

SHORT COMMUNICATION

Bcl-2 deregulation leads to inhibition of sodium butyrate-induced apoptosis in human colorectal carcinoma cellsMahitosh Mandal, Xipu Wu¹ and Rakesh Kumar²

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Epidemiological studies have linked dietary fiber to the prevention of human colorectal cancer and suggest that short chain fatty acids such as butyric acid, which is produced by fermentation of dietary fiber in the large intestine, may be an important mediator of the protective effects of fiber. We investigated the role of Bcl-2 deregulation on the sensitivity of colorectal carcinoma cells to undergo butyrate-induced apoptosis. Here we report an inverse relationship between the levels of Bcl-2 and the sensitivity of colorectal carcinoma cell lines to undergo apoptosis in response to butyrate. Overexpression of Bcl-2 in colorectal carcinoma DiFi cells resulted in suppression of butyrate-induced apoptosis and enhanced cell survival in response to butyrate. Butyrate-induced apoptosis was accompanied by inhibition of expression of a 30 kDa protein (p30, immunorecognized by anti-Bcl-2 mAb) and this cellular effect of butyrate was inhibited by Bcl-2 overexpression. These findings suggest that deregulation of Bcl-2 in human colorectal carcinoma cells confers resistance to induction of apoptosis by butyrate, a dietary micronutrient.

Epidemiological and experimental studies have linked dietary fiber to the prevention of colorectal cancer (1), but the mechanism(s) involved remains poorly understood. Exploration of active micronutrient(s) with growth inhibitory properties could provide new tools for prevention and treatment of human colorectal cancer. One such dietary micronutrient is sodium butyrate (butyrate*), the major short chain fatty acid (SCFA) produced by fermentation of dietary fiber in the large intestine (2). In general, one half of total SCFAs (300 mmol/day) generated from fermentation of fiber by endogenous intestinal bacteria are known to be structurally related to butyric acid (2,3). Studies using *in vivo* models have shown that dietary fiber supplementation led to increased colonic butyrate levels and these changes were associated with reduced colonic cell proliferation (4) and also lower tumor mass (5). Butyrate is a known potent growth inhibitor and differentiating agent for a number of cancer cells, including colorectal, and recently was shown to induce apoptosis in human colorectal carcinoma cells (6–9).

Homeostasis in colonic epithelial cells is regulated by a balance between proliferative activity and cell loss. Apoptosis is a physiological mechanism of cell loss that is dependent on both pre-existing proteins and *de novo* protein synthesis

(10,11). Apoptosis is characterized by nuclear condensation and fragmentation and degradation of DNA into oligonucleosome fragments (10,11). Regulation of apoptosis involves a number of cellular gene products, such as Bcl-2, which suppresses apoptosis, and proteins such as Bax, that promote apoptosis (11). As different members of the Bcl-2 family have been shown to form homo- and heterodimers, it is believed that the ratios of anti- to pro-apoptotic proteins may play a regulatory role in apoptosis (11). Overexpression of Bcl-2 has been shown to suppress the initiation of apoptosis in response to a number of stimuli, whereas inhibition of Bcl-2 expression has been shown to promote apoptosis (10). Thus, the sensitivity of cancer cells to apoptotic signal(s) may depend on the levels of Bcl-2 or related family members. Recent studies have indicated that apoptosis may be an integral part of normal events in the colon *in vivo* (12) and deregulation of apoptosis may be involved in the development of colorectal cancer (13). Since colonic epithelial cells near the top of the crypt and in the lumen have been shown to undergo apoptosis (12) and butyrate is a locally produced SCFA in the large bowel (2), one potential mechanism of the protective effects of dietary fiber against development of colorectal cancer may include modulation of the apoptotic pathway(s) of butyrate. The present investigation was undertaken to investigate the role of Bcl-2 deregulation on the apoptotic sensitivity of colorectal carcinoma cells in response to butyrate.

Human colorectal carcinoma DiFi (14,15) [original source Dr Bruce M.Boman (14)] SW-480 and HCT-116 (American Type Culture Collection) cells were maintained in DMEM–F12 (1:1) supplemented with 10% fetal calf serum. Cell growth was measured by the MTT method (7). Apoptotic cell death was quantified by an ELISA assay (Boehringer) that measures cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptosis (7). To assay DNA fragmentation, low molecular weight DNA was isolated and analyzed by 1.5% agarose gel electrophoresis. Both floating and attached cells were used in apoptotic assays. To overexpress Bcl-2, DiFi cells were plated a density of 10⁶ cells/100 mm diameter plate and transfected with plasma DNA containing the full-length human Bcl-2 cDNA and a selection marker, the neomycin phosphotransferase gene (16), by calcium phosphate procedures as described (7). As a control, we have used DiFi cells stably transfected with a control vector containing the neomycin phosphotransferase gene. For immunoblotting, cell lysates containing equal amounts of total protein were resolved on a 12% SDS–PAGE followed by probing with an anti-Bcl-2 mAb (Dako) or anti-Bax antibody (Santa Cruz). Immune complexes were detected using an alkaline phosphatase-conjugated secondary antibody (7,17). Quantification was performed with a protein scanner (Molecular Dynamics).

