

## The Histone Deacetylase Inhibitor Butyrate Inhibits Melanoma Cell Invasion of Matrigel

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**Abstract.** *Background:* Histone deacetylase (HDAC) inhibitors have anticancer effects. Their effects on expression of cell adhesion molecules might be related to their effects on tumor cell invasion. *Materials and Methods:* Murine B16-BL6 cells were treated with the HDAC inhibitors, butyrate or trichostatin A. Melanoma cell invasion of the artificial basement membrane, Matrigel, was examined by Transwell chamber assay. *Results:* Butyrate as well as trichostatin A inhibited the cell growth mainly by arresting the cell cycle. The cell invasion of Matrigel was inhibited by butyrate and trichostatin A. The butyrate treatment increased the cell-cell aggregation, although neither E-cadherin nor N-cadherin mRNA were up-regulated. Both mRNA expression and protein levels of the immunoglobulin superfamily cell adhesion molecules, Mel-CAM and L1-CAM, were increased in the butyrate-treated cells. *Conclusion:* The HDAC inhibitor butyrate blocked the B16-BL6 melanoma cell invasion of Matrigel, although it increased the expression of Mel-CAM and L1-CAM which are important to the metastatic potential.

Acetylation and deacetylation of histones play important roles in chromatin remodeling and consequently change specific gene expression in eucaryotic cells. Several histone deacetylase (HDAC) inhibitors have been shown to possess anticancer properties (1, 2). Among HDAC inhibitors, the short fatty acid butyrate, which is a physiological metabolite produced by intestinal anaerobic bacteria, has an inhibitory activity on the cell cycle through the Sp1/Sp3-associated p21<sup>Waf1/Cip1</sup> gene activation (3). Butyrate is not as potent an HDAC inhibitor as trichostatin A which is produced by the bacterium *Streptomyces* sp. It is proposed that two molecules of butyrate correspond to one molecule of

trichostatin A for binding to the active site of HDAC (3). Although butyrate is a weak HDAC inhibitor, it is a physiological product but not a synthetic chemical. Recently, the importance of long term exposure to weak or reversible HDAC inhibitors has been reevaluated in the control of colon cancers (4), because potent medicines generally have strong side-effects. We have recently found a new activity of butyrate which can increase the MUC2 mucin production in the colon cancer cell line, LS174T (5). Butyrate might have new activities on other cell types as well as colon cancer cells. Therefore, we examined the effects of butyrate on murine melanoma cells to evaluate the anticancer activities.

B16-BL6 cells derived from murine melanoma have highly metastatic potential to the lung. The lung metastasis is inhibited when the cells are pre-treated with the neuropeptide  $\alpha$ -MSH prior to being injected intravenously in mice, and the  $\alpha$ -MSH treatment reduces the cell motility and adhesion to extracellular matrix proteins *in vitro* (6). Subsequent studies showed that  $\alpha$ -MSH increased the expressions of both E-cadherin and N-cadherin in the B16-BL6 cells, and consequently reduced the cell motility and invasive activity *in vitro*, suggesting the importance of cell adhesion molecules in tumor cell invasion. Here, we examined the effects of butyrate on cell invasion and expression of cell adhesion molecules in B16-BL6 cells.

### Materials and Methods

*Cell culture.* The murine melanoma cell line B16-BL6 was originally provided by Dr. I.J. Fidler (Anderson Cancer Center, Houston, TX, USA). The cell line has been maintained as a monolayer culture in Eagle's minimum essential medium (EMEM; Sigma-Aldrich, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS, Cansera International, Ontario, Canada), 1% non-essential amino acids (Invitrogen, NY, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco Oriental, Tokyo, Japan) in a 5% CO<sub>2</sub> incubator. The adherent cells were subcultured every 3-4 days by treatment with a Trypsin-EDTA solution (Gibco Oriental).

