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Original Article

Inhibition of mouse B16 melanoma by sodium butyrate correlated to tumor associated macrophages differentiation suppression

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Abstract: Objective: As one member of the histone deacetylase inhibitor (HDACi) family, Sodium butyrate (NaB) was found out that could be used as a differentiation inducer of much cancer cell. But its effects on tumor microenvironment cells are not well recognized. The goal of this research is to investigate the effect of NaB on B16 melanoma and analysis its relevant mechanism. Methods: We observed the effect of sodium butyrate on B16 melanoma in *vivo* and in *vitro*. MTT method was performed to detect cell apoptosis rate after treatment. Tumor associated macrophage infiltration condition was detected by flow cytometry. Western-blotting and immunohistochemical method were used to detect the expression of tumor associated macrophage cytokines. Results: A certain concentration of sodium butyrate could effectively inhibit B16 melanoma growth in *vivo* and in *vitro*, and this inhibition effects related to the suppression of tumor associated macrophage differentiation. At the same time we observed the relevant macrophage factors were down-regulated compared to the control. Conclusion: Sodium butyrate could effectively inhibit B16 melanoma growth through suppressing tumor associated macrophage proliferation and reduce relevant pro-tumor macrophage factors expression, which may help to promote the clinical study of melanoma epigenetic therapy.

Keywords: Sodium butyrate (NaB), TAMs, B16

Introduction

Mostly originating from dermal melanocytes, melanoma is known as its high malignance, high differentiation capacity and high metastatic potency. So its insensitivity to radio, chemo therapy always keeps on our clinicians and researchers. The therapy of combined chemotherapy with immunotherapy may achieve a well prognosis. As one member of histone deacetylases inhibitor family, sodium butyrate (NaB), was shown that could be a benefit factor in the treatment of melanoma in recent researches [1, 2]. With the potency of leading to the relaxation of chromatin structure and better access for transcriptional related proteins, sodium butyrate (NaB) exhibits a good anti-cancer ability, which made NaB are widespread used in the clinical treatment of many solid tumors [3]. And, its combination with biological drug may improve the treatment efficiency of HER-2 over-expressing breast cancer [4]. However, the anti-tumor mechanism of NaB remains a big controversial issue. The most well-known mechanism is that NaB could interrupt the bond between DNA and histone by targeting zinc-dependent histone deacetylases, and further inhibit cell mitosis. Also, NaB could induce apoptosis in A375 melanoma cell line by up-regulating p53 protein [5]. Furthermore, NaB may be an effective sensitizer of TRAIL-induced apoptosis by enhancing DR5 gene promoter transcription[6].

Tumor microenvironment is a condition which is made up of kinds of inflammatory cell or immune cell and cytokines. Tumor associated macrophage (TAM) is one of these cells, which display a vital role in the process of carcinogenesis, tumorunedevelopment and angiogenesis. Normally, M1-type TAMs suppress tumor development by releasing anti-tumor cytokines such

as IL-6, TNF, and INF-e promote tumor progress by releasing cytokines like as IL-10, TGF-β, VEGF and so on [7]. So, skewing the differentiation of TAMs to M2-type could be a benefit in the treatment of cancers. As is well known, many cytokines could orient the M2-type plasticity such as CSF-1, IL-10, and CCL-18 [8]. But the effects of some small molecule compound on TAMs plasticity are not well recognized. DMSO (dimethyl sulfoxide), something of the kind, have been proved that could change surface receptor of macrophage [9, 10]. So, study on effects of small molecule compound on TAMs may have an underlying meaning to clinical treatment.

In this research, we focused on the effects of NaB on B16 melanoma tumor microenvironment. We found NaB displayed a good antitumor effect in *vivo* and *vitro* test and TAMs were selectively (M2-type) inhibited compared with control. To further verify this result, we further tested its relevant secretory cytokines. Analogously, cytokines including IL-10, VEGF, and TGF-β released by TAMs were decreased to varying degree. Our data provides evidence that NaB could suppress B16 melanoma growth through impairing capacity of M2-type TAMs.