To examine the possible involvement of apoptosis in the action of butyrate in colon cancer cells, we examined the effect of butyrate on apoptotic cell death in three human colorectal cell lines using a quantitative ELISA assay. The

*Abbreviations: butyrate, sodium butyrate; SCFA, short chain fatty acids.

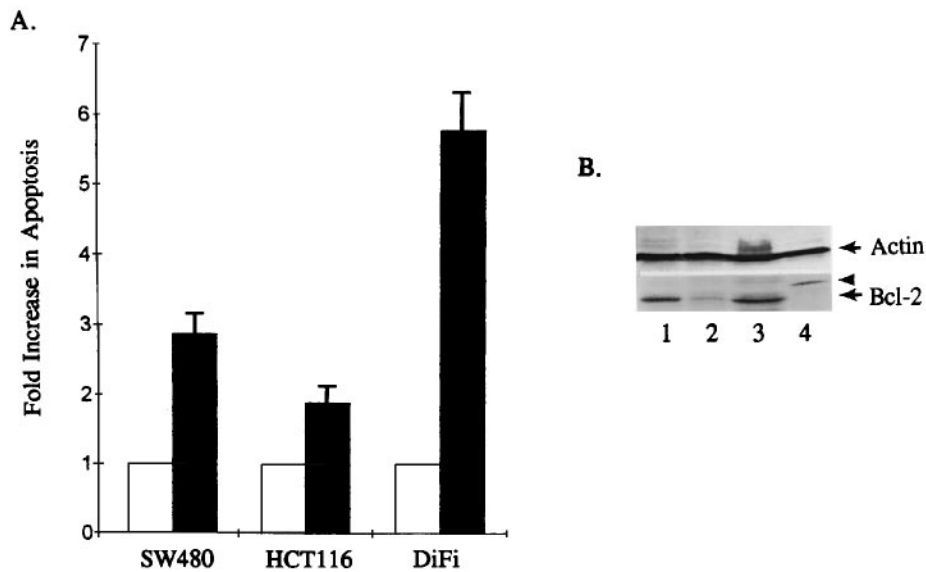


Fig. 1. Butyrate-induced apoptosis in colorectal carcinoma cells depends on the levels of Bcl-2. **(A)** Butyrate induces apoptosis in human colorectal carcinoma cells. SW-480, HCT-116 and DiFi cells were treated with (solid bars) or without (empty bars) 3 mM butyrate for 36 h and apoptosis was quantitated using an ELISA assay. Results are presented as relative fold increase in apoptosis as compared with untreated cells. Bars, SEM ($n = 4$). **(B)** Immunoblot of Bcl-2 expression in human colorectal carcinoma cells using an anti-Bcl-2 mAb. As an internal control, the upper part of the same blot was blotted with an unrelated actin antibody. Lane 1, MCF-7/Bcl-2 as a positive control; lane 2, SW-480; lane 3, HCT-116; lane 4, DiFi. The arrowhead in lane 4 indicates the position of p30.

results in Figure 1A show that HCT-116 cells were least sensitive (1.87-fold compared with untreated cells) and DiFi cells were most sensitive (5.76-fold compared with untreated cells) to butyrate-induced apoptosis. Similar results were also obtained by DNA fragmentation assay (data not shown). Since apoptosis in mammalian cells has been shown to be modulated by Bcl-2, we next investigated whether butyrate-induced apoptosis in colorectal carcinoma cells was related to the levels of Bcl-2. As shown in Figure 1B, the levels of Bcl-2 were very low in DiFi cells (most sensitive to butyrate-induced apoptosis, lane 4) compared with Bcl-2 colonics in HCT-116 cells (least sensitive to butyrate-induced apoptosis, compare lane 3 with lane 4). It was interesting to note that the anti-Bcl-2 mAb, in addition to recognizing Bcl-2, immunoreacted with a protein of ~30 kDa (p30) in DiFi cells (lane 4) but not in SW-480 and HCT-116 cells.

