Effects of sodium butyrate on proliferation and differentiation of human gastric carcinoma cell line AGS

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Key words: sodium butyrate, gastric carcinoma, proliferation, differentiation, P21 protein

Background and Objective: Sodium butyrate (NaBT) can inhibit proliferation and induce differentiation of various tumor cells. This study was to investigate effects of NaBT on the proliferation and differentiation of human gastric carcinoma cell line AGS and the possible mechanism.

Methods: AGS cells were treated with 0, 1.0, 2.0 and 4.0 mmol/L of NaBT. Cell proliferation was detected by MTT assay; cell morphology changes were observed under optical and transmission electron microscopy; cell cycle was detected by flow cytometry (FCM). The expression of cyclin-dependent kinase inhibitor p21 was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Results: After incubation with different concentrations of NaBT for 24 to 72 h, AGS cell proliferation was inhibited dramatically and the highest inhibition rate was 81.54%. The structure of AGS cells changed greatly. NaBT induced an increase of G0/G1 phase cells and a significant decrease of S phase cells, accompanied by changes in DNA ploidy. The expression of p21 was up regulated at both mRNA and protein levels. NaBT exerted its effects in a dose-dependent manner.

Conclusions: NaBT could induce G1 arrest and inhibit cell proliferation in AGS cells by up-regulating the expression of p21. This could reverse the malignant phenotype of AGS to some extents.

Tumorigenesis of gastric cancer is closely related to unlimited cellular proliferation, poor differentiation, and deregulated apoptosis of gastric mucosa epithelial cells under different pathogenic factors. Thus, it is important to correct this unbalanced status using proper methods to reduce the incidence of gastric tumor. Studies have shown that butyric acid, a product of dietary fiber fermentation in the colon, could provide energy for normal colonic mucosal epithelial cells and maintain their renewal. Sodium butyrate can promote proliferation in normal colonic epithelial cells. It is known that proliferation of intestinal mucosal epithelium could be a precancerous. Some researchers suggest that diet fibers may increase the risk of colon cancer. Studies have revealed different biological effects of sodium butyrate on tumor cells compared with on normal cells. Sodium butyrate can inhibit proliferation, induce differentiation and so on in tumor cells. Regarding to these contradictory results, Camarero et al. believe that sodium butyrate inhibits cell growth through inducing the expression of mitochondrial HMG-CoA synthetase, leading to accumulation of mid-products of acetyl-CoA and β-oxidation, and inhibition of acetyl-CoA dehydrogenase. On the other hand, normal colon epithelial cells could effectively catabolize sodium butyrate to provide energy for themselves, thus reduce the inhibitory effect of sodium butyrate on cell growth. Once the normal colon cells undergo carcinogenesis, normal metabolism of sodium butyrate may get lost, which allows this compound to exert its anti-tumor effect in tumor cells. It is also suggested that the anti-proliferation effect of sodium butyrate is tumor-specific.

Currently, most reports on the anti-tumor effect of sodium butyrate are focused on colon cancer. However, there are few studies describing its anti-tumor effect on gastric cancer. This study was designed to observe the effect of different concentrations of sodium butyrate on the proliferation and differentiation of AGS, a poorly differentiated gastric carcinoma cell line, and to discuss its possible mechanism.

Materials and Methods

Materials. Human gastric tumor cell line AGS was purchased from Shanghai Institute of Biochemistry and Cell Biology. Bovine serum and RPMI-1640 culture medium were purchased from Gibco Company. Sodium butyrate and p21 monoclonal antibody were from Sigma Company. Coulter DNA PREP™ Reagents Kit was purchased from Beckman Coulter Company. M-PER Mammalian Protein Extraction Reagent was purchased from Pierce Company. Mouse anti-β-actin antibody was purchased from Santa Cruz Company. Goat anti-mouse secondary antibody and chemiluminescence test kit were purchased from Beijing Zhongshan Biotechnology Company.

