Acquisition of Resistance to Butyrate Enhances Survival after Stress and Induces Malignancy of Human Colon Carcinoma Cells

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ABSTRACT

Acquired resistance to apoptosis by tumor cells remains a major obstacle for cancer treatment, and hence the analysis of resistance to apoptosis constitutes a major goal in the development of antitumoral drugs. We have established a butyrate-resistant human colon adenocarcinoma cell line (BCS-TC2.BR2) from nontumorigenic BCS-TC2 cells to analyze whether the acquisition of such phenotype confers resistance to apoptosis and stress. Although BCS-TC2.BR2 cells exhibited a more differentiated phenotype than the parental BCS-TC2 cells, higher butyrate concentrations remained capable of additionally enhancing their differentiation without inducing apoptosis. Survival rates of BCS-TC2.BR2 cells after glucose deprivation and heat shock were higher than those of parental cells, revealing a stress-resistant phenotype. These findings were accompanied by key differences between parental and butyrate-resistant cells in gene expression profiles and the acquisition of in vivo tumorigenicity. In conclusion, cells gaining resistance to an endogenous physiological modulator of growth, differentiation, and apoptosis concurrently acquired resistance to other agents that influence cell survival.

INTRODUCTION

A growing number of epidemiological studies suggest that a fiber-rich diet exerts a protective role against colorectal cancer development, the third most common cancer and the second leading cause of cancer deaths. It is generally accepted that the end-products of colonic fiber fermentation, particularly butyrate, play an important role in protection against this disease. Butyrate is essential for maintaining the physical and functional integrity of the intestinal mucosa through its stimulatory influence on cell proliferation, differentiation, cation absorption, and colonic blood flow (1–4). Butyrate increases proliferation of nondifferentiated colonocytes from the crypt base, where butyrate concentration is low, whereas it induces differentiation and apoptosis of cells at the neck and top of the crypts, exposed to the higher butyrate concentrations of the intestinal lumen (5). Alterations in butyrate metabolism can lead to inflammatory bowel disease, atrophic colonic epithelium, and colorectal cancer.

Butyrate inhibits cell proliferation and induces differentiation and first apoptosis in transformed cells from colon and other tissues (5–7). It first imposes cell cycle arrest (8, 9), afterward triggering a differentiation program reflected in changes in specific markers such as alkaline phosphatase (ALP) activity, which can subsequently induce cells to undergo apoptosis. Butyrate strongly influences transcriptional activity, causing extensive modifications in gene expression patterns (10, 11). Although the precise mechanisms underlying such alterations have not been fully elucidated, the inhibitory effect of butyrate on histone deacetylase activity appears to be a major influencing factor (3, 12). Butyrate-triggered apoptosis has also been proposed to be a consequence of histone hyperacetylation because such modifications facilitate endonuclease access to chromatin and induce or repress the expression of genes encoding proteins such as caspase-3 and Bcl-2 that are involved in cell death programs (7–9, 13, 14). Other butyrate-driven modifications altering gene expression include alterations in histone phosphorylation, DNA methylation, transcriptional activation of gene promoters bearing butyrate-response elements, and the expression of genes downstream of the β-catenin and Tcf-signaling pathways (4, 15, 16).

Because of its cytostatic and cytotoxic effects on tumor cells, there has been much interest in exploring the use of butyrate and its analogues as anticancer drugs (9, 17, 18). In addition, butyrate has been used to sensitize multidrug-resistant tumor cell lines (19), although in certain instances, treatment with butyrate might itself increase the multidrug-resistant phenotype (20, 21).

Increased proliferation and decreased apoptosis are key features of neoplasia. In the normal colonic epithelium, butyrate is a major player in the regulation of these processes. However, some cells can escape from butyrate-triggered apoptosis and become resistant to this agent, as evidenced by the existence of butyrate-resistant cell lines (21–24). Therefore, the identification of the mechanisms responsible for the acquisition of resistance to butyrate-triggered apoptosis is of great potential value because it could reveal additional targets for therapeutic intervention and new markers for colorectal cancer detection and diagnosis.

In the present study, we have established butyrate-resistant cells from a poorly differentiated, nontumorigenic human colon adenocarcinoma cell line (BCS-TC2) to analyze their biological properties and their resistance to chemical and environmental stress. The degree of induction of apoptosis after exposure to different types of stresses was found to be markedly elevated in the parental cells, suggesting the existence of apoptosis-resistant mechanisms in the butyrate-resistant cells. Importantly, this increase in apoptosis resistance was accompanied by the acquisition of a malignant phenotype in vivo.

