Prolonged Butyrate Treatment Inhibits the Migration and Invasion Potential of HT1080 Tumor Cells

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ABSTRACT  Butyrate, a normal constituent of the colonic luminal contents, is produced by the bacterial fermentation of dietary fibers and resistant starches. It has been hypothesized that butyrate may inhibit the invasion of tumor cells. The purpose of the present study was to investigate the effects of butyrate treatment on the growth, migration, and invasion characteristics of tumor HT1080 cells. HT1080 cells cultured in the presence of 0.5 and 1 mmol/L butyrate for 14 d exhibited an increase in the G1 and G2 fractions with a concomitant drop in the S-phase, thus slowing cell growth. Interestingly, 0.5 and 1 mmol/L butyrate inhibited the migration and invasion rate of the tumor cells compared with the untreated (control) cells. The protein and mRNA levels of the tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 were significantly increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. Enzymatic activities and the mRNA level of the latent forms of matrix metalloproteinase (MMP), pro-MMP-2 and pro-MMP-9, were also increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. In contrast, the active form of MMP-2 was detectable by zymographic analysis in control but not butyrate-conditioned media. Collectively, these results demonstrate that prolonged and low-dose butyrate treatment increases both prometastasis MMP-2, -9 and antimetastasis TIMP-1, -2 expression, and the net effect of these increases is the inhibition of pro-MMP-2 activation and of tumor cell migration/invasion potential. J. Nutr. 135: 291–295, 2005.

KEY WORDS: butyrate • cell cycle • migration • invasion • cancer

The SCFA, butyrate, an energy substrate for colonocytes, is produced in the colonic lumen by the bacterial fermentation of carbohydrate (1). It has been reported that human populations and animal models consuming high levels of dietary fiber or resistant starches have a lower risk of colon cancer (2–6). This may be related to butyrate production in the colonic lumen by the bacterial fermentation of dietary fiber (2–6). Human fecal water containing butyrate was shown to modulate cell differentiation, cell invasion, cell proliferation, cell cycle, and cell apoptosis in in vitro models, particularly in colon cancer cell lines, where butyrate may exert several anticarcinogenic effects (7–9). However, the anticancer effect of butyrate remains the subject of debate, partly because of inconsistencies in the literature (10). For example, several animal studies suggested that although a higher concentration of butyrate was observed in rat colons, there was no benefit of butyrate against aberrant crypt formation (a precursor to colon tumors) (11–13). There are several reasons related to the equivocal effects of butyrate such as timing and amount of butyrate administration (10). Liver metastases are usually responsible for the deaths of colon cancer patients (14). Previous studies demonstrated that the supplementation of 10 mmol/L butyrate (12 to 24 h) inhibited invasive human colon cancer in a cell culture model (7,8,15). It was reported that 10 mmol/L SCFA is similar to the colonic luminal SCFA concentrations of humans consuming moderate fiber diets (16,17). SCFA concentrations exist in gradient distribution in human colonic epithelium even with high-fiber diets. Conceivably, there is a continuous low-dose butyrate exposure in the colonic epithelium due to long-term consumption of a butyrate-producing diet. In view of the putative role of timing and amount of butyrate administration in vivo (10), it is important to characterize the invasive ability of tumor cells as affected by long exposure to low concentrations of butyrate. The development of invasive human colon cancer depends on the capacity of tumor cells to proliferate, migrate, and invade the basement membrane (BM). Matrix metalloproteinase (MMP)-2 (gelatinase A) and MMP-9 (gelatinase B) are key members of the MMP family of zinc-dependent endopeptidases that have been associated with many pathologic phenotypes, in particular cancer metastasis (18). Human tumor HT1080 cells are widely used to study the capacity of tumor cells to invade foreign tissue, and it was documented that the expression of MMP-2 and MMP-9 is high in invasive cells but at a...
low level in noninvasive cells (19). In the present study, we investigated the molecular basis of the effect of longer exposure time (14 d) to low butyrate concentration (~0.5–1 mmol/L) on cell migration and invasion potential.

