# Differentiation of B16-BL6 Melanoma Cells into Microtubule-Associated Protein 2-Positive Cells after Treatment with the Histone Deacetylase Inhibitors Butyrate and Trichostatin A

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**Inhibition of histone deacetylase (HDAC) modulates the expression of many genes and induces cell cycle arrest, apoptosis, and differentiation in several cancer cell lines. Melanoma is a malignant phase of cutaneous melanocytes originally derived from the neural crest and is highly metastatic. A therapeutic agent capable of inducing differentiation of metastatic melanoma cells into a nonmalignant stage would be useful to prevent metastasis. We examined whether HDAC inhibitors can induce differentiation of the murine melanoma cell line B16-BL6 into neural-type cells *in vitro*. A morphologic change accompanied by extended dendrites was induced in melanoma cells after treatment with the HDAC inhibitors butyrate and trichostatin A (TSA). The altered morphology was similar to that of neural cells. Many of the extended dendrites fused with other dendrites of neighboring cells and had synapse-like knobs on their dendrites. These dendrites showed positive labeling with anti-$\alpha$/$\beta$-tubulin and anti-L1 cell adhesion molecule (L1CAM) antibodies but were seldom stained with phalloidin, suggesting that the neural cell-related proteins were major components of the extended dendrites but that actin stress fibers were not. Furthermore, the mature neuron-specific cytoskeletal protein, microtubule-associated protein 2 (MAP2), was detected with the specific antibody in the extended dendrites of cells treated with butyrate and TSA. Butyrate treatment increased the levels of MAP2 and neural cell adhesion molecule (NCAM) mRNA expression in the cells. However, the treatment did not alter the expression of neurofilament light chain (NF-L) mRNA. The observations suggest that the differentiated cells were neural-type cells but not complete neural cells.**

**Key words** —— histone deacetylase inhibitor, melanoma, microtubule-associated protein, differentiation, B16-BL6 melanoma cells

## INTRODUCTION

The local structure of chromatin in eukaryotes is dynamic. Chromatin remodeling is regulated by chromatin remodeling complexes and enzymatic modification of histone proteins. The acetylation and deacetylation of *N*-terminal tails of histones play important roles in chromatin remodeling. Histone deacetylases (HDACs) are essential in remodeling to modulate specific gene expression.[1)] As the inhibition of HDAC modulates the expression of many genes, HDAC inhibitors can show variable effects on cells, such as growth inhibition and cell differentiation. Recently, HDAC inhibitors have been suggested to be promising anticancer agents.[2)]

Melanoma is a serious disease that is difficult to cure because it is highly metastatic. Several HDAC inhibitors or combinations of HDAC inhibitors and other agents may be effective in preventing the progression or metastasis of melanoma.[3,4)] Recently, we have reported that the short fatty acid butyrate, which is a physiologic metabolite in the colon, can inhibit the melanoma cell invasion of Matrigel, an artificial model of the basement membrane, suggesting that HDAC inhibition may be effective in preventing metastasis.[5)] Although butyrate is a weak HDAC inhibitor in comparison with trichostatin A (TSA) from *Streptomyces hygroscopicus*, butyrate is known to induce differentiation as well as inhibition of cell growth.[6)] If melanoma cells can be differentiated into a nonmalignant stage, this will be an effective strategy to prevent metastasis. Melanoma cells are a malignant phase of cutaneous melanocytes originally derived from the neural crest. Therefore melanoma cells have some of the characteristics of neural cells, for example,

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the expression of intermediate filament protein peripherin, muscarinic acetylcholine receptors, and neuron-specific enolase.[7] When neuronal precursors differentiate into mature neurons, the mature neuron-specific cytoskeletal protein, microtubule-associated protein 2 (MAP2), becomes detectable in the cells. Therefore MAP2 is one of the most widely accepted markers of mature neurons.[8] We examined whether HDAC inhibitors can induce differentiation of B16-BL6 melanoma cells, a highly metastatic murine cell line, into neural-type cells. We found that the melanoma cells differentiated into MAP2-positive cells after treatment with the HDAC inhibitors butyrate and TSA *in vitro*.