MATERIALS AND METHODS

Cell Culture, Proliferation, and Treatment. Human colon adenocarcinoma cells were cultured in DMEM (Sigma, Alcobendas, Spain) supplemented with 5% FCS (Bio-Whittaker, Verviers, Belgium). Cell proliferation and viability were measured as described previously (26). Spheroids were obtained by seeding the cells on nonadherent dishes coated with 1% (w/v) agar (Difco, Melville, NY), and their size calculated according to $(a \times b)^{1/3}$, where $a$ and $b$ are the largest diameter and its perpendicular diameter, respectively. Heat shock was performed at 42°C for the indicated times. Glucose-free DMEM was from Biochrom AG (Berlin, Germany). Butyrate (Sigma) was prepared in standard medium and used at the indicated final concentrations. After establishment of the butyrate-resistant cell line BCS-TC2.BR2, cultures were routinely maintained in standard growth medium in the presence of 2 mM butyrate. Unless otherwise specified, all experiments comparing parental and butyrate-resistant cells were performed after trypsinization and reseeding of the cells without butyrate. Plating densities were chosen to achieve exponential cell growth by 2–3 days in culture, whereupon experiments were performed.

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Preparation of Cell Lysates and Western Blot Analysis. Cell lysates were prepared as previously described (27) and protein content determined using the D$_2$ Protein Assay (Bio-Rad, Madrid, Spain). For Western blotting, monoclonal antibodies recognizing the 70-kDa constitutive (HSC70) and inducible (HSP70) heat-shock proteins (Stressgen, Victoria, British Columbia, Canada), p21 (Neomarkers, Fremont, CA), or vinculin (Sigma) were used. Detection was performed using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence (Amersham-Pharmaica-Biotech, Buckinghamshire, United Kingdom). Vinculin was used as loading control for Western blotting data, and densitometric analysis was performed using a photodocumentation system (UVItc, Cambridge, United Kingdom) and the UVIBand V.97 software (28).

Flow Cytometry, DNA Fragmentation, and Caspase and ALP Activity Assays. For flow cytometry, 10$^6$ cells resuspended in 10 mM HEPES (pH 7.4), containing 150 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, and 1.8 mM CaCl$_2$ were incubated for 15 min in the presence of 2 μg/ml annexin A5-FITC (25) or 1 μg/ml rhodamine 123. To discern necrotic cells, 0.005% (w/v) propidium iodide was added. Analyses were performed in a FACScan cytometer (Becton-Dickinson, San Jose, CA), as previously reported (29).

DNA laddering was assessed as described previously (6). Caspase activity was measured in cell lysates obtained as previously described (27) using 10 μM of either acetyl-DEVD- or acetyl-LEHD-7-amino-4-methyl-coumarin (Calbiochem, Darmstadt, Germany) as substrates to detect caspase-3 and caspase-9 activities, respectively. ALP activity was measured with the ALP 10 kit (Sigma) using 20 μl of cell lysates. Absorbance at 405 nm was monitored using a DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

Transmission Electron Microscopy. BCS-TC2 and BCS-TC2.BR2 cells were seeded without butyrate and cultured for 1 week until near-confluence. Cells were then fixed in situ with 0.2% (v/v) glutaraldehyde in PBS for 30 min at 4°C. After washing and centrifugation, cell pellets were resuspended in 0.1 M glucose in PBS and postfixed in 1% (w/v) OsO$_4$ for 30 min, dehydrated with ethanol, and embedded in Epon 812 resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and visualized in a JEOL 101-O electron microscope at 80 kV.

cDNA Array Analyses. Total RNA was prepared from each established cell line growing under standard culture conditions in the absence of butyrate and were collected at ~80% confluence. Cells were lysed, and total RNA was isolated using the ToTALLY RNA kit (Ambion, Austin, TX). RNA samples were reverse transcribed in the presence of [α-32P]dCTP and the radiolabeled products used to hybridize cDNA arrays (MG arrays, 9600 genes), using previously reported methodologies (30, 31). Z ratios were considered significant when ≥ 1.5 or ≤ −1.5; only Z scores from BCS-TC2 and BCS-TC2.BR2 cells in which average was ≥20 were included in the analysis. Significant values were also tested using a two-tailed test (Z values ≥ 2.5; Z value is equal to Z diff divided by the SD of each Z diff) and P ≤ 0.01. The data reflect three independent experiments.