*Cell proliferation assay.* B16-BL6 cells (1x10<sup>3</sup> in 0.1 ml) were precultured in a 96-well plate (Sumilon, Tokyo, Japan) for 2 h to allow them to adhere. Subsequently, 5  $\mu$ l of sodium butyrate (Wako,

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Osaka, Japan) in saline or Trichostatin A (Cayman Chemical, Ann Arbor, MI, USA) in 10% dimethyl sulfoxide were added in triplicate to the cultures at final concentrations of 0, 1, 2 and 4 mM, or 0, 0.1, 0.5 and 1  $\mu$ M, respectively. The cells were cultured for a further 2 or 4 days. Cell Counting Kit-8 solution (8  $\mu$ l, Dojin, Tokyo, Japan) was added to each well and the cells were incubated for a further 4 h. Absorbance at 450 nm was measured with a microplate reader Model 550 (Bio-Rad, Tokyo, Japan) to estimate cell growth. In some experiments, the cell viability was measured by the Trypan blue exclusion method.

**Flow cytometry.** An appropriate density of B16-BL6 cells (1 to  $5 \times 10^4$  in 0.4 ml) were precultured in complete medium (5% FBS) in a 24-well plate (Sumilon) for 24 h and then cultured in incomplete medium (0.1% FBS) for 48 h to starve them. After starvation, the cells were again cultured in the complete medium (5% FBS) supplemented with 4 mM butyrate for 24 h and 48 h. The cell cycles were analyzed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, NJ, USA) by the previously reported method (5).

**Western blotting.** B16-BL6 cells (0.5 to  $1 \times 10^6$ /ml) were precultured in a culture flask (Corning, NY, USA) for 2 h and treated with 4 mM butyrate for 24 h and 48 h. The treated cells were submitted to standard Western blotting with 16% SDS-polyacrylamide gel electrophoresis (PAGE). Histone H1 and acetylated histone H3 were detected with a rabbit anti-histone H1 antibody (1:200; Santa Cruz FL-219, CA, USA) and a rabbit anti-acetylated-histone H3 antibody (1:200; Upstate #06-599, NY, USA), respectively, by the methods reported previously (5). In a second experiment, the butyrate-treated B16-BL6 cells were submitted to standard Western blotting with 7.5% SDS-PAGE. Melanoma cell adhesion molecule (Mel-CAM) and L1 cell adhesion molecule (L1-CAM) were detected with a goat anti-Mel-CAM antibody (1:200; Santa Cruz sc-18942) and a goat anti-L1-CAM antibody (1:200; Santa Cruz sc-31034), respectively.

**Cell invasion assay.** Tumor cell invasion activities were assayed by the previously reported method (6) using Transwell chambers (Costar, MA, USA). Briefly, Matrigel (Collaborative Research, MA, USA) and fibronectin (Asahi Techno Grass, Tokyo, Japan) were coated on the upper and the lower surface of the Transwell filters (8  $\mu$ m pore, Nucleopore, CA, USA), respectively. B16-BL6 cells were cultured with different concentrations of butyrate (1, 2 and 4 mM) or Trichostatin A (0.25 and 0.5  $\mu$ M) for 48 h and then suspended in EMEM supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich). The live cells ( $2 \times 10^5$  in 0.1 ml) were placed on the upper compartment of a chamber and incubated for 7 h in triplicate. After fixing and staining the filters, the lower surfaces of filters were photographed with a digital camera microscope BX51 system (Olympus, Tokyo, Japan). Four fields per filter, randomly chosen, were recorded at  $\times 200$  and then the number of cells was counted using the single blind method.

**Cell migration assay.** Only the lower surface of the Transwell filters was precoated with fibronectin. The live B16-BL6 cells ( $2 \times 10^5$  in 0.1 ml) treated with different concentrations (1, 2 and 4 mM) of butyrate for 48 h were placed on the upper compartment of a chamber and incubated in 0.1% BSA-EMEM for 3.5 h in triplicate.

The number of cells migrated onto the lower surface was counted by the same method as in the cell invasion assay.

**Cell adhesion to fibronectin.** Each well of a 96-well ELISA plate (Sumilon) was precoated with fibronectin (4  $\mu$ g in 50  $\mu$ l) at 37°C overnight and then incubated with 1% BSA in phosphate-buffered saline (PBS) for 30 min to block the surface. The B16-BL6 cells ( $4 \times 10^5$  in 0.1 ml) cultured with different concentrations (1, 2 and 4 mM) of butyrate for 48 h were placed in the fibronectin-coated wells for 30 min. The wells were washed with warm PBS to remove unattached cells. The attached cells were stained with 0.5% crystal violet in 20% methanol for 30 min. After repeated washing, the stained cells were dissolved in 50  $\mu$ l of 30% acetic acid. Absorbance at 570 nm was measured with a microplate reader Model 550 (Bio-Rad).

