in a concentration-dependent manner, as it does in many other colon cancer cell lines (6,7,20,21). In contrast, IGFs inhibit apoptosis (28), and this has been particularly observed in serum and growth factor-deprived colorectal adenoma cells (29). Incubation with IGF-II also inhibited apoptosis of LIM 2405 cells due to serum deprivation. More importantly, IGF-II completely abrogated the ability of butyrate to induce apoptosis of these cells.

Butyrate is physiologically relevant since it is present in the colonic lumen in high concentrations and is, therefore, exposed to colon cancer cells *in vivo*. IGF-II is an autocrine growth factor in many colon cancers (13). The implication of the interaction between IGF-II and butyrate, if it occurs *in vivo*, is that colon cancer cells that produce IGF-II would be resistant to the antitumorigenic, pro-apoptotic actions of butyrate. Thus, a possible mechanism for cancer cells to escape the actions of a physiological control mechanism has been identified. Like most ‘escape’ mechanisms exhibited by cancer cells, it is likely that such a mechanism will not be present in all colon cancers. Indeed, we have found that LIM 1215 cells, a colon cancer cell line that is moderately differentiated and is also sensitive to butyrate-mediated apoptosis, were insensitive to the anti-apoptotic effects of IGF-II in serum-deprived conditions or

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**Fig. 3.** IGF-II inhibits butyrate and trichostatin A-induced apoptosis of LIM 2405 cells. LIM 2405 cells were incubated in serum-free medium (CON) without or with 10 mM sodium butyrate (NaB) or 1 µM trichostatin A (TSA) in serum-free medium for 24 h. In some wells, cells were incubated with 100 ng/ml IGF-II. Floating cells (B) were collected and attached cells (A) trypsinized and counted separately using a haemocytometer. Results are shown as a percentage of control (mean ± SE) of three or four experiments. Within each experiment, results were measured in duplicate or triplicate. In control wells, 3.5 ± 0.4×10⁴ cells were attached whereas 4.7 ± 0.4×10⁴ cells were floating. (C) Apoptosis in cells treated as above was analysed by cell death detection ELISA. Results are shown as a percentage of control (mean ± SE) of three or four experiments. Within each experiment, results were measured in duplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control. ††, P < 0.01, †††, P < 0.001 versus butyrate. ‡‡, P < 0.01 versus TSA. ‡‡‡, P < 0.01 versus TSA.
IGF-II inhibits butyrate-induced apoptosis

Fig. 4. IGF-II enhances butyrate-induced migration of LIM 2405 cells. Cells were grown to confluence and a circular wound made in the monolayer, which was then incubated in serum-free medium (CON) without or with 100 ng/ml IGF-II and/or 2 mM sodium butyrate (NaB) for 24 h. Migration was assessed as the change in wound area from the beginning to the end of incubation. Results are shown as a percentage of control (mean ± SE) of nine (IGF-II) or 10 (NaB ± IGF-II) experiments. Within each experiment, results were measured in quadruplicate. *, P < 0.05, **, P < 0.001 versus control. †††, P < 0.001 versus butyrate.

Fig. 5. Butyrate increases levels of IGFBP-3 in media conditioned by LIM 2405 cells. LIM 2405 cells were incubated with sodium butyrate (0–30 mM) in serum-free medium for 72 h. (A) Conditioned media (100 µl/sample) were separated by 12% SDS–PAGE and analysed by western ligand blotting using [125I]IGF-II. (B) The membrane was then stripped and reanalysed by IGFBP-3 immunoblotting. Migration of molecular weight markers is shown on the left. Lane 1, 0 mM butyrate; lane 2, 1 mM; lane 3, 3 mM; lane 4, 10 mM; lane 5, 50 mM. The figure is representative of five independent experiments.

Fig. 6. Butyrate, trichostatin A and IGF-II increase IGFBP-3 protein levels in LIM 2405 cells. LIM 2405 cells were incubated in duplicate wells with agents as described below in serum-free medium for 24 h. Conditioned media (200 µl/sample) were separated by 12% SDS–PAGE and analysed by IGFBP-3 immunoblotting. Migration of molecular weight markers is shown on the left. Lane 1, control; lane 2, 100 ng/ml IGF-II; lane 3, 10 mM butyrate; lane 4, IGF-II + butyrate; lane 5, 1 µM trichostatin A; lane 6, IGF-II + trichostatin A. The figure is representative of two independent experiments.