## MATERIALS AND METHODS

**Cells and HDAC Inhibitors** —— The murine melanoma cell line B16-BL6 was maintained as previously described[5] in Eagle's minimum essential medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 5% fetal bovine serum (Cansera International, Ontario, Canada), 1% nonessential amino acids (Invitrogen, Grand Island, NY, U.S.A.), penicillin 100 U/ml and streptomycin 100 μg/ml (Gibco Oriental, Tokyo, Japan) in a 5% $CO_2$ incubator. Sodium butyrate was obtained from Wako Pure Chemical Industries (no. 193-01522, Osaka, Japan) and TSA was from Cayman Chemical (no. 89730, Ann Arbor, MI, U.S.A.).

**Staining of Cells** —— B16-BL6 cells ($1–4 \times 10^3$ in 0.2 ml) were precultured in a well of a Lab-Tek Chamber Slide System (Pharmanox slide, 177445, Nalge Nunc, NY, U.S.A.) for 2 days with the culture medium described above. Subsequently, different concentration of butyrate or TSA 0.5 μM was added to the cells, and the cells were cultured for an additional 2 days. The cells were fixed with 4% formaldehyde (Mildform 10N, Wako Pure Chemical Industries) and stained with the conventional hematoxylin-eosin staining method. In another experiment, cells fixed with formaldehyde were permeabilized with 0.2% TritonX-100 in phosphate buffered-saline (PBS) and stained with Alexa-Fluor-phalloidin (A12379, 300 U/ml, Molecular Probes, Eugene, OR, U.S.A.) in PBS supplemented with 1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO, U.S.A.) in the dark. After adding an antifade reagent, ProLong Gold (Molecular Probes), the cells were photographed with a digital camera microscope BX51 system (Olympus, Tokyo, Japan). The experiments were repeated three times for hematoxylin-eosin staining and twice for phalloidin staining with duplicated chamber wells.

**Immunostaining of Cells** —— B16-BL6 cells were precultured and treated with butyrate and TSA for 2 days as described above. The cells were fixed with formaldehyde. In the cases of $\alpha/\beta$-tubulin and MAP2 alone, the cells were permeabilized with Triton X-100 in PBS. The cells were pretreated with 1.5% BSA-PBS for blocking and stained with rabbit anti-$\alpha/\beta$-tubulin antibody (1:50 dilution, Cell Signaling #2148, Danvers, MA, U.S.A.), goat anti-L1 cell adhesion molecule (L1CAM) antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. sc-31034), and anti-MAP2 antibody (1:50 dilution, Cell Signaling #4542) at room temperature for 1 hr. Then, the cells were stained with appropriate second antibodies: fluorescein isothiocyanate (FITC) labeled anti-rabbit IgG (1:50 dilution, Kirkegaard Perry Laboratories, 02-15-16, Gaithersburg, MD, U.S.A.), and FITC-labeled anti-goat IgG (1:200 dilution, Vector Laboratories FI-5000, Burlingam, CA, U.S.A.) at room temperature for 1 hr. One experiment was done for each antibody staining with one or two chamber wells.

**Reverse Transcription (RT)-PCR** —— B16-BL6 cells ($1 \times 10^6$cell/ml) were treated with butyrate for 12 or 24 hr. Total RNA was separated from those cells using a RNeasy Mini Kit (Qiagen, Tokyo, Japan). RT-PCR of neural cell adhesion molecules (NCAMs) was performed as previously described.[9] After one cycle of reverse transcription at 50°C for 20 min, PCR was performed with 30 cycles of 94°C for 15 sec, 60°C for 15 sec, and 72°C for 20 sec. The specific primers of NCAM-140 were: sense 5′-CTGAGCACAGGCGCCATT-3′ and antisense 5′-ATGTCCATGACCACCAGGAGTAG-3′. In the other experiments, a conventional thermal cycler (MyCycler, BioRad, Tokyo, Japan) was used with a OneStep RT-PCR Kit (Qiagen). After one cycle of RT at 50°C for 30 min, PCR of the neurofilament light chain (NF-L) was performed with 36 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 1 min. PCR of MAP2 was performed with 40 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 1 min. The specific primers were: NF-L: sense 5′-AGCAGAATGCAGACATTAGCGCC-3′ and antisense 5′-TGGTCTCTTCGCCTTCCAAGAGT-3′; MAP2: sense 5′-CGGATCAAGAGAAAAAGG-3′ and antisense 5′-CTATTGCATACCTTCCA-3′. PCR products were separated by electrophoresis.