**Cell aggregation assay.** B16-BL6 cells were cultured with different concentrations (1, 2 and 4 mM) of butyrate for 48 h and then suspended in 0.1% BSA in EMEM. The live cells ( $2 \times 10^5$  in 0.4 ml) were transferred into a 24-well plate which had been precoated with 0.1% BSA in EMEM at 37°C overnight. The plate was gently shaken at 37°C for 1.5 h and observed at  $\times 40$  under an Eclipse TE300 microscope (Nikon, Tokyo, Japan).

**RT-PCR.** B16-BL6 cells (0.5 to  $1 \times 10^6$ /ml) were treated with 4 mM butyrate for different times (6, 12, 24, 36 and 48 h). Total RNA was extracted from the cells by a RNeasy Mini Kit (Qiagen, Tokyo, Japan). RT-PCR was performed using a real-time PCR LightCycler System V3 (Roche Molecular Biochemicals, Germany) and a QuantiTect SYBR Green RT-PCR Kit (Qiagen) as previously described (5, 7). After one cycle of reverse transcription at 50°C for 20 min, PCR was performed with proper cycles of 94°C for 15 s, a proper annealing temperature for 15 s and 72°C for 20 s. The specific primers, annealing temperatures and PCR cycles are as follows: (i) E-cadherin: sense, 5'-CGGAGAGGAGAGTCGAAGTG-3'; antisense, 5'-CATGCTCAGCGTCTTCTCTG-3'; annealing, 59°C and 40 cycles; (ii) N-cadherin: sense, 5'-AGTTTCTGCACCAGG TTTGG-3'; antisense, 5'-TGATGATGTCCCCAGTCTCA-3'; annealing, 57°C and 35 cycles; (iii) Mel-CAM: sense, 5'-CTGCGAG GCAGAAAGTAACC-3'; antisense, 5'-ACCCACACCTTCCT CTCCTT-3'; annealing, 62°C and 35 cycles; (iv) L1-CAM: sense, 5'-CCAGCCAGGATCCTAACAACAA-3'; antisense, 5'-ATGTCACC CTTGCACCTTTC-3'; annealing, 64°C and 50 cycles.

**Statistical analysis.** Probability of significances between groups ( $p < 0.05$ ) was determined with Mann-Whitney's *U*-test.

## Results

We examined the effects of the HDAC inhibitors butyrate and trichostatin A on the growth of B16-BL6 melanoma cells. The cell growth was dose-dependently inhibited by butyrate (Figure 1A) and trichostatin A (Figure 1B), but the proliferation was not completely halted by butyrate (Figure 1A). The viability of cells treated with 4 mM butyrate was approximately 97% on day 2 of culture and 90% on day 4. We examined the butyrate-treated cells by the standard Hoechst staining method (5) to detect apoptosis. However, typical apoptotic cells were scarcely