Methods. Cell culture. After recovering, AGS cells were cultured in RPMI-1640 culture medium with 10% bovine serum, 100 µ/
ml of penicillin, and 100 μg/ml of streptomycin at 37°C in an atmosphere of 5% CO2.

Treatment of cells by sodium butyrate. Sodium butyrate was dissolved into sterile PBS solution and diluted to working concentrations before use. AGS cells on the logarithmic phase of growth were treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, and cultured for 24, 48 and 72 h, respectively. Then, cells were collected for future use.

Measurement of cell proliferation by methyl thiazolyl tetrazolium colorimetry (MTT method). AGS cells on the logarithmic phase of growth were digested by 0.25% trypsin and implanted in a 96-well plate at 5 × 10^3 per well. Cells were divided into blank control, solvent control (PBS), and treatment groups. The first well of each row was left as blank control. Cells were cultured for 24 h and the culture media was discarded. Cells were incubated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, respectively. Seven replicates were set up at each time point for each experimental group. After incubation for 24, 48 and 72 h, 20 μL MTT solution (5 mg/mL) was added to each well, following 4 h incubation at 37°C. After discarding the solution, 150 μL of DMSO solution was added to each well. The plate was placed on the shaker for 10 min to completely dissolve crystallized products. The absorbance value of the blank well was adjusted to zero. The value (A-value) of each well was measured at 490 nm wavelength using a ELISA analyzer, and the average was calculated. The inhibitory rate of the blank control was set as zero. The inhibitory rate of cell proliferation of each experimental group was calculated using the following equation: Inhibitory rate of cell proliferation = (1 - A-value of each experimental group / A-value of blank control group) × 100%

Cell cycle measurement by flow cytometry. After incubation with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, AGS cells were cultured for 24 h. The preparation of FCM samples was performed according to the instructions provided by the manufacture. Changes of cell cycle distribution were analyzed by MultiCycle for Windows. This experiment was repeated three times.

Observation of cell morphology under inverse microscopy. AGS cells on the logarithmic phase of growth were obtained and treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, respectively for 24, 48 and 72 h. Changes of cell morphology were observed under inverse microscopy.

Observation of cell ultra-microscopic structures under transmission electron microscopy. AGS cells were treated with or without 2.0 mmol/L of sodium butyrate and cultured for 24 and 72 h. Then the cells were digested by 0.25% trypsin, centrifuged, collected, and prepared for observation according to the routine procedures. Finally, cells were observed under transmission electron microscopy and photographed.

Reverse transcription-polymerase chain reaction (RT-PCR). AGS cells treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate for 24 h were collected. The total RNA was extracted using the Trizol test kit. 2 μg of RNA and the oligo primer were used for reverse transcription reaction. cDNA was synthesized after incubation of the reaction mixture at 42°C for 60 min, followed by at 99°C for 5 min. The primer sequences for p21/WAF1 amplification were as follows: upstream 5'-CCACAGTCCATGCTACAC-3', downstream 5'-TCCACCCACCGTGGCTGTA-3'. Glyceraldehyde-3-phosphate (GAPDH) was used as the internal reference. The primer sequences for amplification of GAPDH were as follows: upstream 5'-ACCACAGTCCATGCTACAC-3', downstream 5'-TCCACCCACCGTGGCTGTA-3'. The volume of each reactive system was 25 μL and the reaction parameters were set as follows: degeneration at 94°C for 5 min, 30 cycles of degeneration at 94°C for 60 s, annealing at 60°C for 60 s, and elongation at 72°C for 60 s, followed by elongation at 72°C for another 5 min. The amplified segments of p21/WAF1 and GAPDH were 316 bp and 452 bp, respectively. The PCR product (8 μL) was run on a 1% agarose gel. The gel was observed under UV-light and photographed. The grey-scale value of each band was measured by the gel image analyzing system. The experiment was repeated for three times. For each experimental group, the average ratio of the grey-scale value of p21/WAF1 to that of GAPDH was compared.