The specific bands were detected with a UV transilluminator and quantitated with a LAS-1000 Plus image analyser (FUJIFILM, Tokyo, Japan).

## RESULTS AND DISCUSSION

We have previously reported that the growth of B16-BL6 cells was inhibited by about 50% after treatment with 4 mM butyrate for 2 days, although the inhibition was not due to cell death but to arrest of the cell cycle in the G0/G1-phase.[5] In this study, B16-BL6 cells cultured with butyrate altered their morphology into the shape of neural-type cells that had extended dendrites. Many of the extended dendrites had synapse-like knobs on them. The top of dendrites often fused with other dendrites of neighboring cells. The morphologic change was induced by treatment with butyrate 1 mM and 4 mM for 2 days (Fig. 1A–1C). A similar morphologic change was induced after the treatment with TSA 0.5 μM for 2 days (Fig. 1D). The appearance of extended dendrites and knobs was clearly detected after treatment with butyrate 1 mM for 12 hr but not for 6 hr (data not shown).

The extended dendrites and knobs were seldom stained with phalloidin but were positively stained with anti-$\alpha/\beta$-tubulin antibody, although the actin stress fibers in the cytosol were positively stained with phalloidin (data not shown), suggesting that tubulins are major components of the extended dendrites but that actin stress fibers are not. Few L1CAM proteins were detected in the cytoplasm of untreated cells, but they became detectable in the extended dendrites and knobs in the treated cells. MAP2 is the most reliable marker of mature neurons.[8] MAP2 proteins were positively stained in the extended dendrites of treated cells, whereas MAP2 proteins were hardly detected in untreated cells (Fig. 2A and 2B). Another HDAC inhibitor, TSA, also induced MAP2 proteins in the extended dendrites of melanoma cells (Fig. 2C and 2D), suggesting the differentiation of melanoma cells into neural-type cells after treatment the HDAC inhibitors.

We examined the up-regulation of MAP2 mRNA and the levels of the essential protein in axon elongation, NCAM mRNA, and the neuron-specific intermediate filament NF-L mRNA using RT-PCR. The levels of MAP2 and NCAM mRNA expression were increased by about 10-fold in the melanoma cells treated with butyrate for 24 hr. However, the level of NF-L mRNA expression was not increased after the treatment (Table 1). These results indicate

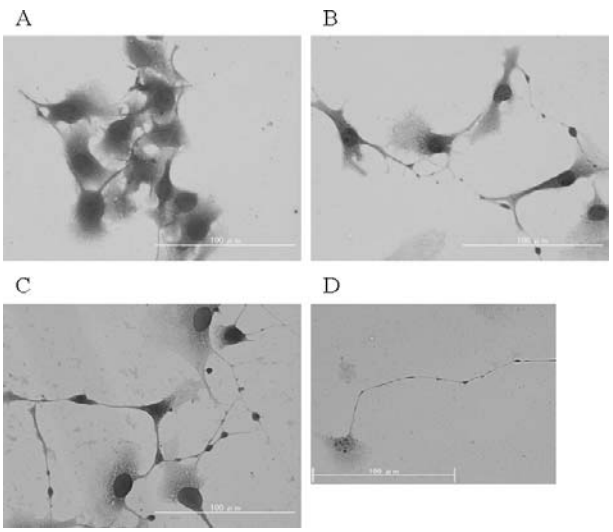


**Fig. 1.** Morphologic Changes Induced with HDAC Inhibitors in Melanoma Cells

B16-BL6 cells were treated with butyrate and TSA for 2 days and stained using the hematoxylin-eosin method. A: saline control; B: butyrate 1 mM; C: butyrate 4 mM; D: TSA 0.5 μM. All magnifications are ×400.