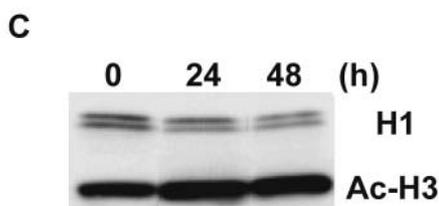
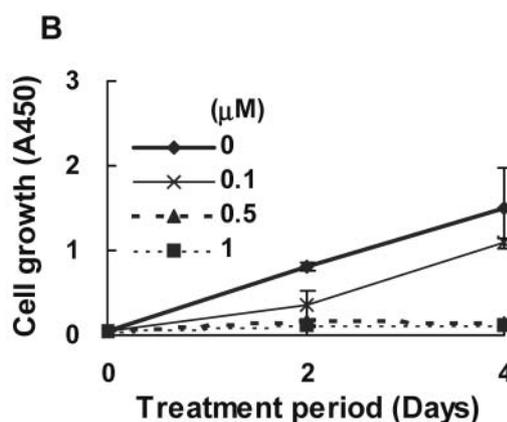
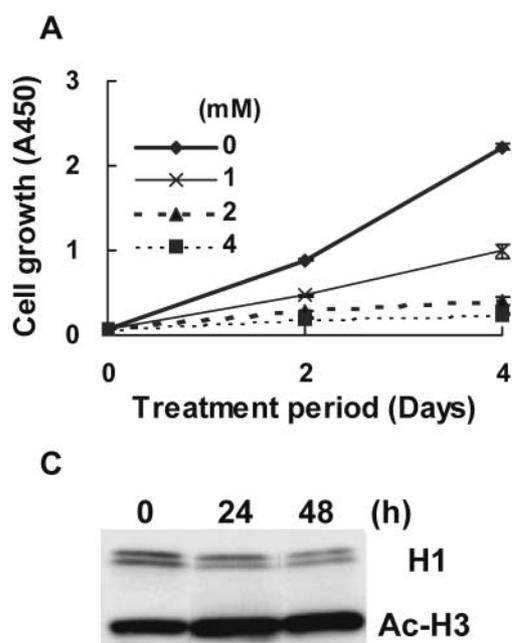


Figure 1. HDAC inhibitors inhibited the growth of B16-BL6 cells. The cells were treated with (A) butyrate and (B) trichostatin A. The cell growth was estimated by colorimetric assay with Cell Counting Kit-8. (C) Accumulation of acetylated histone H3 with butyrate treatment. B16-BL6 cells were treated with 4 mM butyrate for 24 h and 48 h. Histone H1 and acetylated histone H3 were detected with the corresponding specific antibodies by Western blotting.

Table I. Cell cycle phases of starved B16-BL6 cells treated with butyrate at 0 or 4 mM in complete medium.

Time treated (h)	Butyrate (mM)	G0/G1 (%)	S (%)	G2/M (%)
0	0	98.1±0.7	0.7±0.2	0.5±0.2
24	0	89.5±8.1	8.3±7.3	2.1±1.3
24	4	98.9±0.5	0.2±0.1	0.8±0.4
48	0	92.2±1.5	4.8±1.2	2.7±0.2
48	4	99.2±0.3	0.2±0.1	0.2±0.1

The starved B16-BL6 cells were cultured in the complete medium supplemented with 0 mM or 4 mM butyrate for 24 and 48 h. At least 10,000 nuclei were counted for each sample using a flow cytometer. Average % ( $\pm$  S.D) was obtained in triplicate.

observed (data not shown). Therefore, we analyzed the cell cycle distribution of butyrate-treated cells by flow cytometry. The B16-BL6 cells, which had been starved by depleting FBS, consisted of 98% at the G0/G1-phase and 0.7% at the S-phase (Table I). When the starved cells were subsequently cultured in a complete medium supplemented with 10% FBS for 24 h and 48 h, the proportion of cells in the G0/G1-phase decreased and these in the S-phase increased, suggesting an increase in DNA synthesis. When the starved cells were cultured in the presence of 4 mM butyrate for 48 h, cultures still contained 99% of the cells at the G0/G1-phase and 0.2% at S-phase, suggesting that butyrate treatment arrested the cell cycle.

We subsequently examined the effects of butyrate on the inhibition of HDACs. If deacetylation of histones is inhibited by butyrate treatment, acetylated histone proteins should be accumulated in the nucleosomes. The acetylated histone H3 levels in the B16-BL6 cells increased following 24 h and 48 h of butyrate treatment (Figure 1C). These results imply that butyrate inhibited the HDACs and consequently inhibited cell growth not by inducing apoptosis in the B16-BL6 cells but by arresting the cell cycle.