Tumor Development in Nude Mice. Athymic nude male mice (8 weeks old) were from Harlan Ibérica (Barcelona, Spain). In vivo tumorigenicity of BCS-TC2 and BCS-TC2.BR2 cells was assessed by s.c. inoculation of 10$^6$ cells in 0.2 ml of DMEM in the lumbar region. Experiments were carried out from time zero, the culture was composed of a homogeneous population of small cells with stable morphology and growth rates, displaying no significant DNA fragmentation (Fig. 1B) or other apoptotic features. The resulting butyrate-resistant cell population presented cytometric characteristics similar to those of the parental cells regarding membrane asymmetry and mitochondrial membrane potential (Fig. 1, C and D). The new butyrate-resistant cells maintained these characteristics along subsequent subculture in the presence of 2 mM butyrate and were then deemed an established cell line and named BCS-TC2.BR2. BCS-TC2.BR2 cells were routinely cultured in the presence of 2 mM butyrate, and their overall characteristics remained stable. BCS-TC2.BR2 cells cultured in standard growth medium without butyrate retained apoptotic-resistant features as shown in routine monthly tests that verified their continued ability to grow in the presence of 2 mM butyrate.

Characterization of Butyrate-Resistant BCS-TC2.BR2 Cells. First, we compared the proliferation rates of butyrate-resistant BCS-TC2.BR2 cells with those of parental BCS-TC2 cells in the absence or presence of butyrate (Fig. 2A). The doubling time for BCS-TC2 cells was moderately long (35–40 h) in the absence of butyrate, but this agent dramatically decreased their growth rate. By contrast, BCS-TC2.BR2 cells presented a 1.6-fold shorter doubling time than the parental cells, and the addition of butyrate affected proliferation only slightly. In this regard, expression of the cell cycle inhibitor p21 after butyrate treatment was found to increase in both cell lines, but this elevation was more pronounced in the parental cells, likely contributing to their slower growth after butyrate treatment (Fig. 2B). Cleavage of p21 in the parental cells was detected after treatment with 4 and 8 mM butyrate.

The morphological characteristics of the butyrate-resistant cells were examined by transmission electron microscopy (Fig. 2C). Compared with the parental cells (Fig. 2C, top panel), BCS-TC2.BR2 cells exhibited features of enhanced cellular differentiation. Some cells showed cellular polarization with an apical pole and a basolateral membrane (Fig. 2C, a) but without the characteristic epithelial brushborder. They also displayed intracellular cysts (Fig. 2C, b) and increased intermediate filaments and desmosomes (Fig. 2C, c and d). The presence or absence of 2 mM butyrate in the growth medium of BCS-TC2.BR2 cells did not modify the morphological and ultrastructural characteristics of these cells.

A well-established colon differentiation marker, ALP activity, is higher and increases with cell proliferation in differentiated cells. Basal ALP activity in BCS-TC2.BR2 cells, determined in the absence of butyrate, was found to be 2.9-fold higher than in BCS-TC2 cells, increasing 14-fold after being cultured for up to 11 days (Fig. 2D). Taken together, these results revealed a higher degree of differentiation of BCS-TC2.BR2 cells compared with the parental cells.

RESULTS

Establishment of Butyrate-Resistant BCS-TC2.BR2 Cells from the Human Colon Adenocarcinoma BCS-TC2 Cell Line. BCS-TC2 cells were cultured in continuous presence of 2 mM butyrate, and changes in cell morphology and viability were monitored (Fig. 1). Treatment of parental BCS-TC2 cells with butyrate decreased cell viability, causing the detachment of cells from the monolayer, and induced profound morphological changes, as evidenced by the presence of multinucleated cells and by increases in cell volume and vacuolization (Fig. 1A). The decrease in cell viability was a consequence of the induction of apoptosis, as revealed by the detection of internucleosomal DNA fragmentation (Fig. 1B), the exposure of phosphatidylserine (Fig. 1C), and the dispersion and loss of mitochondrial membrane potential (Fig. 1D). All of these changes documented a time-dependent induction of apoptosis in BCS-TC2 cells. However, after treatment with butyrate for 30 days (Fig. 1A), the number of cells released from the monolayer began to decrease, and the remaining cells retained high levels of viability and eventually resumed proliferation. The resulting population was initially heterogeneous, displaying both large and small cells. After reaching confluence, the cells were subcultured in the presence of 2 mM butyrate and cultured for 30 additional days under these conditions. From this time on, the culture was composed of a homogeneous population of small cells with stable morphology and growth rates, displaying no significant DNA fragmentation (Fig. 1B) or other apoptotic features. The resulting butyrate-resistant cell population presented cytometric characteristics similar to those of the parental cells regarding membrane asymmetry and mitochondrial membrane potential (Fig. 1, C and D).