Materials and methods

Cell line and reagents

Mouse melanoma cell lines-B16, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were regularly cultured in RPMI-1640 (Sigma-Aldrich, Shanghai, Trading Co. Ltd.) containing 10% fetal bovine serum (Sigma chemical) under the condition of 5% CO_2 in incubator at 37°C. NaB was purchased from Sigma Company. Fluorescent antibodies for flow cytometry were purchased from (Beck, dicksion. Ltd).

MTT assays

B16 cells of logarithmic phase were harvested and paved into 96-well plates at a concentration of 5×10^3 cells/well to touch overnight. Then, the investigated factors were put in at specific concentrations. After B16 cells were cultured for 24 h and 48 h, MTT assays were performed to evaluate cells' viability. 20 μl MTT solutions (5 mg/ml) were added to keep incubating for 1-4 h. Supernatant was abandoned carefully and plates were washed with PBS for 3 times. Every well was added in 150 μl DMSO and plates were put on shaker for 15-20 min to make the formazan crystal violet dissolved completely. Absorbance of cells was tested at 570 nm wave length with the enzyme-linked immunosorbent assay reader (Thermo scientific). Suppression ratio was indicated as: relative suppression ratio $=(A_e-A_b)\times100/(A_c-A_b)$. A_c represents the absorbance of control. A_e and A_b mean absorbance of experimental groups and background absorbance respectively.

Animal model

C/57 female mice of 5-7 weeks' age were purchased from HFK Bioscience Co, Ltd. China. The mice were raised according to institutional guidelines approved by North Sichuan Medical College in line with the current regulations and standards of ministry, labor and welfare. 1×10^6 logarithmic phase B16 cells in 100 μl serum free medium were inoculated into the back subcutaneous of mice. Mice were randomly divided into five groups when the tiny tumor can be touched. Then, NaB was diluted in sterile saline according to the concentration as given and injected into mouse peritoneal cavity every other day. Control group was injected with sterile saline. Tumor size was tested every 3 days. After the fifth measurement, mice were executed to pick off tumors and visceral organs integrally. Some fresh tumor tissue was stored in liquid nitrogen.

Flow cytometry

Part of fresh tumor tissue was digested in collagenase-1 (Gibco, Ltd, 1 mg/ml) diluted in RPMI-1640 culture without serum and antibiotic for 1.5-2 h. Then, tissue homogenate was centrifuged (1500 rpm) for 5 min, supernatant was removed and subside was washed by PBS for 3 times. Sediment was resuspended with 5-8 ml PBS (pH=7.4). Single-cell suspension was achieved and calculated with red blood cells count instrument; cell suspension concentration was modulated to 1×10^5/100 μl. Then, 100 μl cell suspension was extracted to incubate with flow cytometry antibodies. Next, double stain technique was applied to observe the macrophage surface markers. CD11b-FITC and F4/80-PE (BD Biosciences Pharmingen, CA, and USA) were applied to incubate with speci-

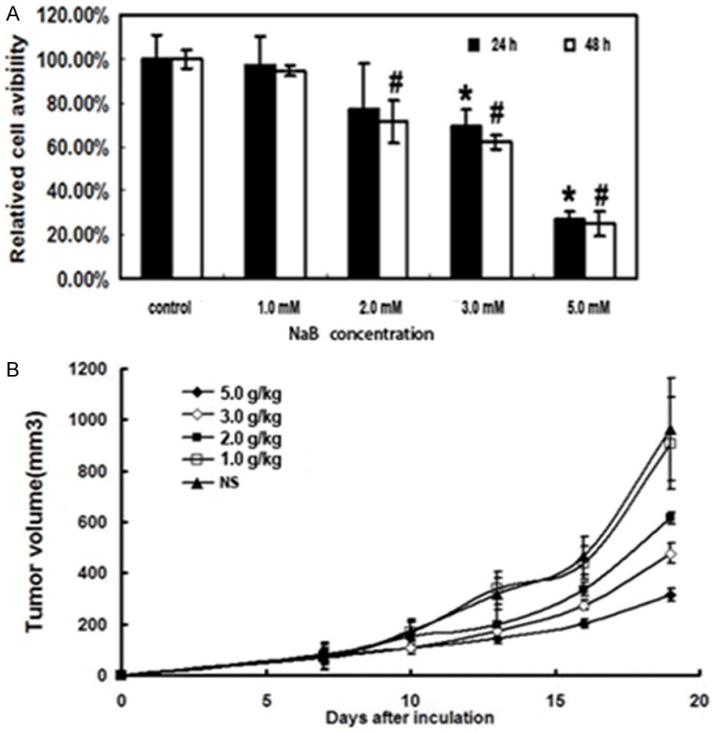

Figure 1. Inhibition effect of melanoma by sodium butyrate in vitro.