B16-BL6 cells are a highly invasive and metastatic murine melanoma. We investigated the effects of HDAC inhibitors on the invasive activity using the Matrigel invasion assay. The cell invasion was significantly inhibited ( $p < 0.05$ ) by butyrate at 2 and 4 mM (Figure 2A) and trichostatin A (TSA) at 0.5  $\mu$ M (Figure 2B). The inhibition of invasion of Matrigel suggested that the motility of cells was suppressed by the butyrate treatment. We therefore examined the motility of cells by a simple migration assay using Transwell chambers. The cell migration toward fibronectin was inhibited by butyrate at 4 mM ( $p < 0.05$ ; Figure 3A). Furthermore, the adhesive activity to fibronectin, which is required for the haptotactic migration of cells, was also inhibited by butyrate at 4 mM ( $p < 0.05$ ; Figure 3B). Besides the interaction between cells and extracellular matrices, the cell-cell interaction might influence the cell invasion in the chamber assay. Therefore, we examined the effects of butyrate on cell aggregation. Cell aggregation was enhanced by butyrate (Figure 4), suggesting the modulation of expression of cell-cell adhesion molecules. Those results

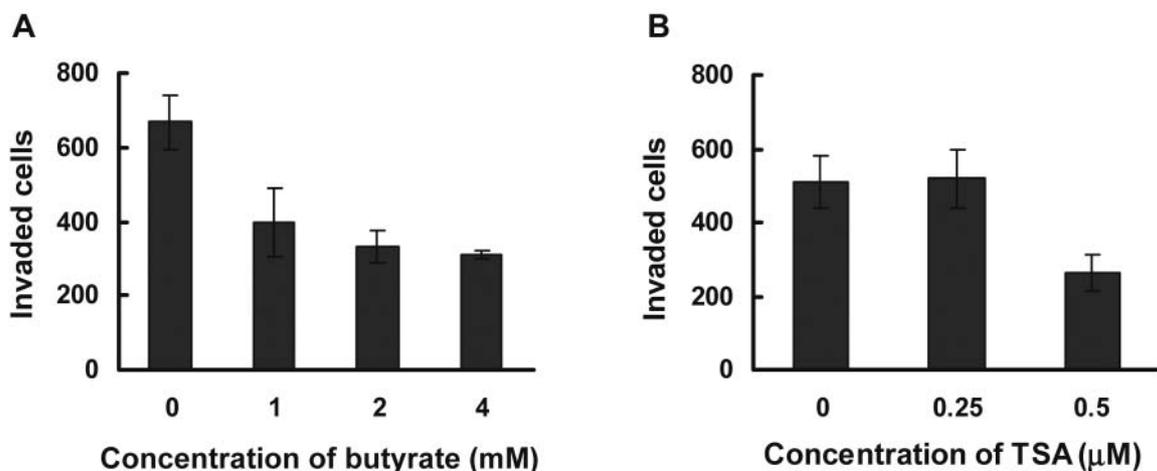


Figure 2. HDAC inhibitors inhibited the B16-BL6 cell invasion of Matrigel. The upper surface of a Transwell filter was coated with Matrigel and the lower surface with fibronectin. The cells were treated with (A) butyrate and (B) Trichostatin A: TSA for 48 h and the live cells were placed on the upper compartment of a chamber and incubated for 7 h. The number of cells invaded into the lower surface was counted in triplicate by the single blind method. Average ( $\pm$  S.D) is shown.

