Bcl-2 Expression Regulates Sodium Butyrate-induced Apoptosis in Human MCF-7 Breast Cancer Cells

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Abstract
Sodium butyrate (butyrate) is a potent growth inhibitor and differentiating agent for many cell types, including breast cancer cells. Programmed cell death, or apoptosis, is a physiological mechanism of cell death that is dependent on both preexisting proteins and de novo protein synthesis. In the studies presented here, we investigated the role of apoptosis in the growth regulation of human MCF-7 breast cancer cells by sodium butyrate. We report that butyrate treatment of breast cancer MCF-7 cells causes a nonreversible growth inhibition by inducing apoptosis in a time- and dose-dependent manner. Treatment of MCF-7 cells for as little as 12 h with butyrate caused a 5.6-fold induction in apoptotic cell death, which continued to increase up to 27-fold by 48 h treatment. The butyrate-induced apoptosis in MCF-7 cells was closely linked with the down-regulation of expression of Bcl-2 mRNA and Bcl-2 protein, a gene product known to be involved in the regulation of apoptosis in mammalian cells. The observed relationship between the down-regulation of Bcl-2 and induction of apoptosis was not causal because stable overexpression of Bcl-2 resulted in protection of MCF-7 cells from the cytotoxic morphological changes and growth-inhibitory effects of butyrate (15% growth inhibition compared to 60% growth inhibition in the parental cells). In addition, Bcl-2-overexpressing MCF-7 cells exhibited a significant suppression in butyrate-induced stimulation of apoptosis (5-fold increase in apoptosis compared to 27-fold in parental MCF-7 cells). These findings demonstrate that the levels of Bcl-2 expression regulate the butyrate-induced apoptosis in breast cancer cells and that butyrate may potentially be useful in sensitizing the breast cancer cells to chemotherapy-induced apoptosis.

Introduction
Programmed cell death, or apoptosis, is a physiological mechanism of cell death that is dependent on both preexisting proteins and de novo protein synthesis (1–3). Apoptosis plays an important role during development, metamorphosis, organ involution, and in many diseases, including cancer (1, 2). Apoptosis is characterized by nuclear condensation and fragmentation and degradation of DNA into oligonucleosome fragments (1). Regulation of apoptosis is a complex process and involves a number of cellular genes, including Bcl-2 (4, 5) and Bcl-2-related family members such as Bcl-XL, Bcl-Xs, and Bax (6–8). The Bcl-2 (B-cell leukemia/lymphoma 2) gene was first identified at the breakpoint of a chromosomal translocation (14;18) in B follicular lymphoma (9). The Bcl-2 gene encodes a protein of M, 26,000 that protects cells against apoptosis in a variety of experimental systems. Overexpression of Bcl-2 has been shown to suppress the initiation of apoptosis in response to a number of stimuli, including anticancer drugs (10–14). Furthermore, inhibition of Bcl-2 expression by antisense oligonucleotide (15, 16), dominant-negative inhibitor Bcl-Xs (8), and dexamethasone (17, 18) has been shown to promote apoptosis and also sensitize cells to chemotherapy-induced apoptosis. Thus cancer cells may primarily depend on Bcl-2 or related family members to prevent cell death. Recent studies have indicated that cells from a variety of human cancers including breast may have a decreased ability to undergo apoptosis in response to some physiological stimuli (2, 19), and thus a defect in apoptosis may be involved in the aberrant survival and/or development of cancer. Therefore, identification of agent(s) that can negatively regulate the expression of the Bcl-2 pathway in breast cancer cells, and thus trigger apoptosis, will provide a therapeutic approach to inhibit breast cancer cell growth.