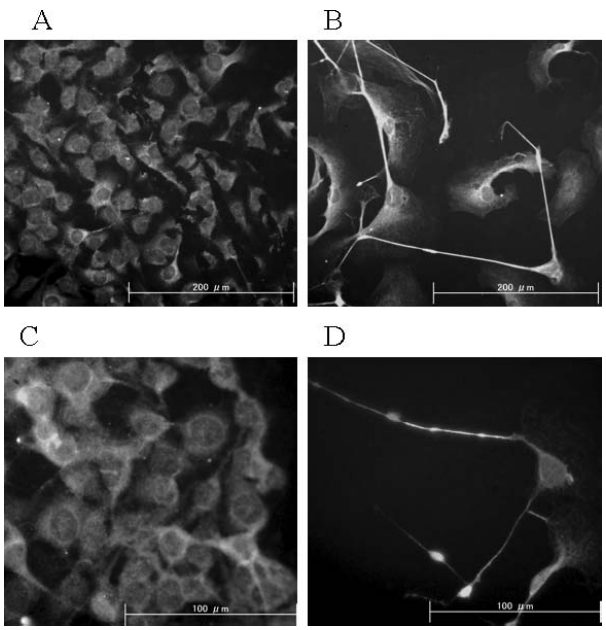


**Fig. 2.** MAP2 Induction with HDAC Inhibitors in Melanoma Cells

B16-BL6 cells were treated with butyrate and TSA for 2 days and stained with anti-MAP2 antibody. A: saline control; B: butyrate 4 mM; C: 0.05% dimethyl sulfoxide (DMSO) control and D: TSA 0.5 μM in a final concentration of 0.05% DMSO. Magnifications are ×200 in A and B, and ×400 in C and D.

**Table 1.** Expressions of mRNA in Butyrate-Treated Melanoma Cells

| mRNA | Butyrate (mM) | Fold increase, 12 hr | Fold increase, 24 hr |
|---|---|---|---|
| MAP2 | 0 | 1.0 | n.d. |
| | 4 | 3.6 ± 1.2 | 10.2 ± 0.3 |
| NCAM | 0 | 1.0 | 1.3 ± 0.5 |
| | 2 | 11.0 ± 0.8 | 9.2 ± 2.5 |
| NF-L | 0 | 1.0 | 2.2 ± 0.3 |
| | 2 | 1.4 ± 0.1 | 2.2 ± 0.3 |

Total RNA was separated at 12 hr or 24 hr from B16-BL6 cells treated with butyrate. The average (± S.D.) of 2, 5, and 3 samples is shown in MAP2, NCAM, and NF-L, respectively. The mRNA level without butyrate at 12 hr was used as a control.

that the differentiated melanoma cells were of the neural type, but were not complete neural cells.

Previously, we reported increased levels of L1CAM mRNA in butyrate-treated B16-BL6 cells.[5] MAP2, NCAM, and L1CAM are essential in neurogenesis and axon elongation.[8, 10, 11] The results suggest that B16-BL6 cells can be induced to differentiate into neural-type cells by the inhibition of HDAC. However, the differentiated cells would not be complete neural cells because the level of the neuron-specific intermediate filament NF-L, which is important for the composition of axons,[12] was not increased by the treatment.

To the best of our knowledge, this is the first report of the appearance of MAP2 in melanoma cells associated with HDAC inhibition. However, the mechanism of MAP2-positive differentiation with HDAC inhibitors in melanoma cells is not yet clear. It has been reported that the differentiation inducer hexamethylene bisacetamide induces MAP2 in cultured human melanoma cells.[7] Melanoma cells are malignantly differentiated from melanocytes, which are cutaneous pigment cells originally derived from the neural crest.[7] HDAC inhibition may induce the reprogramming of melanoma cells to the original neural cells, such as the effects of the microenvironment.[13] To determine the mechanism of neural differentiation in melanoma cells, the HDACs and specific genes involved in MAP2-positive differentiation should be identified.

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