Sodium butyrate induces senescence and inhibits the invasiveness of glioblastoma cells

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Received July 19, 2017; Accepted October 20, 2017

DOI: 10.3892/ol.2017.7518

Abstract. Sodium butyrate (SB), a short chain (C-4) saturated fatty acid, is present in the human bowel at increased concentrations (~2 mM) as a food metabolite. It has been demonstrated that SB exerts an anti-tumor effect as a histone deacetylase inhibitor; however, its precise mechanism of action remains to be elucidated. The present study focused on the mechanisms underlying the effects of SB on glioblastoma (GB) cell proliferation, motility and invasion. In human GB A172 cells, flow cytometry and a Boyden chamber assay demonstrated that physiological concentrations of SB (0.25-4.00 mM) dose-dependently inhibited cell proliferation and invasion. SB also affected cellular morphology, with increases in cell area and the number of focal adhesions observed. However, the phosphorylation (Y397 site) of focal adhesion kinase (FAK) was increased, while that of myosin light chain (S19 site) was unaltered. All of these SB-induced effects were reversible and attenuated following SB withdrawal. In addition, A172 cells treated with SB exhibited positivity for senescence-associated (SA) β-galactosidase (gal) staining and elevated protein expression of p21 and p16 in a time- and dose-dependent manner, whereas the expression of p21 mRNA decreased. Knockdown of p21 expression using small interfering RNA reversed the inhibition of cell growth inhibition and positivity for SA β-gal staining, but did not reverse the inhibition of cell motility and enhanced phosphorylation of FAK. This suggests that cells require p21 to induce senescence but not for SB-mediated decreased motility. Therefore, the current study demonstrated that SB inhibits GB cell proliferation, induces cells to senescence and inhibits tumor cell invasion, indicating that it may be developed as a novel therapeutic strategy to treat GB.

Introduction

The prognosis of patients with glioblastoma (GB) remains poor despite the use of multidisciplinary treatments, which consist of maximal surgical resection, radiotherapy and concomitant/adjuvant chemotherapy with temozolomide (1). The median time-to-recurrence following standard therapy is ~7 months (2). Once tumor recurrence has occurred (generally within 1 year), further clinical deterioration may occur, along with expansive tumor growth, as identified by contrast-enhanced imaging (3) and the dissemination of the tumor into the cerebrospinal fluid (4). Patients with GB typically succumb within 2 years of diagnosis, despite multiple types of therapy available to treat GB (5). Therefore, in order to combat this devastating disease, novel targeted therapies to treat patients with GB are strongly required. In addition, since GB appeared in the brain, therapies are required to minimize adverse effects on the neuronal cells to keep host brain function.

Sodium butyrate (SB), a short chain (C-4) saturated fatty acid, is present in the human bowel at increased concentrations (~2 mM) as a food metabolite (6). It has been reported that SB exerts an anti-tumor effect as a histone deacetylase (HDAC) inhibitor in colon (6,7), pancreas (8) and liver cancer (9) as well as in glioma (10); however, its precise mechanism of action remains to be fully elucidated (10-12). The present study focused on the underlying mechanism of SB regarding its effects on human GB cell proliferation, motility and invasion.

In the present study, SB induced dose-dependent growth inhibitory effects in vitro with G1/S phase arrest and stabilization of p21 expression. SB also increased senescence-associated (SA) β-galactosidase (gal) levels and exhibited a significant inhibitory effect on tumor cell invasion in vitro. All these biological effects of SB were reversible and were attenuated following SB withdrawal.

Materials and methods

Materials and reagents. SB (cat. no. 193-01152; reagent grade), trichostatin A (TSA; cat. no. 203-17561), methotrexate (cat. no. 139-13571) and N-acetyl-L-cysteine (NAC; cat. no. 015-05132; ROS scavenger) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-focal adhesion kinase (FAK) polyclonal antibody

Key words: sodium butyrate, glioblastoma, senescence, invasion, targeted therapy

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RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted and purified using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. A total of 1 µg RNA was subsequently used as a template for reverse transcription using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. PCR was performed using Taq DNA Polymerase (Promega Corporation) with the following gene-specific primers: p21, forward, 5'-TTATGA AATTCTACCCCCCTTTT-3'; and reverse, 5'-GGCCCTTCTA AAGTGGCCATCT-3'; p27, forward, 5'-AGATGTCACACCC TGCGAGTG-3'; and reverse, 5'-TCAGTCTTGGGTTGCC ACCAA-3'; p53, forward, 5'-GGGGCCACCTTTAGCCGTAC TA-3'; and reverse, 5'-CTCCACTAACCAGCTGCACCA-3'; and GAPDH, forward, 5'-AAGGGAGCTGACAGGATG-3' and reverse, 5'-TTGCGGTATTCACCGAGAAGT-3'. GAPDH functioned as the reference gene. Thermocycling conditions were 95°C for 30 sec for denaturation, 60-65°C for 30 sec for annealing and 68°C for 1 min for extension and cycle numbers were 30-35 cycles.

Immunofluorescence microscopy. Immunofluorescence analysis was performed following a previously described protocol (13). Briefly, A172 cells were cultured with 0-4 mM SB on type I collagen-coated 2-well chamber slides (BD Biosciences). After 48 h, the cells were fixed with 1% paraformaldehyde at room temperature for 1 h in PBS and then permeabilized with 0.2% Triton-X-100 in PBS. Non-specific blocking was performed in 0.1% bovine serum albumin (Wako Pure Chemical Industries, Ltd.) at room temperature for 1 h. Cells were incubated with primary antibody, Anti-p-tyrosine pY397-FAK (dilution, 1:100) at 4°C overnight, followed by incubation with Alexa 488 anti-rabbit immunoglobulin G (dilution, 1:1,000; cat. no. A-11088; Molecular Probes; Thermo Fisher Scientific, Inc.) secondary antibody, at room temperature for 1 h in dark room. Images were obtained using the Olympus BX50 fluorescence microscope (magnification, x40; numerical aperture, 0.6; Olympus Corporation, Tokyo, Japan). Images were analyzed and processed for presentation by adjusting brightness and contrast using ImageJ 1.38e software (National Institutes of Health, Bethesda, MD, USA).

Effects of SB on cell motility and invasion. A172 cells were pretreated with 0, 0.25, 0.5, 1, 2 and 4 mM of SB for 48 h. The cell motility assay was performed for 16 h using 24-well Bio-Coat cell migration chambers (BD Biosciences) using pY397FAK, pY-577FAK and p-MLC20 (all 1:100) at 4°C overnight. The membranes were then incubated with were alkaline phosphatase (AP)-conjugated anti-rabbit IgG (dilution, 1:7,500; cat. no. S3731; Promega Corporation, Madison, WI, USA) or AP-conjugated anti-mouse IgG (dilution, 1:7,500; cat. no. S3721; Promega Corporation) secondary antibodies for 30 min at room temperature. Final detection was performed using Western Blue stabilized substrate for alkaline phosphatase (cat. no. S3841, Promega Corporation). Band intensity was analyzed using ImageJ 1.38e software (National Institutes of Health, Bethesda, MD, USA).
0.5% FBS as a chemoattractant, following a previously described protocol (13). The cell invasion assay was performed using Matrigel-coated polyethylene terephthalate membranes for 16 h. Cells at a density of 2x10^5 cells/well were inserted into the upper chamber. The migrating cells on the lower side of the filter were fixed with 70% ethanol at room temperature for 1 h, prior to being stained with Giemsa solution (Wako Pure Chemical Industries, Ltd.) at room temperature for 10 min and counted using Olympus IX70 phase contrast microscopy with a x10 objective lens. For cell area analysis, cell images were captured using Olympus IX70 phase contrast microscopy with a x10 objective lens. Cell periphery was circled then the inner area was calculated with ImageJ 1.38e software. A minimum of 100 cells/each experiment were measured.

β-gal staining assay. The β-gal staining assay was performed using a Senescence β-Galactosidase Staining kit (cat. no. 9860; Cell Signaling Technology, Inc., Danvers, MA, USA). Briefly, A172 cells were cultured with 0, 0.25, 0.5, 1, 2 or 4 mM SB, or 25, 50 or 100 nM TSA at 37˚C for 4 days in 35 mm plastic culture plates, and fixed with 10% formaldehyde at room temperature for 1 h. Plates were rinsed twice with PBS, subsequently the β-gal staining solution containing X-gal as a substrate was added and plates were incubated at 37˚C overnight in a dry incubator. β-gal-positive cells were then counted using Olympus IX70 phase contrast microscopy with x10 objective lens and the percentage of positive cells was calculated.

Proteasome activity assay. The proteasome activity assay was performed using a Proteasome Activity assay kit (cat. no. ab107921; Abcam, Tokyo, Japan) following the manufacturer's protocol. A172 cells were treated with 2 mM SB for 48 h. The chymotrypsin-like function of A172 cell lysate for a 7-Amino-4-methyl-coumarin-tagged peptide substrate (proteasome substrate Succ-LLVY-AMC) was compared with that of the positive control Jurkat cell lysate in the presence of the proteasome inhibitor MG132 (2 µM).

Transient RNA interference. Small interfering (si)RNA against human p21 (CDKN1A, SignalSilence p21 Waf1/Cip1 siRNA) was obtained from Cell Signaling Technology, Inc. A siRNA-control was designed for Pho tinus Pyralis GL3 luciferase as previously described and obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan) (14). This gene is not present in mammalian cell transcripts and therefore the luciferase siRNA-control minimizes the off-targeting effect (14). A172 cells were transfected with siRNA-p21 or siRNA-control at a final concentration of 50 nmol/l using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cell lysates were prepared for western blotting 72 h after transfection, performed as aforementioned. Transfected cells underwent staining for β-gal. Cell motility and invasion assays, as well as cell cycle analyses were also performed.

Statistical analysis. Data are expressed as the mean ± standard deviation. Differences were determined by analysis of variance with Bonferroni's test as a post-hoc test in JMP software (version 11; SAS Institute, Inc., Cary, NC, USA). P<0.01 was considered to indicate a statistically significant difference.

Results

Effect of SB on the proliferation of A172 human glioblastoma cells. Physiological concentrations of SB (0.5-4 mM) inhibited the proliferation of the human GB A172 cells in a dose-dependent manner (Fig. 1A). In addition, cell cycle analysis identified an increase in the percentage of cells in the G0/G1-phase following SB treatment, suggesting G1/S arrest. This inhibitory effect of SB on A172 cell proliferation and cell cycle progression was reversible and attenuated following wash-out of the drug (Fig. 1B (orange line) and Fig. 1C). No sub-G1 apoptotic population was observed following FACS analysis of the A172 cells treated with SB. By contrast, FACS analysis identified apoptotic A172 cells following the treatment of cells with the HDAC inhibitor TSA or the chemotherapy drug methotrexate (data not shown).

Effect of SB and the HDAC inhibitor TSA on SA β-gal staining. SB also induced positive staining for SA β-gal in A172 cells (Fig. 2A). This positive staining for β-gal, indicating cellular senescence, was SB dose-dependent (Fig. 2B). However, the HDAC inhibitor TSA did not induce any positive staining for β-gal (Fig. 2B). To elucidate the mechanism associated with this cell cycle arrest, the expression of cell cycle regulator proteins was assessed.

SB increased the p21 protein level. A172 cells treated with 2 mM SB exhibited elevated levels of p21, p27 and p53 and this increase in expression was time-dependent (Fig. 3A); however, levels of p21 mRNA were decreased 24 h after treatment with SB (Fig. 3B). Since A172 cells harbor wild-type p53 (15), it was deduced that the p53-p21 axis functioned in the cells. The results of the present study suggest that 24 h treatment with SB stabilizes the expression of the three cell cycle regulator proteins p21, p27 and p53 in A172 cells. Although levels of p21 mRNA decreased, the levels of p27 and p53 mRNA were unaltered. Therefore, it is likely that the post-translational protein stabilization of p21, p27 and p53 induced by SB treatment is the primary mechanism responsible for the results of the present study. The present study therefore assessed the potential inhibitory activity of SB against the proteasome compared with that of MG132, a specific proteasome inhibitor. SB did not exhibit any direct inhibitory effect on the proteasome in this proteasome activity assay (data not shown).

Effect of SB on the motility, invasion and morphology of A172 cells. SB also inhibited cellular motility and invasion in a dose-dependent manner and affected the morphology of A172 cells (Fig. 4). Increased cell area and adherence to the extracellular matrix was observed following treatment with SB (Fig. 4C). Since 48 h SB treatment did not affect cellular proliferation or apoptosis (Fig. 1), it could be concluded that the decreased number of infiltrated cells was due to a decrease in cell motility following SB treatment and not due to a decrease in total cell number. In addition, the phosphorylation of FAK (Y397 site) was increased, while that of MLC20 (S19 site) remained unaltered (Fig. 4B).
The results of the immunofluorescence analysis indicated that SB dose-dependently induced an increase in the amount of focal adhesions at the cell peripheries (white arrows, stained with anti-pY397-FAK antibodies; Fig. 5) and increased cell spreading area on the substratum. The effects of SB on the expression and phosphorylation of FAK were reversible and were attenuated following the washing out of SB from the cell culture (Fig. 6A). Cellular motility also recovered 3-4 days following SB wash out (Fig. 6B).

**Figure 1.** Effect of SB on the proliferation of A172 human glioblastoma cells. (A) *In vitro* proliferation assay of A172 cells following treatment with different concentrations of SB (0.0, 0.5, 1.0, 2.0 and 4.0 mM). Results are presented as the mean ± standard deviation (n=3). *P<0.01 vs. treatment with 0 mM SB. (B) Reversibility of SB inhibitory effect. A total of 4 days following treatment with 2 mM SB, A172 cells were washed with media without SB and cultured for 4 additional days. The results are presented as the mean ± standard deviation (n=3). *P<0.01 vs. treatment with 2 mM SB. (C) Cell cycle analysis of the A172 cells in (B) using a BD FACSCalibur system. SB, sodium butyrate.

**Figure 2.** Effect of SB and the HDAC inhibitor TSA on SA β-gal staining. (A) A172 cells treated with SB (0-4 mM) or TSA (25-100 nM) for 4 days were stained with SA β-gal. β-gal-positive cells are indicated by white arrows (scale bar=20 µm). (B) β-gal-positive cells in (A) were analyzed and counted. Results are presented as the mean ± standard deviation (n=4); *P<0.01 vs. control. SA β-gal, senescence-associated β-galactosidase; SB, sodium butyrate; HDAC, histone deacetylase; TSA, trichostatin A.

**p21 involved SB induced A172 cellular senescence but not involved cellular motility.** The present study focused on the underlying mechanism of SB regarding its effects on cellular senescence and motility. Knockdown of p21 expression was achieved by transfection with siRNA specific for the human p21 gene CDKN1A. The inhibition of p21 expression was confirmed by western blotting and assessed following the treatment of cells with 2 mM SB (Fig. 7A). Knockdown of p21 expression did not affect the expression levels of p53, p27 and p16 (Fig. 7A). In contrast, knockdown
of p21 expression partially reversed the inhibition of proliferation and positive SA β-gal staining, but cell motility remained inhibited (Fig. 7B). Knockdown of p21 expression increased S phase in the control and SB treated cells using cell cycle analysis (Fig. 7C). However, the increase in FAK phosphorylation induced by 2 mM SB was unaffected following knockdown of p21 (Fig. 7A). These results suggest that upregulation of p21 is implicated in the cellular senescence, but not the inhibition of cell motility, that is induced by SB.

**Discussion**

The results of the present study indicated that SB reversibly inhibited A172 cell proliferation and induced cellular senescence by upregulating and stabilizing the cell cycle regulator proteins p21, p27 and p53. SB also reversibly inhibited cellular motility and invasion. Downregulating p21 using specific siRNA partially reversed the proliferation-inhibiting effect of SB on cell cycle analysis and β-gal staining. By contrast, cell motility, cell spreading and the phosphorylation
of FAK remained decreased following p21 knockdown in A172 cells.

SB exhibits HDAC-inhibiting activity. However, TSA (a typical HDAC inhibitor) did not promote cellular senescence or reversibly inhibit cellular proliferation in A172 cells, and did not induce cell apoptosis. Previous studies by our group used similar assays to evaluate the effect of SB in various other tumor cell lines, including human fibrosarcoma (HT-1080), human breast cancer (MDA-MB-231; unpublished) and rat mammary cancer (Walker 256; manuscript in preparation). In all cases, SB reversibly inhibited cell proliferation, increased cell adhesion, and inhibited cell motility and invasion. Furthermore, the effect of SB on normal primary cultured cells was assessed and it was identified that SB did not affect cell proliferation much. Therefore, SB may potentially be used as an anti-cancer therapy, although continuous treatment may be required. Using an osmotic pump to maintain the drug concentration,

Figure 5. Effect of SB on A172 cell morphology and intracellular FAK and MLC20 phosphorylation. Immunostaining of A172 cells treated with the indicated concentrations (0–4 mM) of SB for 48 h using phospho-specific pY397 FAK antibody. White arrows indicate the focal adhesions at the cell peripheral regions. Scale bar=20 μm. SB, sodium butyrate; FAK, focal adhesion kinase; MLC, myosin light chain.