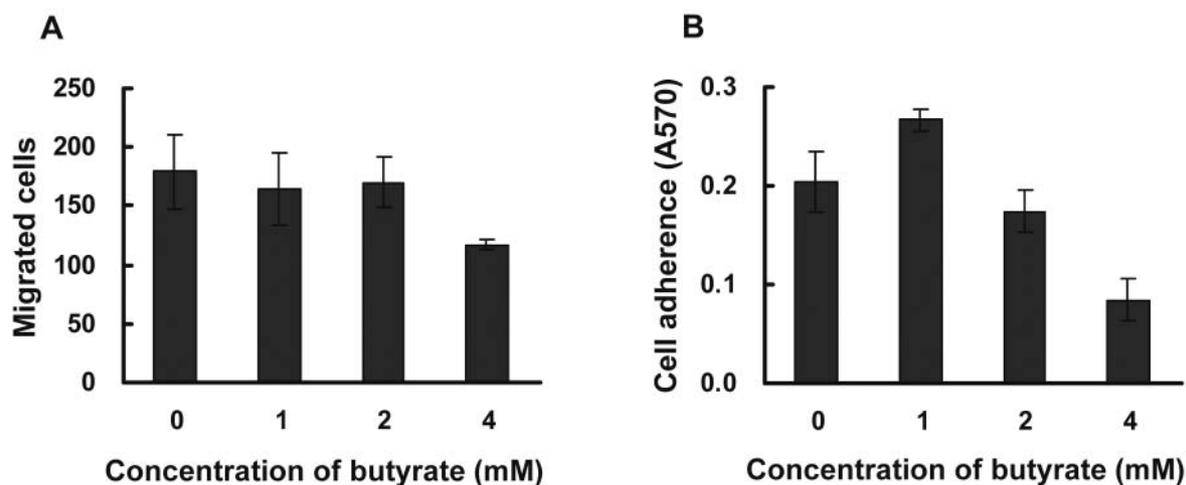


Figure 3. Butyrate inhibited the motility and adhesion to fibronectin in B16-BL6 cells. (A) The butyrate-treated live cells were placed on a Transwell chamber with a filter whose lower surface was coated with fibronectin and incubated for 3.5 h. The number of cells migrated to the lower surface was counted in triplicate by the single blind method. Average ( $\pm$  S.D) is shown. (B) The butyrate-treated live cells were placed in wells coated with fibronectin and incubated in triplicate for 30 min. After removing the unattached cells by washing, the attached cells were stained with crystal violet and dissolved in an acidic solution. Absorbance at 570 nm was measured with a microplate reader. Average ( $\pm$  S.D) is shown.

indicate that butyrate can modulate the expression of cell-cell and cell-matrix adhesion molecules in B16-BL6 cells.

Cell-cell interaction is generally influenced by cadherin molecules. We examined the butyrate effects on mRNA expression of cadherin family molecules in the cells. The mRNA expression of N-cadherin did not changed with time in the butyrate-treated cells (Figure 5A), however that of E-cadherin decreased (Figure 5B). These results cannot explain the increased cell aggregation brought about by butyrate.

Therefore, we then examined expression of the cell adhesion molecules of the immunoglobulin superfamily, Mel-CAM and L1-CAM, in the melanoma cells. The mRNA expression of Mel-CAM increased more than 10-fold (Figure 5C) and that of L1-CAM rose more than 2-fold upon the butyrate treatment of cells (Figure 5D). The up-regulation of Mel-CAM and L1-CAM was also confirmed with specific antibodies by Western blotting. Both Mel-CAM and L1-CAM proteins increased in the butyrate-treated cells (Figure 6). The

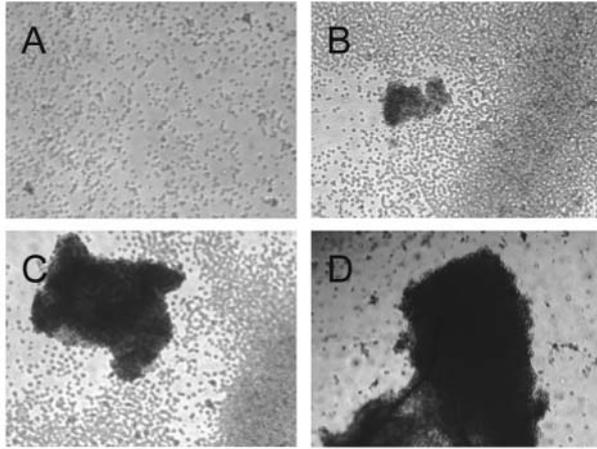


Figure 4. Butyrate enhanced the cell aggregation of B16-BL6 cells. The cells were treated with different concentrations of butyrate for 48 h and suspended in 0.1% BSA in EMEM. The live cells were transferred to the BSA-coated plate. After shaking the plate for 1.5 h, the cells were observed at  $\times 40$  with a microscope. A: 0 mM, B: 1 mM, C: 2 mM and D: 4 mM butyrate.

increase of expression of the immunoglobulin superfamily, Mel-CAM and L1-CAM, implies an increased cell-cell aggregation in the butyrate-treated B16-BL6 cells.