Dietary factors play a vital role both in the development and prevention of human cancers, including breast cancer (20, 21). Exploration of dietary factors and their active micronutrients with growth-inhibitory properties on human tumor cells could provide new tools for prevention and treatment of human cancer. One of such dietary micronutrient is sodium butyrate, the major short-chain fatty acid produced by fermentation of dietary fiber (22, 23). Sodium butyrate and its more stable derivatives, such as monobut-3 (24, 25), are known to influence several physiological processes, inhibit the growth of many cell types, including breast cancer cells (24, 25), and induce markers of differentiation, such as milk-related glycoprotein and lipid deposition in breast cancer cells (26, 27). In addition, studies in recent years have demonstrated the ability of sodium butyrate to cause apoptosis in human colorectal cancer and lymphoid cells (28–30). Although sodium butyrate has been shown to be a potent...
growth inhibitor and inducer of apoptosis in some cancer cell types, the biochemical mechanism of sodium butyrate-induced apoptosis and its role in human breast cancer remains unexplored.

In the studies presented here, we investigated the possible role of apoptosis in the growth inhibition of human breast cancer cells by sodium butyrate. We report that the sodium butyrate-induced apoptosis in MCF-7 breast cancer cells is mediated by down-regulation of Bcl-2, and overexpression of Bcl-2 leads to suppression of sodium butyrate-induced apoptosis and growth inhibition. In addition, treatment with butyrate sensitizes MCF-7 cells to chemotherapy-induced apoptosis.

**Results**

In our initial experiments, three human breast cancer cell lines were examined for growth inhibition by 48-h treatment with butyrate. Fig. 1A shows that MCF-7 cells were most sensitive (75% growth inhibition compared to the growth of untreated cells), and ZR-75-R cells were least sensitive (24% growth inhibition compared to the growth of untreated cells) to growth inhibition by sodium butyrate. The growth inhibition of breast cancer cells by sodium butyrate was not related to the presence or absence of estrogen receptor because sodium butyrate also inhibited the growth of estrogen receptor-negative MDA-MB-231 cells. During these experiments, we consistently observed that a significant number of MCF-7 cells started rounding-up and changing their morphology between 36–48 h after sodium butyrate treatment. We were intrigued by the possibility of apoptosis in the action of sodium butyrate in MCF-7 cells. To test this, we explored the effect of sodium butyrate on apoptotic cell death using a quantitative ELISA assay that measures cytoplasmic histone-bound DNA complexes generated during apoptotic DNA fragmentation (31–33). In the past, classical DNA laddering had not been observed in mammary epithelial cells (such as MCF-7) undergoing apoptosis (34, 35), and ELISA assay used here has been a method of choice to quantitate apoptosis in breast cancer cells (32, 33). As shown in Fig. 1, B and C, sodium butyrate treatment of MCF-7 stimulated apoptosis in a dose- and time-dependent manner. As little as 12 h treatment with sodium butyrate caused a 5.6-fold increase in apoptotic cell death, which continued to increase up to 27-fold by 48 h after treatment. To examine whether the observed growth inhibition of breast cancer cells by sodium butyrate was related to the induction of apoptosis, the quantitation of apoptotic cell death was also performed in ZR-75-R cells treated with or without sodium butyrate. Results...
indicated that treatment of ZR-75-R cells with sodium butyrate (3 mM; 24 h) resulted in only a 3.8-fold increase in apoptotic cell death, suggesting that the inhibitory effect of butyrate in MCF-7 cells was probably associated with its ability to induce apoptosis.

Because apoptosis in mammalian cells has been shown to be regulated by Bcl-2 (4) and because of the fact that Bcl-2 inhibition promoted apoptosis (17, 18), we investigated whether the sodium butyrate-induced apoptosis in MCF-7 cells was related with the possible modulation in the levels of Bcl-2. To test this possibility, we examined the expression of Bcl-2 by Western immunoblotting in MCF-7 cells treated with or without butyrate. As shown in Fig. 2, A and B, it was interesting to note that treatment of MCF-7 cells with sodium butyrate resulted in down-regulation of Bcl-2 expression in a time-dependent manner starting 3 h after treatment. Treatment with sodium butyrate for 6–12 h was sufficient to inhibit the levels of Bcl-2 to a significant extent (40–60% inhibition compared to the levels in untreated cells), and this cellular effect of sodium butyrate was also dose dependent (Fig. 2B).