Western blot. AGS cells treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate for 24 h were collected. Cells were digested by trypsin, centrifuged, and washed twice with PBS. For every 1×10^6 cells, 150 μL of protein lysis solution was used. The mixture was well-mixed to ensure complete cell lysis, centrifuged for 15 min at 12000g at 4°C. The supernatant was collected and the protein concentration was measured using the Bradford colorimetric assay. An equal amount (25 μg) of protein was loaded per well and then separated using SDS (100 g/L) polyacrylamide gel electrophoresis, followed by protein blotting. The membrane was sealed with 50 g/L skim milk for 2 h at room temperature, incubated with p21 antibody (1:200) and mouse anti-β-actin antibody (1:1000) diluted in TBST at 4°C overnight, followed by incubation with secondary antibody (1:5000) for 60 min at room temperature. The protein signal was detected using the chemiluminescence kit. Anti β-actin polyclonal antibody was probed as an internal loading control. The film image was scanned and stored in computer. The grey-scale values were measured by BanScan software. The experiment was repeated three times.

Statistical analysis. All data are presented as mean ± SD, and analyzed by single-factor variance using SPSS 11.0 software. The comparison among each average was performed using Student-Newman-Keuls’s test (SNK). P value of less than 0.05 was considered statistically significant.

Results

Effects of sodium butyrate on cell proliferation of AGS. After 48 h of exponential growth, the cell growth of AGS reached the peak,
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Figure 2. Changes of cell cycle distribution in AGS cells after 24 h treatment of sodium butyrate (A) The DNA ploidy of control cells is tetraploid or near-tetraploid; B–D: After incubation with 1.0 mmol/L (B), 2.0 mmol/L (C) and 4.0 mmol/L (D) sodium butyrate, diploid or near-diploid DNA appeared in cells.

Changes of AGS cell cycle distribution. After incubation with 1.0, 2.0, and 4.0 mmol/L sodium butyrate for 24 h, the percentage of S-phase cells decreased from 57.1% to 44.3%, 29.0%, and 21.6% respectively (p < 0.01), while the cells at G0/G1-phase increased to 33.5% (p > 0.05), 45.8%, and 54.5% (p < 0.01), respectively. Before drug treatment, DNA ploidy of AGS cells was tetraploid or near-tetraploid. After incubation with sodium butyrate for 24 h, diploid or near-diploid DNA appeared in AGS cells (Fig. 2).

Changes of morphology in AGS cells under inverse microscopy. Control AGS cells were polygonal, with a large nuclear-cytoplasmic (N/C) ratio; cells had irregular nucleus, most of which had large nucleoli. All these were accordant with the characteristics of malignant tumor cells (Fig. 3A). After treated by sodium butyrate, the morphology of AGS cells changed dramatically. Most cells spread out to become fibroblast-like cells with enlarged volumes at 24 h. After 48 h, most cells became longer and slender, in long oval shape; the nucleolus of cells became rounder and the N/C ratio was reduced. At 72 h, most cells were round, became smaller and denser, and finally fell off (Fig. 3B–D).

Changes in ultra-microscopic structures of AGS cells under transmission electron microscopy. Control cells were large and irregular, rich in microvilli. Cells had poly-nucleus, with large N/C ratios and varied appearances of nucleoli. The mitochondria were few in number, with irregular shapes (Fig. 4A). After treated by 2.0 mmol/L sodium butyrate for 24 h, in comparison with the control, cells revealed obvious changes, including increased cell volume and mitochondria, decreased microvilli, regular nuclei with smaller N/C ratios and fewer and regular nucleoli (Fig. 4B). At 72 h, some AGS cells started to show classical changes of apoptosis, such as appearance of apoptotic bodies (Fig. 4C).