Since the above results suggested a possible inverse relationship between the sensitivity of colorectal carcinoma cells to butyrate-induced apoptosis and the levels of Bcl-2, we examined whether deregulation of Bcl-2 would modulate the sensitivity of DiFi cells to butyrate-induced apoptosis. For these studies, a number of clonal cell lines stably expressing either high levels of Bcl-2 (DiFi/BCL2 cells) or control neomycin phosphotransferase (DiFi/Neo cells) were generated. Figure 2 shows the levels of Bcl-2 protein in six such clones which overexpress 6- to 10-fold higher levels of Bcl-2 compared with control DiFi cells. Reprobing of the Bcl-2 blot with an anti-Bax antibody (lower blot) indicated that there was no effect of Bcl-2 overexpression on the levels of expression of pro-apoptotic Bax protein, and also on another anti-apoptotic Bcl-X_L protein (Pame B).

Results in Figure 3 show the effect of overexpression of Bcl-2 on the sensitivity of two independent DiFi/BCL2 clones to the growth inhibitory effect of butyrate. Both Bcl-2 overexpressing clones were protected from the growth inhibitory action of butyrate in a time- and dose-dependent manner compared with growth inhibition of DiFi cells or DiFi/Neo-2

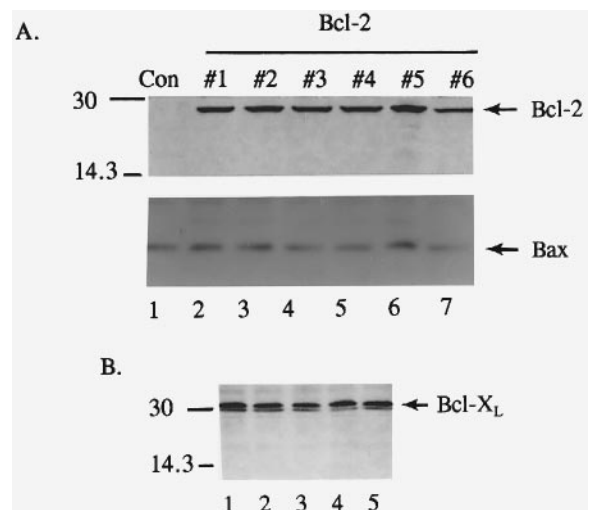


Fig. 2. Overexpression of Bcl-2 in DiFi cells. **(A)** Cell lysates (20 µg protein) from control DiFi cells (lane 1) and six independent Bcl-2 clones (lanes 2–7) were immunoblotted with an anti-Bcl-2 mAb. As an internal control, the Bcl-2 blot was reprobed with anti-Bax antibody and the results are shown in the lower panel. **(B)** Control (lane 1) and clones 2–4 (lanes 2–5) were immunoblotted with an anti-Bcl-X_L mAb.

cells (Figure 3). The observed survival of Bcl-2-overexpressing cells was dependent on the duration of treatment, with maximum protection observed by 48 h after treatment (4–15% growth inhibition in clones compared with 48% growth inhibition at 1 mM butyrate in DiFi cells). Deregulation of Bcl-2-protected DiFi cells from the growth inhibitory effects of butyrate at both 1 (Figure 3A) and 3 mM (Figure 3B) concentrations.

Next we examined the effect of Bcl-2 overexpression in DiFi cells on the ability of butyrate to induce apoptosis using quantitative ELISA and DNA fragmentation assays. Figure 4A shows the results of a quantitative ELISA assay, demonstrating that Bcl-2 overexpression in DiFi/BCL2-2 and DiFi/BCL2-5

cells leads to a significant suppression of butyrate-induced apoptosis (1.4- to 1.9-fold increase in apoptosis compared with 4.2-fold apoptosis in the control DiFi cells by 48 h treatment with 1 mM butyrate). As shown in Figure 4B, there was significant suppression of butyrate-induced DNA fragmentation in both DiFi/BCL2-2 (lanes 3 and 4) and DiFi/BCL2-5 cells (lanes 5 and 6) compared with the extent of DNA fragmentation observed in either DiFi cells (lanes 1 and 2) or DiFi/Neo-2 cells (data not shown). These results suggest that overexpression of Bcl-2 suppresses butyrate-induced apoptosis and enhances cell survival.