ANTIAPOPTOTIC PHENOTYPE OF BUTYRATE-RESISTANT CELLS

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Effect of Butyrate on BCS-TC2.BR2 Cell Differentiation and Apoptosis. It was important to assess whether BCS-TC2.BR2 cells remained responsive to higher butyrate concentrations regarding cell differentiation and induction of apoptosis. We first analyzed the influence of 4 and 8 mM butyrate on ALP activity (Fig. 3A). Butyrate increased ALP activity in both cell types in a time- and concentration-dependent manner, but BCS-TC2.BR2 cells consistently displayed higher ALP activity (after 4 days in 8 mM butyrate, ALP activity was 9-fold higher in BCS-TC2.BR2 cells and 33-fold higher in BCS-TC2 cells).

We detected a significant number of floating cells in butyrate-treated BCS-TC2 cultures only, possibly indicating the activation of apoptotic pathways in these cells (6). To confirm this hypothesis, we evaluated DNA fragmentation and caspase-3 activity after butyrate treatment. As expected, increasing concentrations of butyrate markedly enhanced apoptosis in BCS-TC2 cells, as revealed by the presence of DNA fragmentation products (Fig. 3B) and the increase in caspase-3 activity in floating cells (Fig. 3C). By contrast, BCS-TC2.BR2 cells showed neither DNA fragmentation nor increased caspase-3 activity even at butyrate concentrations as high as 50 mM.

Differences between Gene Expression Profiles in BCS-TC2.BR2 and BCS-TC2 Cells. Additional characterization of the butyrate-resistant cells was carried out using cDNA array analysis. Genes were considered differentially expressed if the change in expression fulfilled the four criteria outlined in “Materials and Methods.” Of the 126 genes that showed the most significant differences in expression levels, 54 were up-regulated and 72 down-regulated in BCS-TC2.BR2 cells relative to BCS-TC2 cells (Table 1). The complete array analysis is available online.5

A selection of the genes that exhibited the greatest differences in expression levels in butyrate-resistant cells, as well as those associated with the effects of butyrate, resistance to butyrate or apoptosis, and colon tumorigenesis, are shown in Table 2. Interestingly, additional genes involved in glucose metabolism such as glyceraldehyde-3-phosphate dehydrogenase, aldolase A, and pyruvate kinase were found to be down-regulated in BCS-TC2.BR2 cells.

The different gene expression profiles and the strikingly distinct apoptotic responses after butyrate treatment supported the notion that BCS-TC2.BR2 cells might have a broad resistance against different stresses, and thus, we set out to test this possibility directly.

Effect of Glucose Deprivation on BCS-TC2 and BCS-TC2.BR2 Cells. To analyze the response of these cells to a metabolic stress, we cultured the cells in glucose-free medium. Glucose depletion caused a loss of cell viability (Fig. 4A), but the toxic effect was more pronounced in BCS-TC2 cells than in BCS-TC2.BR2 cells (45 versus 90% viable cells after 24 h, respectively). This reduction in cell viability was caused by the induction of apoptosis as shown in Fig. 4B.
Whereas the percentage of apoptotic cells, determined by flow cytometry using annexin A5-FITC, rose significantly in BCS-TC2 cells after glucose deprivation (2.3- and 4.8-fold after 12 or 24 h, respectively), only a small increase was detected in the butyrate-resistant cells. Additionally, no significant caspase-9 or caspase-3 activation was observed in BCS-TC2.BR2 cells after 24 h of glucose deprivation, but a significant caspase-9 activation was detected (2.4-fold) in the parental cells (Fig. 4C).