men for 30 min under 4°C. Besides, isotype controls were set as the negative controls. Cells were resuspended with 200 μl PBS to be tested by Flow Cytometer (Beck, dicksion. Ltd). Total cells to be harvested were set to 1×10^4 and speed of collection was controlled at 200-300 cells/s. The data analysis was completed by CELL Quest software (Beck, dicksion).

Immunoblot analysis

Tumor tissue was taken out from liquid nitrogen and pulverized quickly. The RIPA buffer solution (0.5-1.0 ml/bottle) was added to split cells. Then, all products were transferred into EP tubes to centrifuge for 15 min at 4°C, 12000 rpm. Supernatant was collected and cell lysates concentration was detected by Bradford method. Sample concentration was modulated to 20 μg/20 μl and same concentration β-actin protein was used as the internal reference. Next, samples were added into 2×SDS buffer solutions to boil protein sample for 5 min. Then, 20 μl samples were subjected to immunoblot analysis in SDS-PAGE gels, followed by electro transfer onto PVDF filters. The filters were washed with TBS for 5 min and blocked by skimmed milk for 1 h. After washed by TBS/T for 3 times, filters were incubated with primary antibodies: IL-10, VEGF, and TGF-β (Abcam, Cambridge, UK) and then incubated with peroxidase-conjugated IgG antibodies (MBL, Nagoya, Japan). Then, filters were washed with TBS/T for 3 times and TBS for 1 time again. Immune complex was exposed through chemiluminescence method and visualized by Gel Doc lab™ software (Bio-Rad laboratories, Inc.)

Statistical analysis

The data were showed as Means ± SD. Statistical analysis was performed with the method of one way analysis of variance (ONEWAY-ANOVA) employing SPSS16.0 software. The value of $P<0.05$ was considered to be significant.

Results

B16 melanoma growth was suppressed in vitro and vivo by NaB

Based on the fact that NaB could suppress multiple tumor growth, we applied it as a factor to observe its effects on mouse B16 melanoma in *vitro* and in *vivo*. Except for 1.0 g/kg group, it has been found the tumor growth was initially suppressed from day 10. Furthermore, the suppression effect lasted to the end and displayed a dose-dependent effect from the start of 2.0 g/kg (**Figure 1**). Remarkably, the most prominent growth suppression was obtained when NaB was administrated at 5.0 g/kg, where a statistical significant retardation of tumor growth was observed on days 13-19 as compared with control ($P<0.01$).

Melanoma TAMs polarization inhibition and reduction of cytokines

To figure out the anti-tumor mechanism of NaB, we further detected the immune cells in tumor microenvironment. It has been found TAMs were selectively inhibited. As the results say, $CD11b^+$ $F4/80^{high}$ cells' percentage decreased after treatment, of which values were 37.54±2.34%, 41.62±3.10%, 29.28±4.42%, and 19.92±4.80% for 0.25, 0.5, 0.75, 1.0 mg/g respectively. The percentage was 45.00±3.43%