MATERIALS AND METHODS

Cell cultures. HT1080 (passage 19) were obtained from ATCC and maintained in DMEM (GIBCO Invitrogen) with 10% fetal bovine serum (FBS, Sigma Chemical). Stock cells were passaged twice weekly at <80% confluency [0.25% trypsin (GIBCO), 1 mmol/L EDTA, in Ca/Mg-free HBSS (Sigma)], seeded into new flasks at 2286 or 1143 cells/cm² (viability determined by trypan exclusion on hemacytometer counts), and incubated in a humidified chamber at 36.5°C, 5% CO₂. Cultures were tested and found to be mycoplasma free (20). Experiments were performed between passages 23 and 50. Stock cells were grown in standard media supplemented with 10% FBS and 0, 0.5, or 1 mmol/L sodium butyrate (NaB; Sigma) for 14 d. Control and 0.5 mmol/L butyrate treatments were subcultured 3 times and 1 mmol/L butyrate cells twice during the 14-d cycle.

Cell cycle analysis. Cell cycle was analyzed using flow cytometry with propidium iodide (PI) staining. HT1080 cells were trypsinized and washed once with PBS and incubated in 70% (v/v) ethanol at −20°C. After the incubation, cells were washed with PBS and stained with 50 mg PI/L with 6000 U RNase A/L. The DNA contents of cells were determined by flow cytometry. Data were stored as list mode files of at least 10,000 single cell events and analyzed by EPICS proflie II and ModFit LT software (Coulter).

Cell invasion and motility assays. The invasion and motility of butyrate-treated HT1080 cells were determined using BD BioCoatTM Matrigel® Invasion Chambers, Falcon TM Cell Culture Inserts (8-μm pore size PET membrane), and Falcon TC Companion plates (24 well/plate, BD Biosciences). Matrigel-coated and uncoated inserts were suspended in DMEM and rehydrated for 2 h in a humidified, 36.5°C, 5% CO₂ incubator according to manufacturer’s instructions. Hydrated inserts were transferred to wells containing 0.75 mL of DMEM supplemented with 5% FBS. Control and butyrate-treated cells were harvested by trypsinization, washed with FBS-supplemented DMEM, centrifuged at 110 × g for 5 min, 4°C, resuspended in DMEM, and seeded into matrigel and uncoated inserts at 15,000 cells/0.5 mL DMEM. The chambers were incubated for 14–16 h in a humidified incubator at 36.5°C, 5% CO₂. After incubation, the upper surface of the membranes was washed with a cotton tip swab to remove noninvading/migrating cells. Invading and migrating cells on the lower surface of the membrane were fixed and stained with a Wright-Giemsa quick stain (Camco Stain Pak, Cambridge Diagnostic Products). Dry membranes were immersed in immersion oil on microscope slides and cover slipped. Random fields (12/membrane) representing all regions of the membrane were photographed at 40X magnification.

TIMP assay and MMP zymography. HT1080 cells, cultured for 14 d in DMEM containing 10% FBS and 0, 0.5, or 1 mmol/L NaB, were rinsed and incubated 24 h in the presence of serum and butyrate free DMEM for 24 h. Conditioned media were then collected, held on ice, centrifuged at 110 × g for 5 min at 4°C to pellet any nonadherent cells, and stored at −80°C until analysis. Conditioned media were assayed for tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 (Chemicon) levels by specific ELISA and read at 492 and 450 nm, respectively, on a Spectra Max 190 reader (Molecular Devices). MMP-2 and MMP-9 enzymatic activities of conditioned media were separated by electrophoresis on 10% zymogram (gelatin) gels (Invitrogen) and then visualized as clear bands against a dark background with Coomassie Blue staining.

