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Research Paper

Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells

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ABSTRACT

Butyrate is one of the short chain fatty acids, produced by the gut microbiota during anaerobic fermentation of dietary fibres. It has been shown that it can inhibit tumor progression via suppressing histone deacetylase and can induce apoptosis in cancer cells. However, the comprehensive pathway by which butyrate mediates apoptosis and growth arrest in cancer cells still remains unclear. In this study, the role of miR-22 in butyrate-mediated ROS release and induction of apoptosis was determined in hepatic cells. Intracellular expression of miR-22 was increased when the Huh 7 cells were incubated with sodium butyrate. Over-expression of miR-22 or addition of sodium butyrate inhibited SIRT-1 expression and enhanced the ROS production. Incubation of cells with anti-miR-22 reversed the effects of butyrate. Butyrate induced apoptosis via ROS production, cytochrome c release and activation of caspase-3, whereas addition of N-acetyl cysteine or anti-miR-22 reversed these butyrate-induced effects. Furthermore, sodium butyrate inhibited cell growth and proliferation, whereas anti-miR-22 inhibited these butyrate-mediated changes. The expression of PTEN and gsk-3 was found to be increased while p-akt and β-catenin expression was decreased significantly by butyrate. These data showed that butyrate modulated both apoptosis and proliferation via miR-22 expression in hepatic cells.

1. Introduction

The short chain fatty acids, namely butyrate, acetate and propionate, produced by the gut microbiota during anaerobic fermentation are mainly absorbed by the colon or liver cells [1]. Butyrate exerts several effects including, induction of apoptosis and growth arrest in patients with solid tumors [2,3]. It delays tumor growth and induces apoptosis by mediating expression of histone deacetylase (HDAC), SIRT-1, caspase 3, and NFκB [4]. Furthermore, several HDAC inhibitors including butyrate have been found to be useful in chemotherapy strategy to restrain proliferation, growth, and to provoke cell death in the cancer cells [3,5]. Previously it was shown that butyrate significantly inhibited CD44 expression, thereby inhibiting the metastatic ability of the human colon carcinoma cells [6]. Prolonged butyrate treatment inhibited the pro-MMP-2 activation and tumor cell migration potential of HT 1080 tumor cells [7]. Although several scanty reports show the mechanisms by which butyrate mediates apoptosis in cancer cells, no clear relationship is available between butyrate and the induction of apoptosis.

SIRT-1, a member of histone deacetylase protein family, is widely expressed in the hepatoma cells which helps in the tumor cell survival and growth [8]. SIRT-1 knockdown enhanced the chemosensitivity of H292 cells to cisplatin and reduced the tumor volume and the metastatic ability of the cells in nude mice [9]. Recently it was shown that SIRT-1 expression was associated with nanog expression in patients with colorectal adenocarcinoma [10]. Several anticancer drugs and chemotherapeutic agents target the expression of SIRT-1 to suppress the tumor growth [11]. Butyrate, being a HDAC inhibitor, is not known whether it regulates apoptosis via SIRT-1 in hepatic cells.

miRNAs are small, ~21–23 nucleotides long, non-coding RNAs that inhibit the target mRNAs at post-transcriptional level. Depending on the degree of complementarity, miRNA binding with target mRNAs induces either mRNA degradation or inhibits translation, thereby regulating a variety of cell functions, such as proliferation, apoptosis, senescence, differentiation, and cancer [12]. Several specific miRNAs have been shown to act as tumor suppressors or as oncogenes in

List of abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DMSO, Dimethyl sulfoxide; ECL, Enhanced chemiluminescence; Gpx, Glutathione peroxidase; H_2-DCFHDA, 2′,7′-dichlorodihydrofluorescein diacetate; HDAC, Histone deacetylase; NAC, N-acetyl cysteine; ROS, Reactive oxygen species; SOD, superoxide dismutase; TUNEL, Terminal deoxynucleotide transferase dUTP Nick End Labeling

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cancers [13]. Recently, miRNA-204-5p was shown to regulate hepatocellular progression via down-regulating SIRT-1 [14]. miRNA-34a inhibited cell proliferation in prostate cancer by downregulating SIRT-1 expression [15]. In glioblastoma cells, miR-22 inhibited the proliferation, motility and invasion by targeting SIRT-1 [16]. Although, butyrate had been shown to inhibit the cell proliferation and tumor growth, there is no data available to show the involvement of miRNA in butyrate-mediated apoptosis. In the present study, we demonstrated that butyrate induces apoptosis through up-regulation of miR-22, followed by the downregulation of SIRT-1, resulting in increased ROS production and apoptosis in hepatic cells.