Results of other experiments demonstrated that treatment (48 h) with low doses of sodium butyrate (0.5 or 1 mM) also down-regulated (20–42%, compared to the levels in untreated cells) the Bcl-2 expression in MCF-7 cells. The observed down-regulation of Bcl-2 expression was a specific effect of sodium butyrate because there was no influence of sodium butyrate on the levels of an unrelated HSP-40. Blots in Fig. 2, A and B, were cut into two pieces, and upper portions were immunoblotted with a HSP-40 Ab (36) as an internal control within the same blot. To determine whether the observed down-regulation of Bcl-2 expression in sodium butyrate-treated MCF-7 cells was associated with decreased expression of Bcl-2 mRNA, Northern blot analysis was performed. Fig. 2C shows that 16 h treatment with sodium butyrate inhibited the steady-state levels of Bcl-2 mRNA by 75% compared to the levels in untreated control cells. In brief, results from Figs. 1 and 2 indicated that the observed sodium butyrate-induced down-regulation of Bcl-2 expression was an early event that correlated with the period of induction of apoptosis, suggesting that sodium butyrate can inhibit a gene product known to be involved in apoptosis.

To further examine the possible involvement of Bcl-2 in the induction of apoptosis and growth inhibition of MCF-7 cells by butyrate, we examined whether overexpression of Bcl-2 will modulate the sensitivity of MCF-7 cells to the inhibitory effect(s) of sodium butyrate. For these studies, MCF-7 cells were transfected with a Bcl-2 expression vector (13). A number of clonal cell lines stably overexpressing Bcl-2 were generated. Fig. 3A shows the levels of Bcl-2 protein in two such clones, MCF-7/BCL2-1 and MCF-7/BCL2-2 cells, which overexpress 2.7- and 1.9-fold higher levels of Bcl-2, respectively, compared to the levels in the parental MCF-7 cells. Results in Fig. 3B show the effect of overexpression of Bcl-2 on the sensitivity of MCF-7 cells to butyrate. As shown in Fig. 3C, both MCF-7/BCL2-1 and MCF-7/BCL2-2 cells were protected from the growth-inhibitory action of butyrate to a significant extent compared to growth inhibition in MCF-7 cells. The observed partial protection of cells was dependent on the duration of treatment, with maximum protection observed by 48 h after treatment (15–25% growth inhibition in clones compared to 60–75% growth inhibition MCF-7 cells). The observed suppression of growth inhibition of MCF-7 cells overexpressing Bcl-2 by sodium butyrate was not due to G418 selection because MCF-7 cells transfected with the neo vector alone (MCF-7/neo cells) demonstrated no change in the levels of Bcl-2 and were also sensitive to growth inhibition (72% ± 5) and induction of apoptosis (26-fold ± 7) by 48 h treatment with 3 mM sodium butyrate.

Since Bcl-2 overexpression reduced the growth-inhibitory effect of sodium butyrate, we also examined the sensitivity of Bcl-2-overexpressing MCF-7 cells to the cytotoxic effects of sodium butyrate by the criteria of morphological changes. Results in Fig. 4 show that MCF-7/BCL2-1 cells were distinctively protected from the cytotoxic morphological changes observed in MCF-7 cells treated with sodium butyrate (3 mM; 48 h treatment). Similar results were also obtained with MCF-7/BCL2-2 cells.3 The observed protection

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3 Unpublished observations.
4 The abbreviations used are: HSP, heat shock protein; Ab, antibody; mAb, monoclonal antibody; MT1, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide.
Butyrate-induced apoptosis in breast cancer cells

