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Butyrate regulates the expression of inflammatory and chemotactic cytokines in human acute leukemic cells during apoptosis

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ABSTRACT

Butyrate is a histone deacetylase inhibitor implicated in many studies as a potential therapy for various forms of cancer. High concentrations of butyrate (>1.5 mM) have been shown to activate apoptosis in several cancer cell lines including prostate, breast, and leukemia. Butyrate is also known to influence multiple signaling pathways that are mediators of cytokine production. The purpose of this study was to evaluate the impact of high concentrations of butyrate on the cancer microenvironment vis-à-vis apoptosis, cellular migration, and capacity to modulate cytokine expression in cancer cells. The results indicate that high concentrations of butyrate induced a 2-fold activation of caspase-3 and reduced cell viability by 60% in U937 leukemia cells. Within 24 h, butyrate significantly decreased the levels of chemokines CCL2 and CCL5 in HL-60 and U937 cells, and decreased CCL5 in THP-1 leukemia cells. Differential effects were observed in treatments with valproic acid for CCL2 and CCL5 indicating butyrate-specificity. Many of the biological effects examined in this study are linked to activation of the AKT and MAPK signaling pathways; therefore, we investigated whether butyrate alters the levels of phosphorylated forms of these signaling proteins and how it correlated with the expression of chemokines. The results show that butyrate may partially regulate CCL5 production *via* p38 MAPK. The decrease in p-ERK1/2 and p-AKT levels correlated with the decrease in CCL2 production. These data suggest that while promoting apoptosis, butyrate has the potential to influence the cancer microenvironment by inducing differential expression of cytokines.

1. Introduction

Cytokines are regulatory proteins released from numerous immune and other cells of the body that mediate host defense and repair responses. Cytokines mainly regulate cells of the immune and hematopoietic systems, and modulate inflammatory responses [1,2]. The study of cytokine function is complicated by characteristics which include: pleiotropy, redundancy, synergism, and receptor promiscuity [3]. Cytokines are divided into six subgroups: interferons, colony stimulating factors (CSF), growth factors, tumor necrosis factors (TNF), chemokines, and interleukins. It has become increasingly accepted by cancer researchers that in order to fully understand the tumorigenicity of cancer, the function of each cell type within the tumor and how these cells interact with the tumor microenvironment must be dissected. Exploring the crosstalk between these cells within the microenvironment *via* cytokines may explain their effects on tumor growth, angiogenesis, immune response, and metastasis [4–7].

Butyrate is a short-chain fatty acid produced in the human colon by bacterial fermentation activity [8,9]. Butyrate is a histone deacetylase (HDAC) inhibitor [10,11] implicated in many studies as a potential therapy for prostate [12], breast [13], and other forms of cancer [14] due to its ability at high concentrations (>1.5 mM) to cause cell death. Primarily highlighted for its use as a secondary chemotherapy, clinical use of butyrate holds substantial hope for reducing inflammation, reversing epigenetic aberrations, and suppressing the proliferation of cancer stem cells [15]. Butyrate is a feasible candidate to treat obesity, cardiovascular disease, neurological injury and even inherited diseases [15].

Previously, sodium butyrate (NaB) has been shown to induce apoptosis in leukemia cancer cell lines including U937 [16] and HL-60 cell lines [17]. Some reports imply that butyrate-induced

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apoptosis is associated with inhibition of telomerase activity [16], alterations in pro- and anti-apoptotic proteins, and regulation of signaling pathways involved in apoptosis [14]. A well-known target of butyrate is the $p21^{Waf1/Cip1}$ gene. Activation of p21 results in cell cycle arrest at G1, a phenomenon that may lead to apoptosis or cell differentiation [18–20] independent of p53 [21]. Prior studies have shown that butyrate alters gene expression due to its HDAC inhibitory capabilities. Joseph et al. demonstrated that H460 human lung carcinoma cells treated with butyrate displayed differential expression of genes responsible for cytokine signaling and metastasis [22]. Also, butyrate increases the production of Interleukin-5 (IL-5) *via* epigenetic modifications in Jurkat cells [23] and TGF-β1 in colon carcinoma [24]. All of these studies utilized concentrations of butyrate at or below 2 mM. Often, higher concentrations may be needed to elicit the appropriate response. However, the biological implications of using these higher concentrations are largely unknown. Our goal was to understand whether high concentrations of butyrate that induce apoptosis are able to influence the tumor microenvironment vis-à-vis production and secretion of cytokines. We also elucidated the biological relevance of these alterations and attempted to pinpoint the associated signaling pathways required for butyrate-induced effects on cytokine production in leukemia cells. In summary, we concluded that butyrate alters the expression of cytokines and chemokines during apoptosis in leukemia cell lines.