2. Materials and methods

2.1. Cell culture, butyrate treatment and miRNA transfection

Huh 7 cells (1×10^5 cells/well) were cultured in 6-well plates containing DMEM medium with 10% FBS and 1% penicillin-streptomycin for 24 h. The cells were incubated with varying concentrations (0–8 mM) of sodium butyrate for 24 h under serum-free conditions. Transfection of miR-22 premiRs, anti-miR-22 oligos or non-specific miRNA (NS) (Sigma-Aldrich, St Louis, MO, USA) was performed using siPORT™ NeoFX™ transfection reagent (Thermofisher, Carlsbad, CA, USA) as described by us previously [17]. After the experiments, the cells were collected for either total RNA isolation or protein separation.

2.2. Western blotting analysis

Whole cell lysates prepared in M-PER mammalian protein extraction reagent (Thermofisher) and complete protease inhibitor cocktail (1:100). Equal amount (30–50 μg/well) of protein samples were separated on SDS-PAGE and transferred to PVDF membranes. The membranes first blocked with 10% non-fat dry milk and incubated with the primary antibodies (SIRT-1, PTEN, akt, p-akt, gsk3β, β-catenin, bcl-2, cytochrome c, caspase-9, caspase-3 and β-actin). After incubation with appropriate secondary antibodies, the immunoblots were incubated with ECL plus Western blotting substrate (Thermofisher), exposed to X-ray films and developed. PVDF membranes were reused after removing the antibodies using Restore plus Western blot stripping buffer (Thermofisher).

2.3. miRNA isolation and real-time RT PCR

Total RNA enriched with miRNA was isolated using miRvana RNA isolation kit (Thermofisher), cDNA was synthesized using cDNA synthesis Kit (Exiqon, Vedbaek, Denmark) and real time RT-PCR was performed using SYBRgreen mastermix and respective miRNA primers. miRNA-103a was used as a control RNA in all real time RT-PCR experiments. Intracellular expression of superoxide dismutase 1 and β-actin (as control) was quantitated by real time RT-PCR. The fold change in miRNA expression was determined by the comparative CT method ($2^{-\Delta\Delta CT}$) [18].

2.4. Colony formation assay

A total of 1000 cells per well were plated into 6-well cell culture plates and incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 3–5 days and the colony formation was observed. The colonies were stained with 0.1% crystal violet (Sigma Aldrich; dissolved in 50% methanol and 10% glacial acetic acid) and the colony formation was observed under the inverted phase contrast microscope.

2.5. Cell proliferation assay

Cells were seeded at ~2000 cells/well in 96-well plates. After 24 h, the cells were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. Cell viability was determined using the CellTiter-Blue cell viability assay kit (Promega Corp., Madison, WI, USA) as per the manufacturer's instructions. The luminescence was measured using microplate reader (BioTEK, Vinooski, VT, USA) and the percentage of proliferation was calculated by comparing with the control cells.

2.6. ROS analysis

Huh 7 cells (2×10^5 cells/well) were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. H_2-DCFDA (10 μM; Thermofisher) was added to each well and incubated for a further period of 60 min. The cells were observed under fluorescence microscope and the images were acquired. After incubation, cells were counterstained with DAPI and the images were taken using the fluorescence microscope. The percent of ROS production was calculated by counting in at least 10 different high power fields, averaged and the results were presented.

2.7. Measurement of mitochondrial membrane potential

The cells (4×10^5 cells/well) were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. The cells were fixed by 4% paraformaldehyde, washed and 100 nM $DiOC_6$ was incubated for 15 min in dark as described previously [19]. The cells were washed using phosphate buffered saline and observed under fluorescence microscope.

2.8. ABTS assay

In order to quantitate the total free radicals scavenging activity or the total cellular antioxidants, the cells (2×10^5 cells/well) were plated and incubated with butyrate alone butyrate and anti-miR-22 oligos for 24 h. The cell lysates were prepared and equal amount of protein was incubated with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS, 200 μl of 7 mM) for 5 min at room temperature [20]. The absorbance was measured at 730 nm using water as blank. The percentage of ABTS scavenging activity was determined by the following formula.

$$\%\text{ABTS inhibition} = 1-\frac{Absorbance\ of\ the\ Sample}{Absorbance\ of\ Blank}X100$$

2.9. SOD assay

Huh 7 cells (2×10^5 cells/well) were plated and incubated with butyrate alone butyrate and anti-miR-22 oligos for 24 h. SOD activity was measured in the cell lysates using SOD assay kit (Sigma Aldrich) as per the manufacturer's protocol. The percentage of SOD activity was calculated.

2.10. TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay

Huh 7 cells (2×10^5 cells/well) were incubated with butyrate alone or butyrate and N-acetyl cysteine or butyrate and anti-miR-22 oligos for 24 h. After incubation the cells were washed, fixed using 4% paraformaldehyde and the apoptosis was detected using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) as per the manufacturer's instructions. The cells were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and the cells were counted in at least 10 different high power fields and the percent of apoptosis induction was calculated.