RNA isolation and RT-PCR assay. Total cellular RNA was isolated from HT1080 cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions, and the integrity of RNA samples was checked by electrophoresis. To examine mRNA concentration, an RT-PCR (~21–30 cycles; Promega) assay was performed (21) with the β-actin gene as an internal control. Briefly, 3 independent RNA samples were isolated from HT1080 cells, RNA samples were serially diluted to make sure that RT-PCR products were within a linear range, and amplified products were detected by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and UV transillumination. The intensity signals of the PCR products were analyzed by the UVP Bioimaging Systems. Therefore, the intensity of RT-PCR products correlated directly with the mRNA level of the target gene. The primer pairs were as follows: β-actin primers: 5'-ATG GGT CAG AAG GAT TCC TAT G-3'; 5'-CAG CTC GTA GCT CTT CTC CA-3'; TIMP-1 primers: 5'-TCC TGT TGT TGC TGT GGA TCA-3'; 5'-GGA CTG GAA GCC CTT TTA AGA-3'; TIMP-2 primers: 5'-GCC CGA CCG CTC CAG CTG CTC-3'; 5'-GGG GGG CGC CGC GCC GGC GT-3'; MMP-2 primers: 5'-GAG GAT GAG CTA TGG ACC TTC G-3'; 5'-GCT GTC GCA CAC CAC ACT TTC TTT-3'; MMP-9 primers: 5'-TGG GGT TGG AAA GGC AGA TG-3'; 5'-GAG GAA CAA ACT GTC GCA CAC CAC ACT TTC TTT-3'.

Statistical analysis. Results are given as means ± SEM or SD, as noted. Statistical analyses were performed by one-way ANOVA using experiment as a blocking factor followed by Dunnett’s multiple comparisons to the control group (9). Differences with a P-value < 0.05 were considered significant.

RESULTS

Effect of prolonged butyrate treatment on HT1080 cell growth and cell cycle progression. The doubling time (mean ± SEM, n = 11) of control cells, and cells treated with 0.5 or 1 mmol/L butyrate was 15.8 ± 0.35, 19.6 ± 1.1, and 30.7 ± 2.8 h, respectively. Thus, the doubling time of cells treated with 0.5 or 1 mmol/L butyrate was increased by 24 (P < 0.007) and 94% (P < 0.0001), respectively, compared with control cells. The cell growth arrest was due to the increase in G1 and G2 fractions with a decrease in the S-phase in HT1080 cells treated with butyrate (Fig. 1 and Table 1).

Effect of prolonged butyrate treatment on migration and invasion of HT1080 cells. The migration rate (mean ± SEM, n = 3) of control cells and cells treated with 0.5 or 1 mmol/L butyrate was 456 ± 37, 213 ± 28, and 150 ± 9 cells/membrane, respectively. Thus, the migration potential of cells grown with 0.5 or 1 mmol/L butyrate was inhibited 53 (P < 0.05) and 67% (P < 0.05), respectively. Similarly, the invasion rate (mean ± SEM, n = 3) of control cells, and cells treated with 0.5 or 1 mmol/L butyrate was 267 ± 22, 69 ± 9, and 56 ± 9 cells/membrane, respectively. Therefore, 0.5 and 1

![FIGURE 1](image-url) Flow cytometric profiles show cell cycle phase distribution of HT1080 cells in the presence of 0.5 or 1 mmol/L NaB for 14 d. The data are from 1 experiment of 3 that yielded similar results.
mmol/L butyrate inhibited cell invasion by 74 (P < 0.005) and 79% (P < 0.005), respectively.

**Effect of prolonged butyrate treatment on TIMP-1 and TIMP-2 mRNA level, and their protein levels.** In general, butyrate stimulated TIMP-1 and TIMP-2 mRNA level. Butyrate, 0.5 and 1 mmol/L, increased TIMP-1 mRNA level by 36 and 51%, respectively. Similarly, 0.5 and 1 mmol/L butyrate stimulated TIMP-2 mRNA level by 73 and 128%, respectively (Fig. 2). Consistent with the increasing TIMP mRNA level, TIMP-1 protein level (mean ± SD, n = 3) of control cells, and cells treated with 0.5 or 1 mmol/L butyrate was (2.6 ± 0.4 ng), (11.1 ± 2.4 ng), and (21.4 ± 2.3 ng)/4 × 10⁶ cells, respectively. Thus, TIMP-1 was stimulated 3.4- (P < 0.005) and 7.3-fold (P < 0.001) in response to 0.5 and 1 mmol/L butyrate, respectively. Similarly, TIMP-2 protein level (mean ± SD, n = 3) of control cells, and cells treated with 0.5 or 1 mmol/L butyrate was (5.2 ± 0.4 ng), (29.3 ± 1.5 ng), and (41.1 ± 2.6 ng)/4 × 10⁶ cells, respectively. Therefore, TIMP-2 was increased 46- (P < 0.001) and 6.9-fold (P < 0.001), respectively, due to 0.5 and 1 mmol/L butyrate treatment.

