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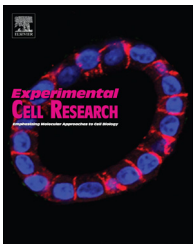

Research Article

Molecular mechanisms for inhibition of colon cancer cells by combined epigenetic-modulating epigallocatechin gallate and sodium butyrate

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ABSTRACT

Bioactive compounds are considered safe and have been shown to alter genetic and epigenetic profiles of tumor cells. However, many of these changes have been reported at molecular concentrations higher than physiologically achievable levels. We investigated the role of the combinatorial effects of epigallocatechin gallate (EGCG), a predominant polyphenol in green tea, and sodium butyrate (NaB), a dietary microbial fermentation product of fiber, in the regulation of survivin, which is an overexpressed anti-apoptotic protein in colon cancer cells. For the first time, our study showed that the combination treatment induced apoptosis and cell cycle arrest in RKO, HCT-116 and HT-29 colorectal cancer cells. This was found to be regulated by the decrease in HDAC1, DNMT1, survivin and HDAC activity in all three cell lines. A G2/M arrest was observed for RKO and HCT-116 cells, and G1 arrest for HT-29 colorectal cancer cells for combinatorial treatment. Further experimentation of the molecular mechanisms in RKO colorectal cancer (CRC) cells revealed a p53-dependent induction of p21 and an increase in nuclear factor kappa B (NF-κB)-p65. An increase in double strand breaks as determined by gamma-H2A histone family member X (γ-H2AX) protein levels and induction of histone H3 hyperacetylation was also observed with the combination treatment. Further, we observed a decrease in global CpG methylation. Taken together, these findings suggest that at low and physiologically achievable concentrations, combinatorial EGCG and NaB are effective in promoting apoptosis, inducing cell cycle arrest and DNA-damage in CRC cells.

Abbreviations: EGCG, epigallocatechin gallate; NaB, sodium butyrate; HDAC, histone deacetylase; HAT, histone acetyltransferase; DMSO, dimethylsulfoxide; DNMT1, DNA methyltransferase 1; DNMT3A, DNA methyltransferase 3A; γ-H2AX, gamma-H2A histone family member X; NF-κB, nuclear factor kappa B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PI, propidium iodide; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; TBS-T, tris buffered saline-tween 20.

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Introduction

Survivin, a member of the inhibitor of apoptosis protein family, plays a bifunctional role, as an anti-apoptotic protein and also as a regulator of cell cycle progression [1,2]. It associates with the mitotic spindle during the cell cycle and serves as a check point for correct association of the spindle with chromosomes in metaphase [2]. In colorectal cancers (CRCs), survivin is over-expressed but its expression in normal adult tissue is undetectable [3]. The expression of survivin has been linked to poor survival, recurrence rate and death due to CRCs [4]. Chemoregulatory expression of this protein is therefore a promising target for cure.

The incidence rate of colon cancer is lower in Asian countries where the diet is predominantly rich in vegetables and fruits [5]. The constituents of these dietary foods provides for a healthy colonic environment. Dietary fiber, an important dietary constituent, ensures that potential carcinogens are removed from the colon and the microbiota within the colon converts the fiber into short chain fatty acids (SCFA) by the process of fermentation [6]. These short chain fatty acids are a major source of energy for the colon cells. Of the SCFAs, butyrate is the predominant energy providing source [7] and is a natural epigenetic regulator functioning as an inhibitor of histone deacetylases (HDACs) [8]. Sodium butyrate (NaB) can induce cell differentiation, apoptosis and histone hyperacetylation [8–10] and these tumor inhibitory properties of butyrate can be exploited as part of a treatment for CRCs.