**Discussion**

In the metastasis of melanoma cells, the motility of cells is an important factor. The highly motile cells migrate in the extracellular matrix, invade the basement membrane and separate from the primary focus. The motility of melanoma cells is influenced by many factors. The cell-cell or cell-matrix interactions are often changed by modulated expression of cell adhesion molecules in melanoma cells. Cadherins are major cell adhesion molecules in  $Ca^{2+}$ -dependent cell-cell adhesion, although melanoma cells generally express quite low levels of E-cadherin on their surface (8). However, the cell adhesion molecules of the immunoglobulin superfamily, Mel-CAM and L1-CAM, are up-regulated in metastatic melanoma cells (8). Mel-CAM is a heterophilic  $Ca^{2+}$ -independent cell-cell

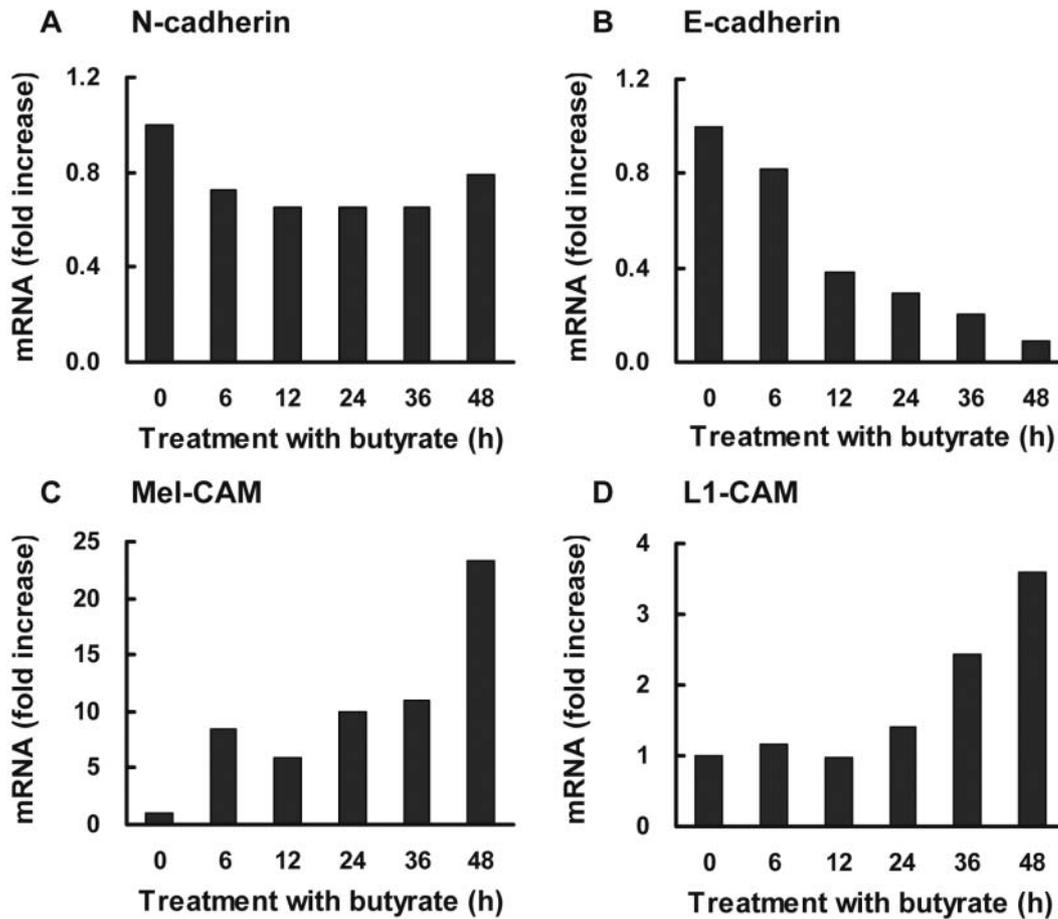


Figure 5. Butyrate modulated the mRNA expression of cell adhesion molecules in B16-BL6 cells. Total RNA was separated from the cells treated with 4 mM butyrate at the time indicated. RT-PCR with equal amount of RNA was performed using a real-time PCR LightCycler System and the amounts of PCR products were measured. Similar experiments were repeated twice and the typical result of two experiments is shown. A: N-cadherin, B: E-cadherin, C: Mel-CAM and D: L1-CAM mRNA.

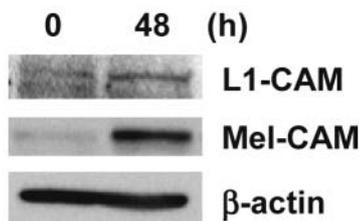


Figure 6. Butyrate increased the amounts of Mel-CAM and L1-CAM proteins in B16-BL6 cells. B16-BL6 cells were treated with 4 mM butyrate for 48 h. The treated cells were submitted to standard Western blotting. Mel-CAM, L1-CAM and  $\beta$ -actin proteins were detected with the corresponding specific antibodies.

adhesion molecule in melanoma cells, although its ligand has not been identified (9). L1-CAM is a homophilic and heterophilic cell adhesion molecule expressed in malignant melanoma cells (10). For the heterophilic association with L1-CAM, integrins  $\alpha$ v and  $\beta$ 3 form a receptor dimer (11).

In our experiments, butyrate and trichostatin A inhibited the B16-BL6 cell invasion of Matrigel, an artificial model of the basement membrane. In addition, 4 mM butyrate inhibited the cell migration and adhesion to fibronectin, and enhanced the cell aggregation. These results suggest that the expression of cell adhesion molecules is modulated by treatment with the HDAC inhibitors, butyrate and trichostatin A, in these melanoma cells. In fact, the butyrate treatment reduced the mRNA expression of E-cadherin, which was probably low in the untreated cells, and did not change the mRNA expression of N-cadherin. Although we did not detect cadherin proteins, the enhanced cell aggregation with butyrate would not be due to the increase of cadherin expression. On the other, both mRNA and protein expressions of Mel-CAM and L1-CAM were increased in the butyrate-treated cells. The mRNA expressions of integrin  $\alpha$ v and integrin  $\beta$ 3, the complex of which is a heterophilic counterpart of L1-CAM, were increased in the butyrate-treated cells (data not shown). Therefore, the increased expression of Mel-CAM and L1-CAM implies increased cell aggregation in the butyrate-treated B16-BL6 cells. The increased cell-cell adhesion would inhibit the cell invasion of Matrigel. Because the cell migration and adhesion to fibronectin were also inhibited in the cells treated with 4 mM butyrate, the cell adhesion molecules which interact with fibronectin would be responsible for the reduced invasion of Matrigel.

Mel-CAM and L1-CAM are well-known surface markers which are related to the progression of melanoma. Either Mel-CAM or L1-CAM expression increases the metastatic potential of melanoma cells (8, 12). Interestingly, the butyrate-treated B16-BL6 cells exhibited reduced invasion of Matrigel, regardless of the increase in Mel-CAM and L1-CAM expressions. The reduced invasion of Matrigel suggests the reduction of metastasis in the melanoma cells *in vivo*. Whether

the butyrate-treated B16-BL6 cells have a reduced metastatic potential or not should be examined in mice. Matrix metalloproteinases play important roles in melanoma cell invasion and metastasis *via* degrading the basement membrane (13). In addition to the modulation of cell adhesion molecules, butyrate and trichostatin A might modulate the matrix metalloproteinase activity in the melanoma cells.

Butyrate can induce apoptosis in some cancer cell lines and also can arrest the cell cycle by modulating cyclins and the cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> in colon cancer cells (14, 15). We reported that butyrate inhibited the growth of the colon cancer cell line LS174T by arresting the cell cycle and stimulating the MUC2 mucin production in the cells (5). This time, we found that butyrate also arrested the cell cycle of B16-BL6 cells, but their apoptosis was not induced in the melanoma cells. Whether butyrate induces apoptosis or not will thus depend on the cell type and the butyrate concentration.

The HDAC inhibitors are hopeful candidates for treating melanoma. A combination of HDAC inhibitors and other medicines might be effective for treatment of malignant melanoma (2). As for butyrate, melanoma cells probably do not interact with a high concentration of butyrate *in vivo* because the blood concentration of butyrate is usually low under physiological conditions (16). However, as this study shows, the weak HDAC inhibitor butyrate, as well as trichostatin A, could inhibit the melanoma cell invasion of Matrigel. For developing proper anticancer medicines which have low side-effects, not only strong HDAC inhibitors but also weak HDAC inhibitors should be considered as candidates.

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