**Effect of prolonged butyrate treatment on MMP-2 and -9 expression.** There was an increase in MMP-2 and MMP-9 levels in HT1080 cells treated with butyrate. In response to 0.5 and 1 mmol/L butyrate, MMP-2 mRNA level increased 66 and 105%, respectively. Similarly, the MMP-9 mRNA level increased by 150 and 540%, respectively (Fig. 3). Consistent with the increasing MMP mRNA level, 0.5 and 1 mmol/L butyrate strongly increased enzymatic activities of latent forms of MMPs (pro-MMP-2 and pro-MMP-9). However, the active form of MMP-2 enzyme was virtually not detectable in butyrate-treated cells, and there was no detectable active form of MMP-9 in either the control or butyrate-treated cells (Fig. 4).

### DISCUSSION

Malignant colon cancers arise from preexisting benign tumors through multiple steps (22). Cancer cell-matrix interaction is a critical step that promotes cell migration, proliferation, and extracellular matrix (ECM) degradation (23,24). Proteolytic degradation of ECM is a critical event during tumor invasion and metastases. Although breakdown of the BM is achieved by several MMPs, MMP-2 and MMP-9 appear to be most important for BM type IV collagen degradation (22–24). Previous studies suggested that butyrate modulates the activities of MMPs and TIMPs in colon cancer cells; however, their precise mechanism of action is not fully understood (7). Recently, there has been considerable interest in the chemopreventive property of tributyrin, a triglyceride analog of butyric acid. Tributyrin, an approved food additive in the

### TABLE 1

**Comparison of cell cycle phase distributions in HT1080 cells treated with NaB for 14 d**

<table>
<thead>
<tr>
<th>Phase-specific cells/total detected cells, %</th>
<th>Control</th>
<th>0.5 mmol/L NaB</th>
<th>1 mmol/L NaB</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁ phase</td>
<td>47 ± 0.65</td>
<td>53.8 ± 0.81*</td>
<td>54.2 ± 0.93*</td>
</tr>
<tr>
<td>S phase</td>
<td>35.2 ± 0.35</td>
<td>24.4 ± 0.78*</td>
<td>19.1 ± 0.46*</td>
</tr>
<tr>
<td>G₂ phase</td>
<td>17.9 ± 0.87</td>
<td>21.8 ± 0.41*</td>
<td>26.6 ± 0.56*</td>
</tr>
<tr>
<td>G₁ + G₂ phase</td>
<td>64.9 ± 0.35</td>
<td>75.6 ± 0.77*</td>
<td>80.9 ± 0.46*</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 3. * Different from control, P < 0.001.
United States, yields 3-fold more butyric acid than NaB, producing and maintaining higher serum butyrate levels than orally administered butyric acid salts (25,26). Therefore, it is important to characterize the effect of butyrate on the expression of MMPs and TIMPs in greater detail. HT1080 cells, a human fibrosarcoma cell line, have been used extensively as a model with which to study the migration and invasion by tumor cells. Similar to various malignant tumors including human colon cancers, HT1080 cells express MMP-2 and MMP-9 at a high level (19). Because butyrate induces growth arrest and apoptosis on a variety of cell lines in vitro (26), the study of butyrate on HT1080 cell migration and invasion will likely further our understanding of functional roles of butyrate in colon cancer cells and other cell types, particularly when serum butyrate levels are high.