Effects of sodium butyrate on the expression of p21WAF1 mRNA. Compared to the control group, the mRNA expression of p21WAF1 was significantly increased after incubation with different concentrations of sodium butyrate for 24 h. The effect was in a dose-dependent manner (Fig. 5). The grey-scale values of p21WAF1 mRNA were significantly higher in groups incubated with sodium butyrate at 1.0 mmol/L (0.77 ± 0.01, p < 0.05), 2.0 mmol/L (0.88 ± 0.04, p < 0.01) and 4.0 mmol/L (0.98 ± 0.02, p < 0.01) than in the control group.

Effect of sodium butyrate on the expression of p21 protein. Compared to the control group, the protein expression of p21 was significantly increased after the treatment of sodium butyrate at different concentrations for 24 h. The effect was in a dose-dependent manner (Fig. 6). The grey-scale values of p21 protein were significantly higher in groups treated with sodium butyrate at 1.0 mmol/L (0.55 ± 0.01, p < 0.05), 2.0 mmol/L (0.95 ± 0.01, p < 0.01) and 4.0 mmol/L (1.24 ± 0.06, p < 0.01) than in the control group.

Discussion

We discovered that sodium butyrate could significantly inhibit proliferation of AGS cells in a time- and dose-dependent manner. After treatment with 4.0 mmol/L sodium butyrate for 72 h, the inhibitory rate reached as high as 81.54%. Other Chinese scholars found that the inhibitory rate of sodium butyrate could...
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Figure 3. Morphological features of AGS cells after the treatment with 2.0 mmol/L sodium butyrate observed under inverted microscopy × 400. (A) Cells were treated without sodium butyrate for 48 h. Cells show polygonal appearances, a large nuclear-cytoplasmic (N/C) ratio, irregular nucleus and varied appearances of large nucleoli. (B–D) After treated by 2.0 mmol/L sodium butyrate, most of the cells spread out to become fibroblast-like cells at 24 h (B); some cells become slender with increased round nucleus and decreased N/C ratios at 48 h (C); some cells become even smaller and denser and fall off finally at 72 h (D).

Figure 4. The ultrastructure changes of AGS cells treated by 2.0 mmol/L sodium butyrate observed under transmission electron microscopy (A) Control cells are large and irregular, rich in microvilli. Cells have poly-nucleus, with large N/C ratios and varied appearances of nucleoli (x 3600). The mitochondria are few in number, with irregular shapes; (B) After treated by 2.0 mmol/L sodium butyrate for 24 h, in comparison with the control, cells show obvious changes, such as increased cell volume and mitochondria, decreased microvilli, regular nuclei with smaller N/C ratios and fewer and regular nucleoli (x 3000). (C) After treated by 2.0 mmol/L sodium butyrate for 72 h, some cells show typical apoptotic changes, such as apoptotic bodies (x 4800).

Figure 5. The expression of p21 mRNA in AGS cells after the treatment of sodium butyrate Lane M: marker; lane 1: control cells; lanes 2–4: cells treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, respectively.

Figure 6. The expression of P21 protein in AGS cells after the treatment of sodium butyrate Lane C: control cells; lanes 1–3: cells treated with 1.0, 2.0 and 4.0 mmol/L sodium butyrate, respectively.