Effect of Heat Shock on BCS-TC2 and BCS-TC2.BR2 Cells. Additional characterization of the stress-resistant phenotype of BCS-TC2.BR2 cells was carried out by studying their response to heat shock. Cells were incubated at 42°C for up to 24 h, whereupon fresh butyrate-containing medium was added, and cells were cultured for an additional 24 h before lysis for Western blot analysis. Vinculin signals (Vin), obtained after stripping of the membranes and reprobing, demonstrate the evenness in loading and transfer of the protein samples. C. transmission electron micrographs of BCS-TC2.BR2 cells showing differentiation features: a, columnar cells; b, intracellular cysts; c, intermediate filaments; and d, desmosomes. A micrograph of BCS-TC2 cells maintained under identical culture conditions is shown in the top panel (bars, 2.5 μm). D. basal and proliferation-associated alkaline phosphatase (ALP) activity in BCS-TC2 and BCS-TC2.BR2 cells in the absence of butyrate. Data represent mean values ± SD.
differentiation also differed between the two cell lines. Whereas ALP activity in BCS-TC2 spheroids was similar to what was observed in cells from different sources, but only few actual butyrate-resistant cell lines have been established, and in some cases, these cells were found to revert their phenotype after butyrate removal (21–24). Here, we describe the establishment of the butyrate-resistant BCS-TC2.BR2 cell line that was derived from a butyrate-sensitive human colon adenocarcinoma cell line (BCS-TC2: Ref. 6) through the indicated butyrate concentrations was added.

In Vivo Tumorigenicity of BCS-TC2.BR2 Cells in Nude Mice.

Fig. 3. Effect of butyrate on BCS-TC2.BR2 cell differentiation and apoptosis. Cells were seeded without butyrate and cultured for 3 days, whereupon fresh medium containing the indicated butyrate concentrations was added. A, alkaline phosphatase (ALP) activity in cells that were incubated in the absence or presence of 4 or 8 mM butyrate for either 2 or 4 days (**, P < 0.01). B, internucleosomal DNA fragmentation analysis after treatment for 3 days with the butyrate concentrations shown. C, caspase-3 activity of BCS-TC2 and BCS-TC2.BR2 cells (a.u., arbitrary units) after 3 days of culture in the presence of butyrate (0–50 mM). Data represent mean values ± SD.

In vitro studies using different colorectal cell lines have demonstrated that butyrate is able to inhibit cell proliferation, induce cell differentiation and apoptosis, and enhance immunosurveillance and anti-inflammatory responses (6, 7, 13, 22, 33). However, the molecular bases of the chemopreventive effects of butyrate remain largely unknown. Moreover, the resistance to butyrate has been analyzed in vitro in cells from different sources, but only few actual butyrate-resistant cell lines have been established, and in some cases, these cells were found to revert their phenotype after butyrate removal (21–24). Here, we describe the establishment of the butyrate-resistant BCS-TC2.BR2 cell line that was derived from a butyrate-sensitive human colon adenocarcinoma cell line (BCS-TC2: Ref. 6) through continuous culture in the presence of 2 mM butyrate.

How can cells acquire this type of resistant phenotype? Mariadason et al. (22) have suggested a correlation between the degree of differentiation along the absorptive lineage of colonic epithelial cells and the resistance to butyrate. Caco-2 and HT29cl.19A cells, which differentiated spontaneously with time in culture, showed increased

![Image of a graph showing ALP activity and caspase-3 activity in BCS-TC2 and BCS-TC2.BR2 cells](https://example.com/graph.png)

### Table 1: Classification of genes differentially expressed between BCS-TC2.BR2 and BCS-TC2 cells

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Up-regulated genes</th>
<th>Down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z ratio</td>
<td>G</td>
</tr>
<tr>
<td>A. Cell cycle control and proliferation</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>B. Apoptosis and stress response</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>C. Protein biosynthesis, folding and degradation</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>D. Transcriptional and post-transcriptional processes</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>E. Signal reception and transduction</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>F. Metabolic pathways</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>G. DNA replication and repair</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>H. Ion transport and physiology, and intracellular membrane trafficking</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>I. Cytoskeleton structure and mobility.</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>J. Differentiation and development</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. Other functions</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>18</td>
</tr>
</tbody>
</table>

*Total RNA was obtained and processed as described in "Materials and Methods." Intensity data from the different genes were analyzed in both cell lines, and only those with significant differences according to the four criteria outlined in the "Materials and Methods" section (Z ratio, Z value, average Z score, and P) were considered. Among these genes, those showing a Z ratio ≥ 2 or ≤ -2 are also indicated. Genes without a clearly assigned biological function, as well as those coding hypothetical proteins, are grouped in K. Each gene appears only in one group, although several biological functions may be assigned to some genes.
The selection of such a subpopulation during the establishment of BCS-TC2.BR2 cells was quite plausible, particularly because BCS-TC2 parental cells are heterogeneous and have previously allowed the selection of a tumorigenic cell subpopulation from tumors induced after s.c. coinjection into nude mice of the nontumorigenic parental cells with specific extracellular matrix components (29, 34).