2. Materials and methods

2.1. Cell culture

U937 leukemia cells were purchased from ATCC, HL-60 leukemia cells were from Dr. Ann Richmond at Vanderbilt University, and THP-1 leukemia cells were from Dr. Chandravanu Dash at Meharry Medical College. Cells were maintained in CO_2 (5%) incubator in RPMI-1640 media (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Kansas City, MO) containing 50 U/mL each of penicillin and streptomycin (Gibco, Grand Island, NY). Cell viability was determined to be better than 95% by trypan blue exclusion assay. Sodium butyrate (NaB) and valproic acid (VPA) (Sigma-Aldrich, St. Louis, MO) were reconstituted in sterile water at a stock concentration of 1 M.

2.2. Isolation of monocytes

Human peripheral blood mononuclear cells (PBMC) were isolated from anonymous donors (New York Blood Center, Long Island, NY). PBMCs were harvested *via* Ficol-Percoll PLUS gradient (GE Health Care, Piscataway, NJ). Monocytes of >85% purity were harvested and suspended in RPMI-1640 media supplemented with 10% FBS containing 50 U/mL each of penicillin and streptomycin as previously described [25].

2.3. Cell viability assay

Cells (5×10^6) were either untreated or treated with various concentrations of butyrate or valproic acid for 24 h, harvested, and then diluted in 0.4% trypan blue at a ratio of 1:5. The cell number was counted with a hemocytometer.

2.4. Caspase-3 activation

Cells (10×10^6) were untreated, treated with butyrate (5 or 10 mM) or valproic acid (2 or 4 mM) for 24 h. Cells were lysed and equal amount of cell lysate proteins (120 μg) were assayed for evidence of activated caspase-3 using a caspase-3 colorimetric protease assay (Medical & Biological Laboratories Co. LTD, Woburn, MA) as previously described [25]. This assay utilized a substrate in which the amino acid sequence aspartic acid-glutamic acid-valine-aspartic acid is recognized by activated caspase-3 resulting in cleavage of substrate and exposure of a chromophore that can be detected with a spectrophotometer at 405 nm.

2.5. Apoptosis detection using Annexin V staining

Cells (5×10^6) were either untreated or treated with butyrate (5 mM) or valproic acid (4 mM) over 24 h. The cells were harvested, and suspended in 600 μL of Binding Buffer containing Annexin V-FITC conjugate as provided by the manufacturer (Raybiotech, Norcross, GA). Stained cells were run on Guava EasyCyte flow cytometer (EMD Millipore, Billerica, MA) and analyzed with FlowJo 7.6 Single Cell Analysis software as previously described [26].

2.6. Analysis of cytokine production

Culture supernatant from untreated or treated cells (5×10^6) was assayed for quantitative and qualitative levels of inflammatory cytokines and chemokines using a multi-analyte cytokine ELISA assay (SABiosciences, Frederick, MD). Briefly, tissue culture supernatants (50 μL/well) or standards (50 μL/well) were incubated in pre-coated 96-well microtiter plates at room temperature for 2 h. After washing, 100 μL of the detection antibody mixture was added for 1 h. Then, 100 μL of Avidin-HRP conjugate was added to each well for an additional 30 min at room temperature. Development and stop solutions were added and absorbance read at 450 nm. To quantify cytokine production, single-analyte ELISAs were used. In this assay, the protocol is previously described above. In addition, a range of 3.2–10,000 pg/mL recombinant cytokines was used to establish a standard curve for protein concentration.

2.7. Cellular migration assay

BD Biocoat Chambers (BD Biosciences, San Jose, CA) were used as a matrix. Media from butyrate-treated cells (5×10^6), FBS, or RPMI media only was added to the bottom wells to act as a chemoattractant (500 μL). Human monocytes (1×10^5) were suspended in 100 μL of RPMI media only and placed in the insert. The chambers incubated for 24 h in the tissue incubator at 37 °C. After the incubation period, the number of migrated cells in the lower chamber was determined by trypan blue exclusion assay.

2.8. Western blot analysis

U937 or HL-60 cells (10×10^6) were cultured overnight in serum-free media containing 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 100 U/mL each of penicillin and streptomycin to eliminate the influence of serum components on the activation of signaling proteins. The cells were then treated at various time points with either individual or varying combinations of the following compounds: butyrate, SB203580 (10 μM) (EMD Millipore, Billerica, MA), Anisomycin (10 μg/mL) (Sigma-Aldrich, St. Louis, MO), DMSO (0.5%) (Sigma-Aldrich, St. Louis, MO), LY294002 (20 μM) (Cayman Chemical, Ann Arbor, MI), and U0126 (20 μM) (Cayman Chemical, Ann Arbor, MI). Cell lysates were prepared and equal amounts of proteins were analyzed by Western blotting as previously described [27]. The following antibodies were used: p-ATF, Caspase-3, GAPDH, total and phosphorylated forms of AKT, p38 MAPK, JNK, and ERK1/2 (Cell Signaling, Danvers, MA). Blots were visualized by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA).