Sodium butyrate restores ASC expression and induces apoptosis in LS174T cells

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Received June 1, 2012; Accepted September 14, 2012

DOI: 10.3892/ijmm.2012.1156

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Key words: sodium butyrate, apoptosis-associated speck-like protein, LS174T cells

Abstract. Sodium butyrate (NaBu) is a short-chain fatty acid (SCFA), which has been proposed as a potential anticancer agent. Apoptosis-associated speck-like protein (ASC) is a pro-apoptotic signaling factor that is subjected to epigenetic silencing in human cancers. Modulation by the aberrant methylation of CpG islands of ASC is a well-characterized epigenetic mechanism, and the methylation-induced silencing of ASC has been observed in several types of tumors. NaBu induces cell cycle arrest, markers of cell differentiation and apoptosis in colon cancer. NaBu promotes transcriptional activation by relaxing the DNA conformation and displays anti-proliferative and differentiating activity in a wide variety of cancers. Thus, we used NaBu to investigate the relationship between the status of cell proliferation and the re-expression of ASC in colon carcinoma LS174T cells. Our experiments determined ASC re-expression at the protein level using western blotting. In addition, we used reverse transcription-polymerase chain reaction to detect the expression levels of ASC mRNA and an MTT assay to detect the inhibitory rate of cell growth. The apoptosis rate was also detected for further validation of the re-expression of ASC. The results showed that ASC re-expression was significantly increased in the LS174 cells following NaBu treatment in a time- and dose-dependent manner. The expression of ASC also induced the apoptosis of LS174T cells. These results suggest that NaBu plays a role in the reactivation of ASC expression and that the latter promotes the apoptosis of LS174T cells.

Introduction

Colon cancer is a common malignant disease worldwide (1). The incidence of colon cancer has increased by 2.1% each year (2), and is increasing in most countries, particularly in developing countries. The mortality rate of colorectal cancer is second only to that of lung cancer in men and breast cancer in women and has shown little sign of decreasing in the past 20-30 years (3). In most parts of China, the incidence of colon cancer has experienced the highest increase among malignant tumors.

It has been confirmed that colorectal cancer is initiated by the accumulation of genetic mutations (4). Chromatin modification strongly affects tumorigenesis due to the dynamic regulation of transcription. The disorganized chromatin structure promotes oncogenesis.

In parallel with the discovery of the importance of chromatin modification in tumorigenesis was the finding that butyrates are potent anticancer agents. Sodium butyrate (NaBu) is a short-chain fatty acid (SCFA), which can influence a variety of physiological functions. The anti-neoplastic activity of SCFAs such as butyrates in colorectal carcinogenesis is produced as a consequence of a high fiber diet (5). Butyrate, as one main end-product of intestinal microbial fermentation, demonstrates the protective role of dietary fiber in the maintenance of colonic homeostasis (6). SCFAs induce cell cycle arrest, differentiation and apoptosis in cancer cells, several also have anti-inflammatory activities, and a number have progressed to clinical trials. These agents, which include NaBu and (R)-trichostatin A (TSA), have displayed anti-proliferative and differentiating activity in a wide variety of cancers (7-9). Research has shown that NaBu induces tumor cell apoptosis in colon, breast, esophageal and prostate cancer (10). Although several SCFAs have been identified as possible anticancer agents, in regards to colon cancer, NaBu is a particularly attractive agent that may have both preventative and therapeutic value. Applied with other chemotherapy drugs, the curative effect was found to be significantly improved. Therefore, some researchers speculate that NaBu may also activate several tumor-suppressor genes and thus play a role in the antitumor mechanism indirectly (11).

Chromatin remodeling plays a vital role in the normal function of tumor suppressors, such as Rb and p53, and this...
function becomes dysregulated in several types of cancer. Furthermore, changes in DNA methylation are prominent characteristics of most cancers, and it is now known that methylated DNA recruits complexes that alter chromatin structure and repress gene transcription (12). It was previously demonstrated that the apoptosis-associated speck-like protein (ASC) is a p53-target gene which regulates the p53-Bax mitochondrial apoptotic pathway. ASC is also known to be a target of methylation-induced gene silencing. It participates in retinoic acid-induced apoptosis and forms tungsten filament structure in cells. ASC can be considered as a tumor-suppressor gene involved in the regulation of apoptosis, caspase-induced inflammation and NF-κB activation (13). The expression of ASC can be regulated at the gene level, mainly by enhancer methylation. In addition, the abnormal expression of ASC has been found in leukemia, bile duct, colorectal and prostate cancer (14,15). Genetic modulations of apoptotic genes affect the development, carcinogenesis, tumor growth, chemoradiosensitivity and inflammatory response. Aberrant hypermethylation in the promoter regions of tumor-suppressor genes has been shown to be a mechanism for the inactivation of many genes. This transcriptional silencing involves methylation of the promoter associated CpG islands and changes in the local chromatin structure resulting in tightly compacted chromatin that inhibits access to transcription factors and thus in the silencing of that gene (16).

We previously investigated colon cancer, particularly the LS174T cell line and found the absence of ASC. We proposed that this may be associated with cell apoptosis. The mechanism of colorectal cancer incidence remains unclear, yet the overexpression of oncogenes and inactivation of tumor-suppressor genes may be involved. We used NaBu-treated human colorectal cancer cell line LS174T and investigated the proliferative status, transcription of the tumor-associated gene ASC as well as their interaction and their effects on the apoptosis rate of colon cancer cells to assess whether NaBu could be effectively applied in the clinical treatment of colorectal cancer in the future.

**Materials and methods**

**Chemicals.** NaBu was obtained from Sigma-Aldrich (St. Louis, MO, USA). For cell proliferation and *in vitro* enzyme inhibition studies, NaBu was diluted to different concentrations with phosphate-buffered saline (PBS) and stored at 4°C until use.

**Cell culture.** Human colon cancer-derived cell line LS174T was maintained in our laboratory. LS174T cells were maintained in RPMI-1640 supplemented with 100 µl/l heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 50 µl/l CO₂ incubator.

**Western blotting of ASC protein.** LS174T cells were cultured with (5 mmol/l) or without NaBu for 12, 24, 48 and 72 h or with 1, 5 and 10 mmol/l for 24 h. Cells were washed twice with ice-cold PBS, scraped and transferred to tubes, and then lysed in 1 ml buffer A (10 mmol/l HEPES, pH 7.4, 10 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.5 mmol/l DTT, 0.2 mmol/l PMSF, 1 µg/ml protease inhibitors, 0.25 g/l NP-40) for 15 min with rotation at 4°C. The nuclear pellet was resuspended in 100 µl buffer B (20 mmol/l HEPES, pH 7.4, 420 mmol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 250 ml/l glycerol, 0.5 mmol/l DTT, 0.2 mmol/l PMSF, 1 µg/ml protease inhibitors) for 30 min, and the soluble nuclear protein was collected by centrifugation. Fifty micromegrams of nuclear extracts was boiled in loading buffer (125 mmol/l Tris-HCl, pH 6.8, 40 g/l SDS, 200 g/l glycerol, 0.05 g/l bromphenol blue) for 5 min and then loaded onto a 150 g/l SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane (0.45 µm). The following antibody was used: goat polyclonal antibody against rabbit IgG (Santa Cruz Biotechnology, Inc.). The binding of the antibody was detected using an ECL system (Sigma-Aldrich), and membranes were then exposed to Kodak BioMax film for 1 min. Antibody against β-actin (Sigma-Aldrich) was used for the western blotting as a control for protein concentration.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** Cells were placed in 25 cm² flasks at a density of 2x10⁶ cells/flask. After 24 h, cells were exposed to complete medium in the presence (5 mmol/l) or absence of butyrate for 1 day. Total RNA was extracted by the RNAsio reagent (Takara). Reverse transcription was performed on 2 µg total RNA in a reaction volume of 20 µl with 2 µl of 10X RT buffer, 4 µl MgCl₂ (25 mmol/l), 2 µl dNTP mix (10 mmol/l), 0.5 µl RNAase inhibitor, 2 µl dithiothreitol (0.1 mol/l), 1 µl AMV reverse transcriptase (5 U/µl) and 1 µl downstream primer (50 µmol/l). The volume was then adjusted to 20 µl with distilled water. PCR amplification was then performed with 10 µl cDNA solution supplemented with 10 µl of 10X PCR buffer, 0.5 µl of upstream primers (50 µmol/l), 0.25 µl Ex Taq HS (Takara) and water to a final volume of 50 µl. PCR conditions consisted of 35 cycles for β-actin primers 5'-cct tcc tgt gag tgg aat ccg gcg tgc cgc ttc-3' (sense) and 5'-gga gca atc tgg atc ttc-3' (antisense); 35 cycles for ASC primers 5'-tgg gcc tgc agg ttc-3' (antisense); 35 cycles for ASC primers 5'-tgcc tgg aag cg-3' (sense) and 5'-att tgg gag tgc cag-3' (antisense). Reactions were initiated at 95°C for 5 min, and each PCR cycle consisted of 30 sec at 95°C, 1 min at 55°C and 1 min at 72°C, followed by one cycle of 60°C for 10 min.

**MTT assay.** Cell proliferation was determined by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, LS174T cells were placed at a density of 10⁴ cells/well in a 96-well plate (Costar, Cambridge, MA, USA) overnight, and different concentrations of NaBu were added to each well. After the preset culture duration, medium was replaced with 50 µl of 1 mg/ml MTT (Sigma-Aldrich) solution and the plate was incubated for another 4 h. The dark-blue formazan crystal precipitate reduced from the yellow soluble MTT by viable cells was dissolved in 100 µl DMSO. The absorbance was read at 595 nm on an enzyme linked immunosorbent assay (ELISA) plate reader (Bio-Red Instruments, Inc.). Proliferation survival rate = (OD value of experimental hole - OD value of blank hole/OD value of control hole - OD value of blank hole) x 100%.

**DAPI staining.** The LS174T cells were incubated in 15-cm² cell culture flasks for 24 h. Then the cells were treated with different concentrations of NaBu for the specified times as
described above. The cells were washed with 60 µl/well of PBS for 3 times, 5 min each time. The cells were then fixed with 60 µl/well of 4% PFA (paraformaldehyde) for 20 min. The PFA was washed out with PBS. Cells were permeabilized with 60 µl/well of 0.1% Triton X-100 for 10 min. The washes were repeated 3 times. Incubation was carried out for 15 min at room temperature, then rinsed 3 times in PBS, and mounted. Finally, the cells were photographed under a fluorescence microscope.

Annexin V-FITC/PI assay. To detect apoptosis, the cells were stained with PI and fluorescein isothiocyanate (FITC)-conjugated Annexin V using the Annexin V-FITC Apoptosis Detection Kit I (Beyotime, China). Annexin V-FITC identifies cells in early apoptosis by detecting externalized phosphatidyserine and PI identifies cells that have lost plasma membrane integrity (i.e., necrotic or late apoptotic cells). LS174T cells were seeded at a density of 0.5 x 10^6 cells/ml/well and treated with NaBu diluted with RPMI-1640 medium for the times indicated at 37˚C in an incubator with a humidified atmosphere containing 5% CO₂. After incubation, 1 ml of the cell suspension was transferred to a 15-ml tube and 1 ml of cold PBS was added for washing the cells. Centrifugation was carried out at 500 x g for 5 min at 4˚C. Cells were re-suspended in binding buffer and 2.5 ml of FITC-labeled Annexin V and 2.5 ml of PI solution were added. Then tube was kept on ice for 10 min and then subjected to flow cytometry (Becton-Dickinson, USA). Cells were gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. The gated cells were then plotted for Annexin V-FITC and PI staining in a two-way dot plot to assess the percentage of apoptotic LS174T cells.

Results

Sodium butyrate induces re-expression of ASC protein. To examine the change in ASC expression in response to the different dosages of NaBu and exposure times, we initially detected the ASC protein by using western blotting. As the results showed, following treatment with different dosages of NaBu, the re-expression of ASC protein increased along with the gradually increased dosages (Fig. 1A). When 1 mmol/l of NaBu was applied to the cells for 24 h, the re-expression of ASC protein was very weak and blurred. Expression was more evident when cells were exposed to 5 mmol/l and significant at 10 mmol/l NaBu when compared with the control groups (Fig. 1A). When using the same concentration of NaBu (5 mmol/l) with different exposure times, the re-expression of ASC protein increased along with prolonged time exposure (Fig. 1B). The results showed that the expression of ASC increased in a time- and dose-dependent manner when exposed to NaBu.

Sodium butyrate upregulates the expression level of ASC mRNA. We next determined ASC mRNA using RT-PCR. The expression level of ASC mRNA was upregulated following the administration of different dosages of NaBu and increased with gradually increasing dosages (Fig. 2A). We then used the same concentration of NaBu (5 mmol/l) with different exposure times. The expression level of ASC mRNA was upregulated along with the time duration (Fig. 2B). The results showed that the mRNA expression of ASC increased in a time- and dose-dependent manner when exposed to NaBu.

Effect of NaBu on the proliferation rate of LS174T cells. ASC is a tumor-suppressor gene. Upregulated expression of ASC inhibits the proliferation of tumor cells or promotes apoptosis. Generally, a variety of genes in the genome are re-expressed by NaBu. Thus, increasing the dosages of NaBu may enhance the possibility of the re-expression of ASC and a reduction in cell proliferation. We exposed LS174T cells to different dosages of NaBu for 24 h (Fig. 3B). The cell proliferation was determined using an MTT assay. The results showed that cell proliferation rates were decreased by 7.57, 13.81 and 24.50% in the presence of 1, 5 and 10 mmol/l NaBu, respectively (each group repeat 8 times). When cells were treated with the same concentration (5 mmol/l) of NaBu (Fig. 3A), the cells...
proliferation rates decrease progressively along with the time exposure. The proliferation rates decreased by 0.56, 13.11, 16.62 and 73.17% at 12, 24, 48 and 72 h. The above results indicated an inverse relationship between the cell proliferation rate and the exposure time and the drug concentration.

**Morphological changes in the LS174T cells as a result of NaBu-induced apoptosis.** The apoptotic morphology in cellular bodies and chromatin condensation were confirmed by DAPI staining (Figs. 4 and 5). Structures of cells in the control group were complete and clear (Figs. 4 and 5). In contrast, in the NaBu-treated groups, the cell nuclei became shrunken and some began to be disrupted or exhibited an abnormal nuclear shape. This phenomenon appeared to become gradually more severe with increased exposure time (Fig. 5B-E) as well as with increased dosages (Fig. 4B-D).