Fig. 3. Effect of sodium butyrate on the growth of MCF-7 cells and MCF-7 cells overexpressing Bcl-2. A, expression of Bcl-2 in clones overexpressing Bcl-2. B, kinetics of growth inhibition of MCF-7 cells and clones overexpressing Bcl-2 by sodium butyrate. MCF-7, MCF-7/BCL2-1, and MCF-7/BCL2-2 cells (3 x 10^5 cells) were plated into each well of a 48-well plate. After 24 h (time 0), some cells were treated in quadruplicate either without (■) or with 3 mM sodium butyrate for 4 days (○), 3 days (△), 2 days (□), or 1 day (△). On day 4, cell growth was determined by MTT assay. Results are presented as a percentage of controls for each cell line. Results shown are representative of four experiments; bars, SEM.

Discussion

Apoptosis is a physiological mechanism of cell death that plays an important role during embryonic development, organ involution, and in diseases like cancer (1-4). Apoptosis is regulated by specific cellular pathways, including Bcl-2 (4, 5). A number of recent studies suggested that the overexpression of Bcl-2 protects cells against apoptosis (11, 14, 37), and inhibition of Bcl-2 expression promotes apoptosis (17, 18) in response to a number of stimuli. Therefore, cancer cells may depend on Bcl-2 or related family members to prevent apoptosis, and a defect in regulation of apoptosis may be possibly involved in the aberrant survival and/or development of cancer. The ability of Bcl-2 to inhibit apoptosis and its relationship with cancer, provided by transgenic studies involving the Bcl-2-Ig locus as a transgene, lend support for this model (38). In recent years, approaches aimed to modulate the expression of cellular apoptotic pathways such as Bcl-2 are subject of active investigation in an effort to control cancer cell growth. The present investigation was undertaken to explore the role of apoptosis and its possible modulation by Bcl-2 expression in the growth inhibition of human MCF-7 breast cancer cells by sodium butyrate, a potent growth inhibitor and inducer of differentiation that is produced by fermentation of dietary fiber (22). We have selected the MCF-7 cell system for experimental convenience as well as to enable us to take advantage of the wealth of knowledge available about its biology.

The results presented here demonstrated that the treatment of MCF-7 cells with sodium butyrate induced apoptosis
and growth inhibition, and this was closely linked with the down-regulation of Bcl-2 expression. In addition, overexpression of Bcl-2 in MCF-7 cells resulted in significant inhibition of sodium butyrate-induced apoptosis and also protected the MCF-7 cells against the inhibitory effects of sodium butyrate. The observed down-regulation of Bcl-2 expression by sodium butyrate may also provide the biochemical basis of some of the known growth-inhibitory actions of sodium butyrate in MCF-7 cells (24–27). The finding that sodium butyrate inhibited the expression of Bcl-2, a gene product involved in apoptosis, is important because it suggests that a dietary micronutrient can modulate the expression of a specific apoptotic regulatory pathway. In the past, there was precedence to correlate the levels of Bcl-2 with the induction of apoptosis. In this context, it is important to note that dexamethasone and interleukin 6 have been shown recently to down-regulate Bcl-2 expression and induce apoptosis in myeloid leukemic cells (17, 18). The mechanism of observed down-regulation of Bcl-2 by sodium butyrate remains to be delineated. This may occur at the transcription level and/or posttranscription level and may also involve reduced Bcl-2 mRNA stability, leading to a decrease in Bcl-2 expression.

The observed temporal relationship between the down-regulation of Bcl-2 expression and induction of apoptosis in MCF-7 cells was not causal because stable overexpression of Bcl-2 in MCF-7 cells resulted in a significant suppression of sodium butyrate-induced stimulation of apoptosis and inhibitory effects. These results are consistent with the previous studies (11, 14, 37), and support the notion of a pro-
A B C D
- + - + - + + Bcl-2
- + Bcl-2
- + Hsp40
- + Actin