reach 59.19% at an intervention concentration of 10 mmol/L. Compared with that, sodium butyrate exerted a more effective effect in gastric cancer AGS cells at lower concentrations in this study. After incubation with sodium butyrate, AGS cells at G1 phase were increased, while cells at S-phase were decreased. It is well known that G1 phase is the pre-DNA synthesis period and S-phase is the DNA synthesis period. At the end of S phase, diploid cells would become tetraploids to ensure adequate and even distribution of genetic materials for mitosis. Sodium butyrate retarded most AGS cells at G1 phase, further confirming its inhibitory effect on cell proliferation. DNA-ploidy of the control group was tetraploid or near-tetraploid. After drug treatment, AGS cells appeared apparent change in DNA-ploidy, displaying diploid or near-diploid. In normal human body, the DNA-ploidy (diploid) is stable. Researches have suggested close correlations of biological behaviors of malignant tumors to DNA-ploidy of tumor cells. The order (from the highest to the lowest) of different DNA-ploidy related to the degree of malignancy was as follows: M (multiploidy) > AN (aneuploid).
nancy phenotype of gastric tumor cells after treatment with sodium butyrate can, through other pathways, cause apoptosis in the cells. As a result, E2F-DP1 bound to Rb in the early G1 phase could inhibit the activity of the complex and prevent its phosphorylation and cell proliferation. Therefore, we believe that sodium butyrate could partially reverse the malignancy phenotype of AGS. We speculate that sodium butyrate could partially reverse the malignancy phenotype of AGS. We speculate that sodium butyrate could partially reverse the malignancy phenotype of AGS.

The morphology and ultra-microstructures of tumor cells are greatly different from the normal cells. Tumor cells are characterized by irregular shapes, enlarged N/C ratios, abnormal nuclear shapes, increased number of cells with large nucleoli, and increased microvilli on the cell surface, and so on. Characterization of the changes in morphology and ultra-microstructures in tumor cells is important for identification of extrinsic substances, especially those that induce differentiation in tumor cells. AGS is a malignant gastric adenocarcinoma cell line with fast proliferation and poor differentiation. In this study, AGS demonstrated typical morphology of tumor cells and ultra-microstructures of the malignancy phenotype. After treatment of sodium butyrate, morphological changes in apoptosis of AGS cells were observed under inverse microscopy and transmission electron microscopy, which were consistent with the findings reported by Shi et al. We suppose that with an increase of treatment duration, sodium butyrate can, through other pathways, cause apoptosis in AGS cells. Our study displayed characteristics of reversed malignancy phenotype of gastric tumor cells after treatment with sodium butyrate.

The biological characteristics of tumor cells are unlimited proliferation and poor differentiation. Differentiation and proliferation have antagonistic effects and differentiation generally occurs during interphase stage. Therefore, cell differentiation and proliferation inhibition are closely correlated to each other. Many scholars believe that cell differentiation is related to retardation or termination of cell proliferation. On the other hand, during differentiation, when the cells mature and stop dividing, cells appear to increase at G0/G1 phase and decrease at S phase. Previous studies have identified sodium butyrate as a polar small molecule compounds with a confirmed effect on inducing differentiation in tumor cells. Taken together, sodium butyrate exerts its inhibition effect on tumor cells through delaying tumor cells in the interphase. Inhibition of proliferation may be the pre-requisite condition for induction of differentiation.

Histone acetylation plays an important role in tumorigenesis. Recently, some researchers have ascribed the effect of sodium butyrate to histone acetylation and regarded sodium butyrate as an inhibitor of histone deacetylase. Once the activity of histone deacetylase is inhibited, histone would remain in a highly acetylated status, leading to the changes in chromosomal structures, as well as in DNA replication and transcription, and thus, resulting in a series of other variations. These changes include regulations on the gene expression, especially on those genes related to cell cycle regulation and apoptosis, such as p21, c-myc, c-myb, and so on. RT-PCR and Western blot studies confirmed the effect of sodium butyrate on the expression of p21 at both mRNA and protein levels. The expression of p21 was increased in cells treated with sodium butyrate, consistent with conclusions drawn from other studies on colon cancer. Increased p21 would bind with the cyclin-CDK complex, thus inhibit the activity of the complex and preventing phosphorylation of Rb. As a result, E2F-DP1 bound to Rb in the early G1 phase could not be released, and entry of G1 phase cells into S phase is blocked, and cell proliferation is inhibited. Therefore, we believe that sodium butyrate, through upregulation of p21, could induce cell cycle arrest at G1 phase, and therefore significantly inhibit the proliferation of AGS cells.

References