**Apoptosis assay.** For further verification of the re-expression of ASC, we applied an apoptosis assay to detect the apoptosis level following treatment with NaBu. To identify apoptosis of the plasma membrane, Annexin V-FITC/PI double staining in the cells was performed by flow cytometry. In the non-apoptotic viable control cells, the Annexin V-FITC staining and PI-negative staining were noted by dots in the bottom left quadrant (Fig. 6A). When cells were exposed to 1 mmol/l NaBu for 24 h, the change in the cell apoptosis rate was slight (Fig. 6B). After exposure of the cells to 5 mmol/l NaBu for 24 h, a small number of cells showed Annexin V-FITC-positive and PI-negative staining, which increased the number of dots in the bottom right quadrant from 0.01±1% in the control cells to 1.33±1% (Fig. 6C). The cells in this stage of apoptosis were still viable. Following treatment with NaBu at 10 mmol/l, cells undergoing advanced apoptosis stained positive for Annexin V-FITC and PI (upper right quadrant) and the numbers were significantly augmented to 15.23±2%
after 24 h (Fig. 6D). The population of cells progressed to advanced apoptosis, indicating that the cells were no longer viable. The relationship between cell apoptosis and NaBu culture time was also investigated. We treated the cells with 5 mmol/l of NaBu for different exposure times. The results showed that the percentage NaBu-treated cells undergoing early apoptosis and secondary necrosis was increased with the increased exposure time (Fig. 7). The rates of cell apoptosis and secondary necrosis were increased to 6.48% at 12 h. At 24 h the rate was 19.5%. The viability of the cells decreased with an increase in exposure time, but the apoptosis rate did not increase at 72 h compared to that of 48 h (Fig. 7D and E). Thus, the results showed a time- and dose-dependent manner in the rates of cell apoptosis following exposure to NaBu.

To investigate the association of transcriptional modification, ASC, a p53-target gene which regulates the p53-Bax mitochondrial apoptotic pathway, was introduced to our experiment.

In the mitochondrial apoptosis pathway, activated p53 promotes apoptosis through Bax, a molecule that stimulates the release of cytochrome-c from mitochondria, activating procaspase-9 and resulting in cell death. Expression of regulators of the p53-Bax mitochondrial apoptosis pathway, ASC/TMS1, can become inactivated by promoter methylation leading to the disruption of the pathway. In addition, ASC-activated caspase-8 can further cleave the cytosolic Bid protein, which can translocate to mitochondria and act as an activator of the mitochondrial apoptotic pathway. Accumulating evidence shows a wide array of functions for TMS1/ASC, from triggering apoptosis to regulating the activity of NF-κB to activating inflammatory caspases (18). Aberrant NF-κB activity can promote tumorigenesis by stimulating the proliferation of tumor cells, enhancing the angiogenic and metastatic potential of tumors and inhibiting apoptosis (19). It was suggested that ASC is a tumor-suppressor gene as noted by its pro-apoptotic function. Induced expression of ASC inhibits cell proliferation and results in DNA fragmentation in a time-dependent manner. It was reported that the methylation-mediated silencing of the TMS1 gene is accomplished through the binding protein MBDs (methyl-CpG binding proteins) (20). CpG island methylation has been shown to be essential for normal development, X-chromosome inactivation, imprinting and the suppression of parasitic DNA sequences (21,22). Gene silencing associated with the aberrant methylation of promoter region of CpG islands is an acquired epigenetic alteration that serves as an alternative to genetic events in the inactivation of tumor suppressor and other genes in human cancers.

Discussion

The SCFA family of transcriptional co-repressors, such as sodium butyrate (NaBu), induces cell cycle arrest, markers of cell differentiation and apoptosis in colon cancer cell lines in vitro (17). At the same time, mechanisms responsible for the resistance of certain cancers to the effect of chemotherapeutic agents are not completely understood. In this research, we used NaBu to investigate the re-expression of ASC in LS174T cells. We demonstrated that the expression of ASC was obviously recovered following treatment with NaBu, which induced the apoptosis of LS174T cells; the proliferation rates decreased which further confirmed the re-expression of ASC. This suggests the NaBu plays a role in the reactivation of ASC expression and the latter promoted the apoptosis of LS174T cells.
NaBu has been applied in cellular and molecular research (23,24), particularly in antimutator research (25-28). It plays a vital role in vitamin D-induced apoptosis through PTEN upregulation, which has potential benefit in gastric cancer therapy (29). Furthermore, NaBu activates Notch1 signaling, reduces tumor markers (e.g. ASCL1 and CgA) and induces cell cycle arrest significantly, alters tumor cell proliferation and apoptosis in pheochromocytoma, which were indicated by the levels of cyclin D1, p21, cleaved PARP and cleaved caspase-3 proteins (30). NaBu also increases expression of the coxackie and adenovirus receptor in colon cancer cells (31). Although some researchers have reported that NaBu induces human colon carcinoma HT-29 cell apoptosis through a mitochondrial pathway (32), we found a new relational molecular, ASC, whose expression was recovered in LS174T cells.