The most prominent feature of BCS-TC2.BR2 cells is their overall resistance to stress-induced apoptosis. It is interesting to note that cells gaining resistance to an endogeneous physiological modulator of growth, differentiation, and apoptosis were found to concurrently exhibit increased survival in response to other stress conditions such as heat shock, glucose deprivation, or attachment-independent growth. It is well known that HSPs confer protection to intestinal epithelial cells and improve cell survival under a variety of stress conditions.

Table 2 Partial list of genes exhibiting significantly different expression in BCS-TC2.BR2 compared to parental cells

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Symbol</th>
<th>Up-regulation</th>
<th>Down-regulation</th>
<th>Biological involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin A1</td>
<td>ANXA1</td>
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<td></td>
</tr>
<tr>
<td>Calmodulin 2</td>
<td>CALM2</td>
<td>3.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleophosmin (nucleolar phosphoprotein B23)</td>
<td>NPM1</td>
<td>2.94</td>
<td>a,b,c</td>
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<td>EBNA1 binding protein 2</td>
<td>EBNA1BP2</td>
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<tr>
<td>Clusterin</td>
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<td>2.51</td>
<td>a,b</td>
<td></td>
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<tr>
<td>Heat-shock 70 kDa protein 8</td>
<td>HSPA8</td>
<td>2.43</td>
<td>a,b</td>
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<td>Thyroid hormone receptor α (oncogene ERBA1)</td>
<td>TRHRA</td>
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<tr>
<td>High mobility group AT-hook 1</td>
<td>HMGA1</td>
<td>2.19</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>Proliferation-associated 2G4</td>
<td>PA2G4</td>
<td>2.19</td>
<td></td>
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<td>Cytochrome c (somatic)</td>
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<td>RNF7</td>
<td>1.76</td>
<td>b</td>
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<tr>
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<td>SRI</td>
<td>1.75</td>
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<td>ATP-dependent DNA helicase II</td>
<td>XRCC5</td>
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<td></td>
<td></td>
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<tr>
<td>Thymosin β4, X-linked</td>
<td>TMSB4X</td>
<td>1.62</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>Actin 1</td>
<td>ACTG1</td>
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<td>Nucleoside-diphosphate kinase 1</td>
<td>NME1</td>
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<td>Calgizzarin</td>
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*The biological involvement refers to genes whose expression changes with (a) butyrate treatment or resistance, (b) resistance to apoptosis and stress, and (c) colon tumorigenesis.

cDNA array analysis (Table 2). Another possible route of acquisition of the resistant phenotype is through the selection of a preexisting cell subpopulation that either spontaneously differentiated to a butyrate-oxidizing phenotype or carried permanent alterations in their genome. The selection of such a subpopulation during the establishment of BCS-TC2.BR2 cells was quite plausible, particularly because BCS-TC2 parental cells are heterogeneous and have previously allowed the selection of a tumorigenic cell subpopulation from tumors induced after s.c. coinjection into nude mice of the nontumorigenic parental cells with specific extracellular matrix components (29, 34).

The most prominent feature of BCS-TC2.BR2 cells is their overall resistance to stress-induced apoptosis. It is interesting to note that cells gaining resistance to an endogenous physiological modulator of growth, differentiation, and apoptosis were found to concurrently exhibit increased survival in response to other stress conditions such as heat shock, glucose deprivation, or attachment-independent growth. It is well known that HSPs confer protection to intestinal epithelial cells and improve cell survival under a variety of stress conditions.