Another dietary epigenetic molecule, epigallocatechin gallate (EGCG), is a predominant constituent of green tea polyphenols, and regulates epigenetic changes by altering methylation profiles of genes through its DNA methyltransferase 1 (DNMT1) inhibitory activity [11]. Combination therapies incorporating EGCG with other bioactive molecules may be very effective in numerous cancers [12]. However, many of these studies employ high concentrations of the compound that may not be achievable *in vivo*. Our rational is that when two effective compounds with potent epigenetic properties are used the combined epigenetic effects may be more effective in reducing *survivin* expression, an upregulated anti-apoptotic molecule in CRCs, and that this may allow lower concentrations of the compounds for therapy. Studies in various other cancer cell lines have shown that EGCG and NaB can effectively inhibit survivin independently, albeit at higher concentrations [13,14]. However, the combination effects of these compounds on colon cells, where the availability of the molecules are at the highest physiological levels, are not known.

In our study, we treated RKO, HCT-116 and HT-29 CRC cells at physiologically achievable concentrations of EGCG and NaB (10 μM and 5 mM, respectively) [15–18] and the combined effects of these epigenetic regulators were observed in terms of survivin down-regulation. RKO and HCT-116 are colorectal carcinoma cell lines and are genetically similar. HT-29 is not genetically similar to RKO or HCT-116 cell lines and is an adenocarcinoma cell line. We sought to determine if the compounds were effective against cell lines that were genetically similar or different, and if p53 would govern the molecular changes observed in the study. We also assessed p21, an important cell cycle regulatory protein that has been reported to regulate survivin expression in other cancer cell types [19,20]. We asked if the combined therapy of EGCG and NaB could have a greater effect at inducing p21 expression with the concomitant down-regulation of survivin in CRC cells, at lower molecular concentrations. NaB alone is potent enough to induce DNA-damage, and when combined with EGCG this damage may be enhanced, stimulating cell cycle arrest in parallel with p21 induction and down-regulation of survivin. We found that the combination of EGCG and NaB arrested cells in the G2/M phase for both the RKO and HCT-116 CRC cells and a G1 arrest was observed in HT-29 cells. All cells had a decreased S phase. p21 induction was observed in the RKO CRC cells which was p53-dependent. Taken together this study provides a novel chemotherapeutic approach in the treatment of CRCs at lower effective doses of natural molecules.

Materials and methods

Cell culture

RKO (CRL-2577), HCT-116 (CCL-247) and HT-29 (HTB-38) CRC cells were obtained from American Type Culture Collection (ATCC). RKO CRC cells were cultured in DMEM 1X medium (Mediatech Inc., Manassas, VA, USA), HCT-116 and HT-29 were cultured in DMEM-F12 (Mediatech Inc., Manassas, VA, USA), and all cell cultures were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA, USA). The cells were cultured as per the manufacturer's protocol and were maintained in a humidified 5% CO_2 incubator at 37 °C. RKO, HCT-116 and HT-29 CRC cells were treated with 10 μM EGCG (Sigma, St. Louis, MO, USA) or 5 mM sodium butyrate (NaB) (Sigma, St. Louis, MO, USA) for 48 h. EGCG was prepared in DMSO with a stock concentration of 20 mg/ml and NaB was at a stock concentration of 100 mg/ml in sterile water. The concentration of DMSO in medium was less than 0.1% (v/v). Cells treated with DMSO served as a vehicle control. During treatments working solutions were freshly prepared and the medium was changed every 24 h with the freshly prepared compound solutions.

Cell viability assessment

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay after treatment with various concentrations of EGCG and NaB and selected concentration of the combined drugs. Approximately 1×10^4 RKO, HCT-116 and HT-29 CRC cancer cells were seeded in each well in 96-well plates. Cells were treated as indicated after 24 h. At the end of each treatment the cells were washed twice with 100 μL PBS and 100 μL of media containing 10 μL of 1 mg/mL MTT (Sigma, St. Louis, MO, USA) was added to each well before incubation for 1 h at 37 °C in a humidified 5% CO_2 incubator. At the end of the incubation period, the medium was aspirated and 200 μL DMSO was added to each well to dissolve the formazan crystals. Dye absorbance in each well was measured at 490 nm with a reference wavelength at 620 nm. Cells treated only with media served as negative control and DMSO at a final concentration of 0.1% was used as experimental control.