Butyrate induces apoptosis in cells by inhibiting the activity of HDAC, as best illustrated by the similar effect of TSA, a specific HDAC inhibitor that is structurally unrelated to butyrate (20,21). TSA-induced apoptosis in LIM 2405 cells, suggesting that the action of butyrate is also likely to be mediated by inhibition of HDAC in this cell line. Incubation with IGF-II inhibited TSA-induced apoptosis of LIM 2405 cells, suggesting that IGF-II abrogated the effects of histone hyperacetylation. There are no reports of IGFs directly inhibiting histone deacetylase and IGF-II does not affect histone deacetylase I gene expression in hepatocellular carcinoma cells (30). It is more likely that IGF-II inhibited butyrate-induced apoptosis downstream of histone hyperacetylation. Caspase-3 activation is implicated in butyrate and TSA-induced apoptosis of LIM 1215 colon cancer cells (20). IGF-II may have inhibited butyrate-induced apoptosis by preventing caspase-3 activation, since IGF-I inhibits apoptosis of cardiac fibroblasts upstream of mitochondrial perturbation and caspase-3 (31). Since apoptosis of LIM 1215 cells due to butyrate was not inhibited by IGF-II, this would suggest differences in the upstream pathways leading to apoptosis in LIM 1215 and LIM 2405 cell lines.

The present study indicates that inhibition of butyrate-induced apoptosis by IGF-II was mediated by the IGF-I receptor. The IGF-I receptor mediates IGF-induced survival in many other cell types (28). In colon cancer cells, other effects of IGF-II, including proliferation (32,33) and up-regulated cyclooxygenase expression (34), are also mediated by the IGF-I receptor. Additionally, inhibition by IGF-I of colon cancer cell apoptosis induced by tumour necrosis factor-α is also mediated by the IGF-I receptor (35).

The IGF-2 gene is maternally imprinted. Treatment with butyrate or TSA promote biallelic expression of this gene, suggesting a role for histone acetylation in imprinting (36). In contrast, TSA decreased overall IGF-2 transcription in Hep3B hepatocellular carcinoma cells (37). LIM 2405 cells express IGF-II mRNA (Leng, S.L., and Bach, L.A., unpublished data), so it is possible that HDAC inhibitors regulate IGF-II expression in these cells. In particular, decreased IGF-II expression may result in increased apoptosis. However, butyrate had no effect on IGF-II levels in conditioned media in the present experiments (data not shown). Although regulation of IGF-I receptor expression by HDAC inhibitors may also contribute to apoptosis, previous studies have shown no effect of TSA on levels of this receptor (38). Since IGFBPs are important modulators of the actions of IGFs and may have IGF-independent effects (18), they may have had a role in the apoptosis observed in the present study. In particular, IGFBP-3 promotes apoptosis by IGF-dependent (39) and IGF-independent (19) mechanisms. To address this
issue, the effects of butyrate and IGF-II on IGFBP-3 expression by LIM 2405 cells were assessed. IGFBP-3 levels were increased by butyrate treatment in a dose-dependent manner. TSA also increased IGFBP-3 levels, suggesting that the butyrate effect was mediated by inhibition of HDAC. A possible mechanism for the effect of butyrate on IGFBP-3 expression is up-regulation of p53-dependent gene transcription since HDACs down-regulate p53-dependent gene activation (40), the IGFBP-3 gene is p53-responsive (41) and LIM 2405 cells express intact p53 (R.Whitehead, personal communication). Effects of HDAC inhibition on IGFBP-3 are, however, variable across different cell lines. For example, TSA increases IGFBP-3 mRNA levels in Hep3B hepatocellular carcinoma cells (38), but butyrate decreases IGFBP-3 protein and mRNA levels in Caco-2 cells (42).