Fig. 6. Effect of sodium butyrate on the levels of Bcl-2 in MCF-7 cells with or without BCL2 overexpression. Cells were treated with (+) or without (−) 3 μM sodium butyrate for 24 h. Cell lysates were made, and equal amounts (20 μg) of protein were analyzed by immunoblotting with a Bcl-2 mAb, followed by signal development using 125I-labeled protein A (A and B resulted from exposure of the same blot for 3- and 12-h exposures, respectively). The upper portion of Bcl-2 blot was immunoblotted with a HSP-40 Ab (C). D, immunoblotting of above samples with a polyclonal actin antibody (Sigma). Lanes 1 and 2, MCF-7 cells; Lanes 3 and 4, MCF-7/Neo cells; Lanes 5 and 6, MCF-7/BCL2-2 cells; Lanes 7 and 8, MCF-7/BCL2-1 cells.

Table 1 Sodium butyrate potentiates doxorubicin-induced apoptosis in MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative fold increase in apoptosis* (over control)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>Doxorubicin (100 μM)</td>
<td>1.13 ± 0.09</td>
</tr>
<tr>
<td>Sodium butyrate (500 μM)</td>
<td>1.28 ± 0.08</td>
</tr>
<tr>
<td>Doxorubicin 9100 μM + sodium butyrate (500 μM)</td>
<td>3.0 ± 0.36</td>
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*DNA fragmentation was measured by quantitative ELISA assay after 72 h treatment; SD (n = 4).

tective role(s) of Bcl-2 against apoptosis in breast cancer cells. It was curious to note that although MCF-7/BCL2-1 cells overexpress only 2.7-fold higher levels of Bcl-2 compared to the levels in MCF-7 cells, they demonstrated a significant suppression in sodium butyrate-induced apoptosis, implying that a small change in the levels of Bcl-2 over the presumptive apoptotic threshold levels could possibly modulate the induction of apoptosis. In addition, it will be interesting to explore whether sodium butyrate can also modulate the expression of other members of genes that either suppress induction of apoptosis, such as Bcl-XL (6), or promote apoptosis, such as Bcl-XS and Bax (7, 8), and such efforts are under way.

Our finding that treatment with butyrate, which down-regulated the Bcl-2 expression, could potentiate the sensitivity of MCF-7 cells to undergo apoptosis in response to doxorubicin provides further support to a regulatory role of Bcl-2 in the modulation of apoptosis in human breast cancer cells in response to a chemotherapeutic agent(s) and opens an interesting area of investigation to further explore the possible thera-and/or a more stable analogue of sodium butyrate in the development of strategies to control the growth of human breast cancer cells.

Materials and Methods

Cell Lines and Growth Assays. Human breast cancer MCF-7 cells (39) were maintained in DMEM-F12 (1:1) supplemented with 10% FCS. Other human breast cancer cells (ZR-75-R and MDA-MB-231) were obtained from the American Type Culture Collection and were grown in L-15 medium supplemented with 10% FCS. Cell growth was measured by the MTT dye (Sigma Chemical Co.) uptake procedure, as described previously (40, 41). About 2 × 104 cells/0.5 ml culture medium were seeded into each well of a 48-well plate. After 24 h, the medium was changed, and appropriate cultures were supplemented with sodium butyrate. For each point, the medium was removed from triplicate wells, MTT dye (5 mg/ml in PBS) was added, and the plate was wrapped in aluminum foil and incubated at 37°C for 4 h. Dye taken up by living cells was extracted in isopropryl alcohol:1 N HCl (96:4) for the determination of absorbance at a wavelength of 570 nm, and results are presented as a percentage of control.

Quantitation of Apoptosis. To measure apoptotic cell death, we used a "cell death" ELISA (Boehringer Mannheim, Indianapolis, IN) that measures cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptotic DNA fragmentation (31–33) and not free histone or DNA that could be released during nonapoptotic cell death (42). Results obtained by this ELISA assay has been shown to correlate with those obtained by other methods (31). MCF-7 cells or MCF-7 clones overexpressing Bcl-2 (2 × 104) cells were plated into each well of a 48-well plate. After desired treatment, cytoplasmic extracts were made from both floating and attached cells, according to manufacturer’s protocol. Control and drug-treated cell extracts were equalized on the basis of equal cell number as well as protein in extracts. Briefly, wells were first coated with anti-histone antibody, loaded with cytoplasmic extracts, and followed by incubation with anti-DNA second antibody conjugated with peroxidase. ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured using Microplate autoreader (Bio-Tek Instruments).