Figure 6. Reversibility of the effects of SB in A172 cells. (A) At 4 days after treatment with 2 mM SB, A172 cells were washed in media without SB and cultured for 4 additional days. The effects on the expression of p21 (FK506 binding protein like), p27 (cyclin dependent kinase inhibitor 1B), p53, SIRT1, pY397/pY577 FAK and p-MLC20 were determined by western blotting. (B) Cell motility was measured in A172 cells treated as in (A). Results are presented as the mean ± standard deviation (n=6). *P<0.01 vs. control. SB, sodium butyrate; SIRT, sirtuin; FAK, focal adhesion kinase; p-, phosphorylated; MLC, myosin light chain.
As reported previously (13), the intrathecal administration of SB into rat cerebrospinal space was performed in a previous study by our group, which inhibited the invasion of Walker 256 cells (a syngeneic rat mammary cancer cell line) into the brain parenchyma and increased the survival rate of rats (manuscript in preparation).

The mechanism corresponding to the inhibition of motility induced by SB is currently under investigation. It has been reported that cancer motility and invasion is primarily regulated by the Rho family of small GTP-binding proteins (including RhoA and cell division cycle 42) and Rho-associated kinase (ROCK), and that the downstream molecular motor activity of myosin, determined by the phosphorylation of MLC20, is crucial (16). The specific ROCK inhibitor Y-27632 inhibits the motility, invasion and adhesion of hepatoma (16,17) and breast cancer cells (13,18). Our group has also demonstrated that the invasive phenotype of murine osteosarcoma cells is positively associated with increased FAK levels (19). In the present study, the effects of SB were unusual, particularly regarding the increases in FAK phosphorylation, cell spreading and cell area associated with decreased cell motility. Therefore, novel molecular targets of SB within tumor cells should be evaluated. SB induced multiple metabolic changes in A172 cells; therefore, metabolic pathways associated with isocitrate dehydrogenase 1 and branched chain aminotransferase, which have been implicated as critical targets in oncogenesis and progression of malignant glioma (20), may be associated with the effect of SB on glioma cell motility. Further studies by our group aim to analyze the metabolome alterations induced by SB.

Previous studies have focused on the effect of butyrate on microRNAs (miRs). Pant et al (21) reported that miR-22 and SB inhibited sirtein 1 (SIRT-1) expression, enhanced reactive oxygen species (ROS) release in hepatic cancer cells and induced cellular apoptosis. Similarly, the present study demonstrated that treatment with SB reversely inhibited the expression of SIRT-1 in A172 cells; however, an ROS scavenger (N-acetyl-L-cysteine (NAC)) did not affect SB-induced A172 cell senescence (data not shown). Han et al (22) reported that SB induced miR-203 expression and that the miR-203 target gene neural precursor cell expressed developmentally downregulated 9 was associated with the SB-induced inhibition of colony formation and invasion in colorectal cancer cells. Further studies into the targeting activity of SB besides its HDAC-inhibiting function are required.

It was previously reported that irradiation induced GB cells to senescence (23) and that anti-vascular endothelial growth factor A-targeting therapy induced senescence-associated cell death (24). p21 protein (FK506 binding protein like)-dependent cytotoxicity was suggested in these cases (23,24). Therefore, the present study hypothesized that SB induces senescence via a similar pathway.

In conclusion, SB inhibited A172 cell proliferation, induced cellular senescence and inhibited cell invasion, potentially via a mechanism associated with upregulation of p21. It may therefore be developed as a novel treatment for GB.

Acknowledgements

The authors acknowledged that the preliminary work of the present study was reported at the American Association for Cancer Research (AACR) annual meeting on April 9, 2013, and appeared in an AACR article as the meeting abstract (25). The authors would like to thank Dr Kiyoko Yoshioka (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan) for her technical assistance. The present study was supported by the Japan Society for the Promotion of Science (grant no. 15K15560 to KI).
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