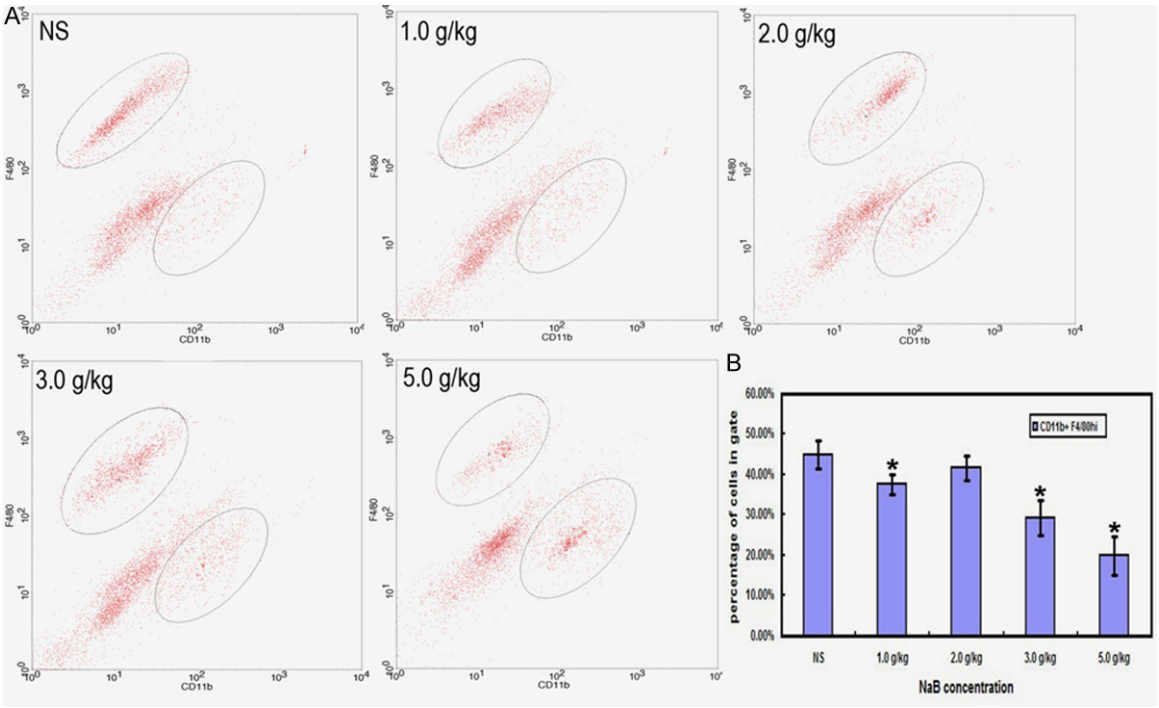

Figure 2. Changes in tumor-associated macrophages by Flow cytometry.

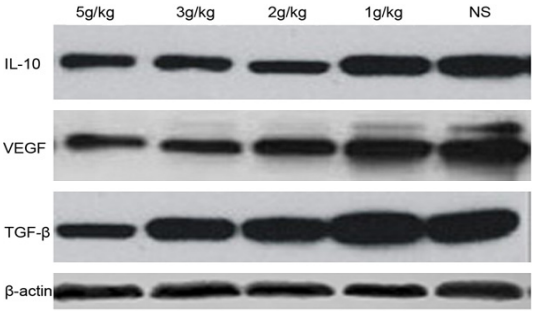

Figure 3. Changes in related cytokines of tumor microenvironment.

in control (**Figure 2A**). The most remarkable reduction of $CD11b^{+}$ $F4/80^{high}$ cells was observed at 5.0 g/kg ($P<0.01$) (**Figure 2B**). We also tested relevant cytokines released by the TAMs. Similarly, much kind of cytokines including IL-10, VEGF, and TGF-β released by TAMs were decreased compared with control (**Figure 3**).

Discussion

HDAC inhibitors can induce apoptotic cell death in a number of tumor cell types, including melanoma [11, 12]. Although report of the exact mechanism by which this process occurs varies in different tumor types, apoptosis induced in melanoma cell seems to have some uniform parts. Firstly, HDAC inhibitors can induce apoptosis in many melanoma cell lines, while there is no effect on some cell lines and normal human melanocytes [13]. Further, Bcl-2 overexpression in melanoma cell lines, or treatment with caspase inhibitors, reverses the apoptosis induced by HDAC inhibitors, in particular pancaspase inhibitor [14]. Accordingly, all HDAC inhibitors studied to date also induce PARP cleavage in melanoma cell lines. As a result, there exist some different molecular mechanisms in apoptotic pathway induced by various HDAC inhibitors.