resistance to butyrate because of their enhanced ability to oxidize it, thereby decreasing its intracellular levels. Consequently, histone deacetylase was not inhibited as potently as it was in the nonresistant undifferentiated Caco-2 cells, which showed histone hyperacetylation. Our data are in accordance with this model because BCS-TC2.BR2 cells not only showed a higher degree of differentiation than did the poorly differentiated parental cells but were also able to further differentiate either with time in culture or in the presence of higher concentrations of butyrate. These cells were also able to grow under conditions of glucose deprivation probably because they can efficiently use butyrate as an alternative energy source. This hypothesis is supported by the fact that several genes linked to the glycolytic pathway were down-regulated in BCS-TC2.BR2 cells according to
through the stabilization of cellular components and processes (pro-
teins, cytoskeleton, mitochondrial function, and so forth) or the inhi-
bition of apoptotic pathways (35). Among these proteins, inducible
HSP70 has been shown to be strongly involved in cellular protection
against stress. Heat shock induced the expression of this protein in
both cell lines, but this increase appeared to be insufficient for
maintaining cell viability in the parental cells probably due to their
lower basal HSP70 levels. Moreover, BCS-TC2.BR2 cells are also
resistant to metabolic stress induced by glucose starvation. Whereas
the parental cells showed clear apoptotic features after glucose dep-
"rivation, including loss of membrane asymmetry and caspase-9 acti-
vation, butyrate-resistant cells were almost unaffected by these culture
conditions. These results, taken together with the lack of caspase-3
activation even at high butyrate concentrations, suggest that BCS-
TC2.BR2 cells presented an impairment or blockage in the apoptotic
pathway upstream of the activation of effector caspases. In addition,
when cultured on nonadherent substrates, butyrate-resistant cells were
able to form more stable multicellular aggregates than parental cells.
This type of growth additionally increased the resistance of these cells
to apoptosis, probably by restricting the accessibility of this agent into
the inner cell layers of the spheroids, suggesting that cell-cell interac-
tions between these cells were tighter than in the parental cell aggregates.

Most notably, attaining a resistant phenotype was accompanied by

the acquisition of tumorigenic capacity, as revealed by the ability of
BCS-TC2.BR2 cells to develop tumors after inoculation into nude mice. A study by Williams et al. (36) supports this notion by provid-
ing experimental evidence that during the adenoma–carcinoma pro-
gression sequence in vitro, butyrate contributed to the selection of
cells with a more malignant phenotype. Butyrate did not directly
induce tumorigenicity in the butyrate-resistant cells, but it did increase
their susceptibility to transformation by carcinogens. These findings
are particularly interesting in light of increasing in vivo evidence of a
progressive inhibition of apoptosis during malignant transformation.
In this regard, in the adenoma–carcinoma transition model of colon
carcinogenesis, tissue specimens of increasing malignancy showed
decreased rates of apoptosis (32).

The molecular events underlying the resistance of BCS-TC2.BR2
cells to apoptosis are likely to be complex, as suggested by the
extensive differences in gene expression profiles between the parental
and the resistant cell lines. These changes in gene expression patterns

Fig. 5. Influence of heat shock on BCS-TC2 and BCS-TC2.BR2 cells. A, cell viability
after incubation at 42°C for up to 24 h (100% corresponds to ~1.5 x 10^5 cells/cm^2 in both
cell lines). Western blot analysis of the expression of 70-kDa heat-shock cognate (HSC70)
and heat-shock protein 70 (HSP70) in cell lysates: basal expression (B) and expression (C)
after incubation for different time periods at 42°C. Vinculin signals are shown to
demonstrate the evenness in loading and transfer of the protein samples (Vin). Protein
expression (in a.u., arbitrary units) is normalized to the intensity values of untreated
BCS-TC2 cells. Data correspond to mean values (± SD) from three different experiments
(**, P < 0.01). Representative Western blot analyses are shown.

Fig. 6. Effect of butyrate on BCS-TC2 and BCS-TC2.BR2 cell spheroids. A, representa-
tive micrographs showing multicellular spheroids growing on adherent surfaces. B, alkaline phosphatase (ALP) activity in monolayers (M) or spheroids (S) from cells treated
with 4 mM butyrate for 7 days. Data correspond to mean values (± SD) from three
independent experiments.

Fig. 7. In vivo tumorigenicity of BCS-TC2 and BCS-TC2.BR2 cells in nude mice. Tumor
size measurements were carried out with a caliper at the time points shown; data represent mean values (± SD).

Fig. 4. A, representative micrographs of butyrate-resistant cell aggregates formed on
nonadherent substrates. B, representative micrographs of butyrate-resistant cell
spheroids. C, representative micrographs of butyrate-resistant cell spheroids formed
on adherent surfaces.
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Acquisition of Resistance to Butyrate Enhances Survival after Stress and Induces Malignancy of Human Colon Carcinoma Cells

Isabel López de Silanes, Nieves Olmo, Javier Turnay, et al.

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