Since butyrate has been shown to down-regulate the levels of Bcl-2 in other cell types (7,18), we investigated the effect of butyrate on the levels of Bcl-2 in DiFi/BCL2-5 versus DiFi cells or DiFi/Neo-2 cells (as negative controls). As shown in Figure 5A, there was no significant effect of 1 (Figure 5A, lanes 6–10) or 3 mM butyrate (Figure 5B, lanes 3 and 4) on the levels of Bcl-2 in DiFi/BCL2-5 cells. There was also no effect of butyrate on the levels of Bcl-2 expression in DiFi/BCL2-2 or HCT-116 cells (data not shown). These results suggest that butyrate may utilize different mechanisms to induce apoptosis in different cell types. However, the anti-

Bcl-2 mAb used here distinctively recognized a protein of ~30 kDa (p30) in DiFi cells (Figure 5A, lanes 1–5) but not in Bcl-2 overexpressing cells (Figure 5A, lanes 6–10). The levels of p30 were down-regulated in a time-dependent manner in butyrate-treated DiFi cells (Figure 5A). In brief, these results suggest that p30 may be either post-translationally modified Bcl-2 or a related protein present in cells such as DiFi with very low levels of Bcl-2.

In summary, the results presented here demonstrate an

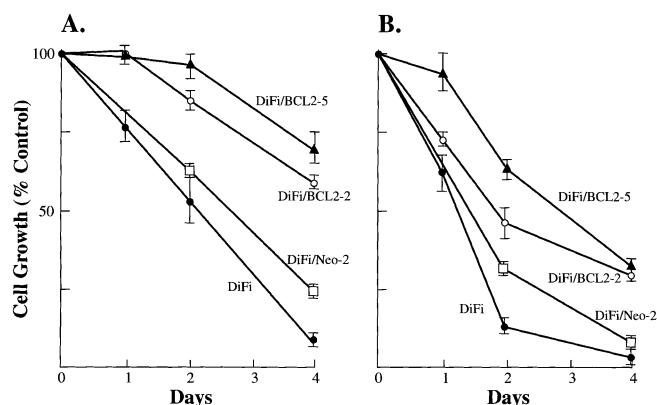


Fig. 3. Effect of butyrate on the growth of DiFi, DiFi/Neo-2 and DiFi cells overexpressing Bcl-2. Cells (3×10^4) were plated into each well of a 48-well plate. After 24 h (0 time), some cells were treated or not in quadruplicate with 1 (A) or 3 mM (B) butyrate for the indicated periods. Cell growth was determined by MTT assay. The growth of control DiFi cells was increased by 2.8 ± 0.3 -fold during the 4 days. Results are presented as percentage of controls for each cell line. Bars, SEM ($n = 4$).

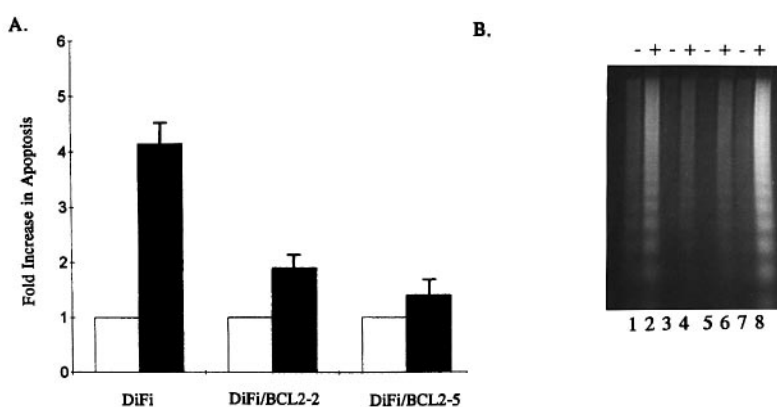


Fig. 4. Overexpression of Bcl-2 suppresses butyrate-induced apoptosis in DiFi cells. (A) Quantitative ELISA assay. Equal numbers (2×10^4) of DiFi, DiFi/BCL2-2 and DiFi/BCL2-5 cells were treated with 1 mM butyrate for 24 h. Cytoplasmic extracts were made to quantitate apoptotic cell death using ELISA. All results are presented as fold increase in apoptotic cell death compared with the respective untreated control cells as a base value. Bars, SEM ($n = 4$). (B) DNA fragmentation. Lanes 1–6, DiFi (lanes 1 and 2), DiFi/BCL2-2 (lanes 3 and 4) and DiFi/BCL2-5 (lanes 5 and 6) cells were treated (+, lanes 2, 4 and 6) or not (–, lanes 1, 3 and 5) with 1 mM butyrate for 24 h. Low molecular weight DNA was analyzed. Lanes 7 and 8, DiFi cells treated (+, lane 8) or not (–, lane 7) with anti-EGF receptor mAb 225 (20 nM, 24 h) were used as a positive control (15).