Preparation of Cell Extracts. All experiments were performed with cells in logarithmic phase by controlling the plating density. The viability of cells was assessed by trypan blue dye exclusion. Cells were treated with or without sodium butyrate (Sigma); extracts were prepared as described (43). Briefly, cells were washed twice with cold PBS on ice and lysed in buffer (50 mV Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μM NaVO3, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) for 15 min on ice. The lysates were centrifuged at an Eppendorf centrifuge at 4°C for 15 min. The protein concentration in extracts was determined by using the Bio-Rad kit.

Immunoblotting. Cell lysates containing an equal amount of total protein (40 μg) were resolved on a SDS-PAGE, followed by transfer onto nitrocellulose (41, 44). Membranes were blocked in 2% BSA in TBS [10 mM Tris-HCl (pH 7.5), 150 mM NaCl], followed by probing with either anti-Bcl-2 mAb (Dako or Neomarkers, Inc.) or anti-HSP-40 Ab (36), and immune complexes were detected by using a secondary antibody-based alkaline phosphatase color reaction or 125I-labeled protein A. Quantification of specific protein bands was performed by using a protein databases scanner (Molecular Dynamics). As a internal control, the blot was always cut into two pieces after transfer of proteins. The lower portion of blot was probed with anti-Bcl-2 mAb and the upper portion with an unrelated antibody. Low-molecular-mass markers (Amersham Corp.) were used as standard.

Northern Analysis. Total cellular RNA was isolated and analyzed by Northern blot methods as described previously (44). Briefly, 20 μg RNA denatured in the presence of formaldehyde were fractionated on a 1% agarose formaldehyde gel and transferred to a nitrocellulose filter, with hybridization and washing as described previously (44). Loading of the RNA was monitored by intensities of ethidium bromide-stained rRNA bands (28 S and 18 S). The quantification of the specific signal was performed by densitometric scanning of the autoradios with necessary precautions to ensure a linear response between different autorad exposures of the same blot being taken. The specific cDNA clone (clone pB4; Ref. 45) for human Bcl-2 mRNA was from the American Type Culture
Collection, and the 0.91-kb EcoRI fragment was used for hybridization experiments. As a negative control, the filter was rehybridized with an unrelated actin cDNA probe (43).

Expression of Bcl-2 in MCF-7 Cells. MCF-7 cells at a density of 10^6 cells/100-mm diameter plate were transfected with plasmid DNA containing the full-length human Bcl-2 cDNA and a selectable marker, neomycin phosphotransferase gene (pSSBcl-2; Ref. 13) by calcium phosphate precipitation procedures (46). At 24 h after transfection, selection medium containing 1 mg of G418, a neomycin analogue, per ml was added. Seven to 10 days of culture in this medium killed most of the cells, but a few colonies of cells, one to five per plate, grew. Well-separated colonies were harvested by trypsinization in cloning cylinders and were propagated in the presence of G418. Expression of Bcl-2 in individually isolated colonies was determined by immunoblotting with Bcl-2 mAb. As a control, we have used MCF-7 cells stably transfected with vector containing only neomycin. Once a stable cell line from each clone had been established, the drug was removed from the culture medium. The clonal lines have been maintained in drug-free medium since then.

Acknowledgments
We thank Stanley J. Korsmeyer for providing Bcl-2 expression vector, C. Kent Osborne for MCF-7 clones, Kenzo Ohtsuka for HSP-40 Ab, Neo-markers for anti-Bcl-2 mAb, and Neeta Sharma for technical assistance.

References