In this study, we investigated the effect of NaBu on ASC expression and the tumor proliferation in LS174T cells. According to the results, the expression of ASC increased in a time- and dose-dependent manner when exposed to NaBu. The cell proliferation rates decreased and the apoptosis rates correspondingly increased. Heerdt et al proposed that the apoptosis noted may be the result of the effect of NaBu on mitochondria (33). It was established that mitochondrial activities are essential for the initiation of butyrate-induced apoptosis as well as cell cycle arrest in colon carcinoma cells (34). These resulted in changes to the membrane potential of mitochondria and led to the activation of caspases. Recent observations showed that apoptosis by NaBu was dependent on the activation and translocation to the mitochondria of the pro-apoptotic Bcl-2 family member Bid, also NaBu downregulates expression of Bcl-2 in colon cancer cells (35). By regulating Bcl-2 the mitochondrial membrane potential changes, the NaBu-induced activation of the caspase cascade may be an important part of apoptosis induction.

NaBu can directly inhibit cell growth and differentiation, promote apoptosis or inhibit tumor growth by inhibiting angiogenesis indirectly. Chopin et al (36) showed that NaBu-induced apoptosis in MCF-7 cells was independent of the G1-phase blockage, suggesting that the underlying mechanisms of cell cycle blockage and induction of apoptosis occurs through different pathways. At present, it is well established that NaBu can initiate apoptosis through several different pathways. Nonetheless, all primarily execute cell death through activation of the intrinsic mitochondria-mediated pathway. For example, an early p53-dependent and a late p53-independent form of cell death have been associated with exposure to these compounds. Although most studies have focused on its induction of G1 cell cycle arrest, butyrate also induces a G2 arrest and the present study was undertaken to examine this aspect of butyrate action as a sensitive G2 cell cycle checkpoint and the consequential occurrence of aberrant mitosis (37).

NaBu and other SCFAs are naturally occurring products produced by fiber fermentation in the colon and have been found to inhibit the growth of colon cancer cells both in vivo and in vitro (38). In addition, when SCFAs were applied in combination with a variety of chemotherapy drugs, better co-treatment efficacy was noted. Yang et al (39) reported that the combination of using DNA methyltransferase inhibitors and SCFAs can reactivate MLH1, TIMP3, CDKN2B, CDKMN2A, ARHI and other tumor-suppressor genes. These events promoted apoptosis in tumor cells. Nimnanapalli et al (40) found that the combination of using SAHA with Gleevec enhanced chemotherapy sensitivity of Gleevec-resistant chronic myeloid leukemia cells. Compared to transformed cells, normal cells are not sensitive to SCFAs. NaBu causes changes in gene expression and the regulation of proteins. Furthermore, it alters physiological functions of drug-induced transformed cells (41,42).

Co-treatment of NaBu with a variety of chemotherapy drugs may be beneficially utilized in clinical practice. The present study revealed its important clinical value. Research on NaBu and ASC expression may help to develop a strategy to improve drug safety and efficacy and significantly enhance the efficiency of drugs applied in cancer therapy.

Acknowledgements

This study was supported by grants from the Chinese National Natural Science Foundation Projects (31270867) and the Chinese State Key Program in Basic Research (2012CB822103).

References