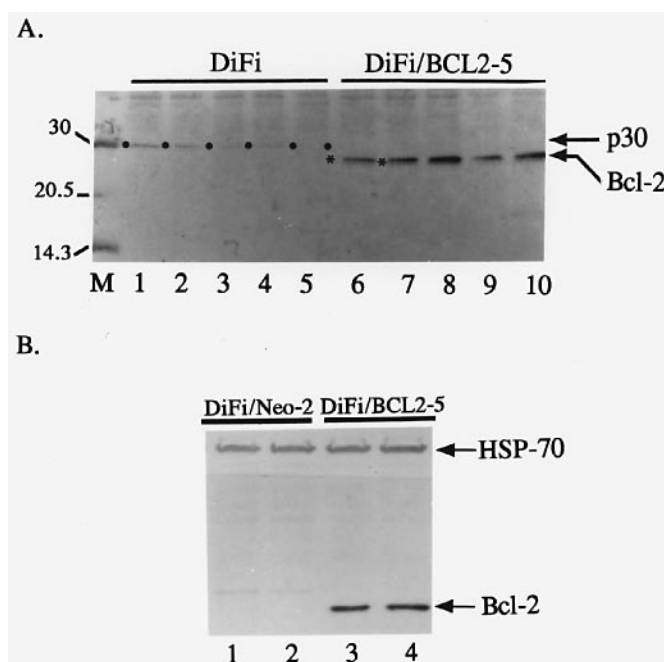


Fig. 5. Effect of butyrate on the levels of Bcl-2 in Bcl-2-overexpressing DiFi cells. (A) DiFi (lanes 1–5) and DiFi/BCL2-5 (lanes 6–10) cells were not treated (lanes 1 and 6) or were treated with 1 mM butyrate for 36 (lanes 5 and 10), 24 (lanes 4 and 9), 12 (lanes 3 and 8) or 6 h (lanes 2 and 7). Cell extracts were immunoblotted with anti-Bcl-2. Results shown are representative of four experiments with similar results. (B) DiFi/Neo-2 (lanes 1 and 2) and DiFi/BCL2-5 (lanes 3 and 4) cells were not treated (lanes 1 and 3) or were treated with 3 mM butyrate (lanes 2 and 4) for 24 h and immunoblotted with an anti-Bcl-2 mAb. As an internal control, the upper part of the blot was immunoblotted with an unrelated mAb against heat shock protein 70.

inverse relationship between the levels of Bcl-2 and the degree of butyrate-induced apoptosis in colorectal carcinoma cell lines. In addition, overexpression of Bcl-2 in DiFi cells which have very low levels of Bcl-2 resulted in significant suppression of butyrate-induced apoptosis and enhanced cell survival in response to butyrate. These results are consistent with previous studies (8,9) and support a potential role of Bcl-2 deregulation in modulation of apoptosis by butyrate in human colorectal carcinoma cells. These results, coupled with the fact that transformation of colorectal epithelium to carcinomas has been shown to be associated with inhibition of apoptosis (13) and butyrate is a locally produced major SCFA in the large bowel (2), suggest that butyrate-induced apoptosis may play a physiological role in normal colonic epithelium cells and deregulation of Bcl-2 may influence the protective effect(s) of SCFA such as butyrate. The evidence that DiFi cells, which have extremely low levels of Bcl-2, express a 30 kDa protein (p30) that was recognized by anti-Bcl-2 mAb is intriguing, as it raises the possibility that p30 may be a Bcl-2-related protein or a mutated form of Bcl-2 present in DiFi cells. This view is further supported by the data in Figure 5 showing that the levels of p30 were reduced in Bcl-2-overexpressing DiFi cells compared with the levels in DiFi cells (Figure 5A, lanes 1 and 6). Chao *et al.* have shown that overexpression of a Bcl-2 transgene can down-regulate endogenous Bcl-2 levels (19). We do not know whether the presence of p30 is a phenomenon restricted to colorectal cells or could be demonstrated in other cell types with no and/or low levels of Bcl-2. Additional investigations are required to further characterize the biochemical and molecular nature of p30. In addition, as DiFi cells express low levels of Bcl-2 and its deregulation in DiFi cells has distinct effects on the survival of cells. These overexpressing clones may serve as a useful system to elucidate the influence of Bcl-2 deregulation on *in vivo* regulatory interactions between the specific pro- and anti-apoptotic proteins, as well as to explore the mechanism of apoptotic pathway involvement in the action of other chemopreventive agents in colorectal cancer.

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