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Acquisition of resistance to butyrate induces resistance to luminal components and other types of stress in human colon adenocarcinoma cells

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Abstract

Butyrate, naturally produced by anaerobic fermentation of diet-fiber, is the major nutrient of colonocytes and also an important regulator of colonic epithelium renewal and physiology. Other luminal components, such as bile acids or bacterial products, influence these processes. The model system we used to analyze the influence of several luminal stressors is composed of a previously established cell line resistant to the apoptotic effects of butyrate and their parental butyrate-sensitive cells. Viability of butyrate-resistant cells is unaffected by mild heat-shock (2 h, 42 °C) and only slightly reduced by severe heat-shock (2 h, 45 °C) in contrast to their butyrate-sensitive counterparts. The higher constitutive expression of HSP70 and HSP60 could contribute to this resistance. In addition, expression of HSP70 follows a different pattern after heat-shock in both cell lines. Butyrate-resistant cells are quite unaffected by treatment with deoxycholic acid but apoptosis is induced by chenodeoxycholic acid although to a lower extent than in butyrate-sensitive cells. These resistant cells are also less sensitive to lipopolysaccharide and show differences regarding the activation of ERK following osmotic stress. Thus, the cell model herein reported is a useful tool for investigating the molecular mechanisms of resistance to apoptosis, as well as to analyze specific targets for butyrate-resistant tumors.

Keywords: Bile acids; Butyrate; Colon adenocarcinoma; Heat shock; Stress

1. Introduction

The crypt-villus axis of the intestinal mucosa is composed of a dynamic cell population that changes from proliferative undifferentiated crypt cells, to mature differentiated surface epithelial cells. Epithelial cell migration from the crypt base to the surface is accompanied by cellular differentiation, which is influenced by luminal components including several physiological and environmental stressors. Among them, butyrate is a major player in the regulation of these processes and induces apoptotic cell death, promoting in this way the physiological turnover of the mucosa (Andoh et al., 2003; Miller, 2004; Scheppach and Weiler, 2004). Colon cells may transiently survive the cytotoxic effects of these luminal stressors by triggering different molecular responses, such as the induction of the expression of heat-shock proteins (HSPs). Acquisition of resistance to apoptosis by tumor cells allows the accumulation of DNA damage that may lead to malignant transformation constituting a major obstacle for cancer therapy (Rupnarain et al., 2004).

We have previously established a butyrate-resistant tumorigenic human colon adenocarcinoma cell line (BCS-TC2.BR2) from the non-tumorigenic butyrate-sensitive BCS-TC2 parental cells (López de Silanes et al., 2004). The analysis of the differences between gene expression profiles in BCS-TC2.BR2 and BCS-TC2 cells reveals alterations in several genes involved in resistance to apoptosis

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and stress. Thus, we have analyzed the potential resistance of BCS-TC2.BR2 cells against different types of stress and heat-shock treatment. Moreover, as the intestinal epithelium surface is continually exposed to a high intraluminal concentration of diverse bacteria and to bile acids, we have also studied the effect of different bile acids and bacterial lipopolysaccharide (LPS) on the behavior of both cell lines. The aim of this study is to determine whether the acquisition of resistance to apoptosis induced by luminal agents such as butyrate is also accompanied by an overall stress resistance and to validate our model system to obtain further information concerning the molecular mechanisms involved in the acquisition of the resistant and more tumorigenic phenotype.

2. Materials and methods

2.1. Cell culture

The establishment and characterization of BCS-TC2 and BCS-TC2.BR2 cells have been previously described (López de Silanes et al., 2004; Turnay et al., 1990). These human colon adenocarcinoma cells were cultured in Dulbecco's modified Eagle's medium, containing 4.5 g/l glucose, and supplemented with 5% heat-inactivated fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) and glutamine (300 μg/ml). Routinely, cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was replaced every three days and BCS-TC2.BR2 cells were routinely maintained in standard growth medium in the presence of 2 mM butyrate. Cells were routinely weekly subcultured by trypsinization [0.05% (w/v) trypsin, 0.02% (w/v) EDTA].

2.2. Treatment of cells under different stress conditions

Experiments were carried out after trypsinization and seeding of the cells at 5×10^4 cells/cm^2 in supplemented medium; the cells were allowed to attach and grow for 2 days before the different treatments. Heat shock was performed under mild or severe conditions (2 h at 42 or 45 °C, respectively), allowing afterwards cells to recover for different periods of time. Deoxycholic (DCA) or chenodeoxycholic acid (CDCA) was added for 2 h at 500 μM. Incubations with LPS were performed at different concentrations for 24 h. Hyperosmotic stress was induced by addition of 80 mM NaCl excess.

2.3. Cell viability

Cell viability was measured by evaluation of the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) after each treatment (Pérez-Ramos et al., 2005). At the different indicated conditions and times, cells were incubated at 37 °C for 4 h in the dark in the presence of 0.5 mg/ml MTT and washed with PBS. The dye was extracted in 0.04 M HCl in isopropanol, and absorbance was registered at 570 nm.

2.4. Confocal microscopy

Cells were stained with 10 μg/ml acridine orange and with 50 μg/ml propidium iodide (Pérez-Ramos et al., 2005). After 15 min incubation at room temperature, cultures were analyzed by confocal microscopy using a Bio-Rad Laboratories MRC-1024 laser scanning microscope equipped with a Nikon Eclipse TE300 inverted microscope. Quantification of the number of apoptotic/necrotic cells was carried out by microscopic evaluation of cell and chromatin morphology, and differential dye-staining (Chu et al., 2003). This analysis was performed on twelve fields from three different preparations of untreated or DCA-, or CDCA-treated cells (500 μM, 2 h). Within the primary apoptotic cell group, we have considered cells that present initial apoptotic nuclear features (IP-negative); apoptotic cells showing IP-staining (last stages of the apoptotic cell death pathway) were included in the secondary apoptosis group. Necrotic cells showed no nuclear apoptotic features and were IP-positive.

2.5. Western blot analysis

Equal amounts of protein from cell lysates obtained under phosphatase free conditions were analyzed by PAGE-SDS after heat denaturing in the presence of 5% β-mercaptoethanol, transferred to nitrocellulose membranes and analyzed (Guzmán-Aránguez et al., 2005). Anti-human HSC70, HSP70, HSP60, HSP27 (Stressgen) and vinculin (Sigma) monoclonal antibodies or anti-ERK and phospho-ERK polyclonal antibodies (Cell Signaling Technology) were used to detect the corresponding proteins, and detection was performed by ECL. Densitometric analysis of the membranes was performed as previously described (Guzmán-Aránguez et al., 2005) using the signal corresponding to vinculin (control of even protein load) to normalize protein levels as the expression of vinculin does not vary under the experimental conditions used.

2.6. Other procedures

Protein content was determined using the D_C Protein Assay (Bio-Rad). Alkaline phosphatase (ALP) activity was measured using the ALP 10 kit (Sigma). The differences between the mean values were analyzed using Student's *t*-test; statistical significance was considered to be achieved at the $P < 0.05$ level.

3. Results

3.1. Heat-shock treatment: viability and HSP levels

We have studied whether butyrate resistance is accompanied by alterations in the cell sensitivity to heat shock