Despite the induction of IGFBP-3 expression by butyrate in LIM 2405 cells, it is unlikely that this binding protein mediated the effects of butyrate, since apoptosis was apparent within 24 h of butyrate treatment whereas the peak of IGFBP-3 induction occurred at 48–72 h. Furthermore, incubation with IGF-II, which inhibited apoptosis, increased IGFBP-3 levels within 24 h of butyrate treatment, and the effects of butyrate and IGF-II were additive. This is consistent with the additive effects of IGF-II on TSA-induced IGFBP-3 mRNA expression in Hep3B hepatocellular carcinoma cells (38). Nevertheless, IGFBP-3 expression in response to butyrate might accelerate cell death either by sequestering IGFs, thereby preventing IGF-induced survival (39) or by an IGF-independent mechanism (19).

To further explore the interaction between IGF-II and butyrate, the effect of IGF-II on a potentially pro-tumorigenic effect of butyrate, enhancement of cell migration, was also examined. Butyrate increased cell migration following wounding of LIM 2405 monolayers, as previously reported (4). TSA did not increase cell migration, suggesting that this effect of butyrate is independent of HDAC inhibition. IGFs have been reported to promote cell migration (43) and indeed IGF-II also stimulated migration of LIM 2405 cells. IGF-II had an additive rather than antagonistic effect when cells were exposed together with butyrate. This observation suggests that the effect of IGF-II is specific for the anti-tumorigenic apoptotic pathway and strengthens the contention that IGF-II acts downstream of histone hyperacetylation.

The intracellular pathways by which butyrate and IGF-II increase cell migration are not completely understood. The butyrate effect requires protein and RNA synthesis but is independent of intracellular acidification or β-oxidation (4). It acts largely via activation of the epidermal growth factor receptor and is independent of protein kinase C, another potent activator of migration (22), whereas protein kinase C isoforms have been implicated in IGF-mediated colonic epithelial cell migration (43). In most cells studied, the IGF-I receptor has been implicated in IGF-induced motility (44), and IGF-I-induced migration of colonic epithelial cells is mediated by the IGF-I receptor (43). However, the IGF-II/mannose 6-phosphate receptor has been implicated in IGF-II-stimulated migration of rhabdomyosarcoma cells (45) and fibroblasts (46). Using IGF-II mutants which selectively bind the IGF-I and IGF-II/mannose 6-phosphate receptors, the results of the present study showed that IGF-II-stimulated migration of LIM 2405 cells is mediated by the IGF-I receptor. The effects of both butyrate and IGFs involves mitogen-activated kinases, which act downstream of the epidermal growth factor and IGF-I receptors. The additive effects of IGF-II and butyrate on cell migration, therefore, suggest that they act via independent proximal pathways.

On the basis of the findings of the present study, a model of the interactions between butyrate and IGF-II is proposed (Figure 7). Butyrate induces apoptosis and IGFBP-3 expression by inhibition of HDAC. IGF-II does not act upstream of HDAC since it also inhibits TSA-mediated effects and TSA acts by directly binding to HDAC (47). Since IGF-II modulates butyrate-induced apoptosis and IGFBP-3 expression, both of which are HDAC-dependent, as well as butyrate-mediated cell migration, an HDAC-independent effect, the actions of IGF-II are not entirely due to direct modulation of histone deacetylation. Rather, inhibition of HDAC, which affects expression of a wide range of genes, results in modulation of a number of intracellular pathways which are differentially modulated by IGF-II, as suggested by its inhibitory effect on apoptosis but additive effect on IGFBP-3 levels induced by butyrate and TSA. Many of the issues raised by this model are directly assessable and warrant further investigation.

In conclusion, IGF-II abrogates the pro-apoptotic effects of butyrate in LIM 2405 colon cancer cells, but stimulates butyrate-mediated promotion of cell migration. IGF-II exerts its effect downstream of the inhibition of HDAC. Although butyrate induced IGFBP-3 expression, it is unlikely that this IGFBP mediates the proapoptotic effect. Since IGF-II is an autocrine growth factor in many colon cancer cells, it may promote tumour growth by mediating the development of resistance to butyrate found in the colonic lumen without affecting tumorigenic effects of butyrate, such as the promotion of cell migration. IGF-II expression may be one mechanism whereby colon cancer cells acquire resistance to the anti-tumorigenic effects of butyrate and may explain at least in part the relatively modest effects of butyrate on tumorigenesis in vivo.

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References


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