The intestine is a continuously regenerating epithelium, and the timing and amount of butyrate administration have been hypothesized to play a critical role in the anticancer effects of butyrate (10). The current experimental approach using cells cultured in the presence of prolonged and low butyrate dose may bring us closer to the in vivo situation. Our data showed that 0.5 or 1 mmol/L butyrate treatment for 14 d inhibited cell growth by increasing the G1 and G2 fractions with a concomitant drop in the S-phase, and greatly decreased tumor cell migration and invasion potential. This observation suggests that a constant low butyrate concentration in vivo may play an important role in the anticancer effect of a butyrate-producing diet. Our RT-PCR and gelatin zymographic analysis demonstrated a parallel increase in pro-MMP-2 and pro-MMP-9 levels and enzyme activities but a significant decrease in active-MMP-2 enzyme activities, in the presence of prolonged butyrate treatment. It was reported that both pro-MMP-2 and pro-MMP-9 were elevated in human colon cancer (22). The increase in the expression of pro-MMP-2 and pro-MMP-9 appears to be paradoxical to the inhibitory effect of prolonged butyrate treatment. However, a further study demonstrated that a significant correlation between metastatic phenotype and MMP-2 and MMP-9 concentrations was noted only with the activated forms (22), and their activities were regulated by natural specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (27). Therefore, the ratio between MMPs and TIMPs may influence the metastatic phenotype, as well as other physiologic processes. The seemingly paradoxical effects of butyrate on the expression of both prometastatic and antimetastatic genes may be related to the inconsistency of chemopreventive effect for butyrate in vivo in some studies (10), and warrant further study of butyrate’s molecular effects. TIMP-1 and TIMP-2 are natural inhibitors of MMP-9 and MMP-2 (27); the significant increases in protein and mRNA levels of TIMP-2 and TIMP-1 suggest at least 2 important molecular roles of prolonged butyrate treatment. First, the upregulation of TIMP-2 and TIMP-1 suppresses the activation of pro-MMP-2 and pro-MMP-9, respectively, and may account in part for the basis of the inhibitory effect of prolonged butyrate treatment on migration and invasion of tumor cells. This is supported by the fact that there were no detectable active-MMP-2 and active-MMP-9 forms in butyrate-treated cells even though pro-MMP-2 and pro-MMP-9 were also upregulated. Second, TIMP-2 may inhibit tumorigenesis via a MMP-independent pathway. It was reported that epidermal growth factor receptor is highly expressed in human cancers but at a low level in normal tissues, and TIMP-2 can directly suppress activation of a mitogenic response through inhibition of tyrosine kinase receptor activation (28,29).

In summary, our data demonstrate that prolonged butyrate treatment increases the expression of both prometastatic genes, MMP-2 and MMP-9, and antimetastatic genes TIMP-1 and TIMP-2, and that the net effect of these increases is the inhibition of pro-MMP-2 and pro-MMP-9 activation and tumor cell migration/invasion capacity (Fig. 5).

ACKNOWLEDGMENTS

We are grateful to Tom Johnson and Jun Ren for critical review of the manuscript, and to Gerald Combs and Janet Hunt for helpful discussion. The technical support given by James Botnen, Brenda Skinner, Joseph Idso, LuAnn Johnson, Karen LoneFight, Matt Soule, and Christine Bogenreif is greatly appreciated.

LITERATURE CITED


FIGURE 4 Effect of 0.5 and 1 mmol/L NaB on MMP-2 and MMP-9 enzymatic activities. To test the activities of MMP-2 and MMP-9, HT1080 cells were cultured in the presence of NaB for 14 d. Conditioned media were collected, and gelatin zymography was performed. The following changes were consistently seen in 3 experiments: 1) stronger signals of MMP-2 and MMP-9 in the cells treated with 0.5 and 1 mmol/L NaB compared with untreated (control) cells; 2) active MMP-2 existed only in control cells but not NaB-treated cells.

FIGURE 5 Proposed mechanism of the role of NaB in the expression and activation of MMPs and TIMPs in HT1080 tumor cells. Treatment with low butyrate concentrations for a prolonged time period increases the expression of pro-MMP-2, -9 and TIMP-1, -2, but inhibits pro-MMP-2, -9 activation and tumor cell migration/invasion potential. Arrows represent induction, and capped lines represent inhibition of pathways.