As previously suggested, a number of HDAC inhibitors have entered clinical trials [15]. Although some inhibitors have reached the phase II level, none have been directly targeted at melanoma. Otherwise, the original phase I trial of depsipeptide included five patients with melanoma [16]. HDAC inhibitors require sufficient period of exposure to achieve maximized tumor cell killing in culture, presumably because of their actions as cell cycle agents. Sequestration and elimination may also be problems in *vivo*. Therefore, continuous administration should be required to achieve efficacy in the clinic.

In this study, we found NaB, one of the HDAC inhibitors, displayed anti-tumor effect in *vivo* and *vitro* test and TAMs of melanoma were selectively (M2-type) inhibited compared with control. Besides, we further found cytokines including IL-10, VEGF, and TGF-β released by TAMs was decreased to varying degree. Though, the exact molecular mechanisms involved in this suppressing effects induced by NaB remain unclear. Further, the reasons why HDAC inhibitors could also induce the reduction of specific cytokines remain to be elucidated. HDAC inhibitors may need to be applied with other agents to successfully treat malignant melanoma.

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Disclosure of conflict of interest

None.

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References

[1] Demary K, Wong L, Spanjaard RA. Effects of retinoic acid and sodium butyrate on gene expression, histone acetylation and inhibition of proliferation of melanoma cells. Cancer Lett 2001; 163: 103-108.

[2] Fernandes BF, Maloney SC, Antecka E, Di Cesare S, Granner T, Burnier MN Jr. Sodium Butyrate (NaB) Induces KAI-1 Expression And Apoptosis In Uveal Melanoma. Invest Ophtalmol Vis Sci 2011; 52: 1447.

[3] Jazirehi AR. Regulation of apoptosis-associated genes by histone deacetylase inhibitors: implications in cancer therapy. Anticancer Drugs 2010; 21: 805-813.

[4] Chen W, Wei F, Xu J, Wang Y, Chen L, Wang J, Guan X. Trastuzumab enhances the anti-tumor effects of the histone deacetylase inhibitor sodium butyrate on a HER2-overexpressing breast cancer cell line. Intl J Mol Med 2011; 28: 985.

[5] Bandyopadhyay D, Mishra A, Medrano EE. Overexpression of histone deacetylase 1 confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway. Cancer Res 2004; 64: 7706-7710.

[6] Kim YH, Park JW, Lee JY, Kwon TK. Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells. Carcinogenesis. 2004; 25: 1813-1820.

[7] Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A.Macrophage polarization in tumour progression. Semin Cancer Biol 2008; 18: 349-55.

[8] Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-555.

[9] Marthyn P, Beuscart A, Coll J, Moreau-Gachelin F, Righi M. DMSO Reduces CSF-1 Receptor Levels and Causes Apoptosis in v-myc immortalizedmouse macrophages. Exp Cell Res 1998; 243: 94-100.

[10] Takasaki S, Leive L. Induction by dimethyl sulfoxide of Fc fragment-mediated phagocytosis in the mouse macrophage-like cell line P388D1. Proc Natl Acad Sci U S A 1982; 79: 4980-4.

[11] Medina V, Edmonds B, Young GP, James R, Appleton S, Zalewski PD. Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res 1997; 57: 3697-3707.

[12] Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, Gabrielli BG. Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. Mol Biol Cell 2000; 11: 2069-2083.

[13] Zhang XD, Gillespie SK, Borrow JM, Hersey P. The histone deacetylase inhibitor suberic bishydroxamate regulates the expression of multiple apoptotic mediators and induces mitochondria-dependent apoptosis of melanoma cells. Mol Cancer Ther 2004; 3: 425-435.

[14] Flørenes VA, Skrede M, Jørgensen K, Nesland JM. Deacetylase inhibition in malignant melanomas: impact on cell cycle regulation and survival. Melanoma Res 2004; 14: 173-181.

[15] Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005; 45: 495-528.

[16] Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J, Brooks R, Piekarz RL, Tucker E, Figg WD, Chan KK, Goldspiel B, Fojo AT, Balcerzak SP, Bates SE. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin Cancer Res 2002; 8: 718-728.