Effect of Sodium Butyrate on Cell Differentiation and Neurofilament Expression in Neuroblastoma

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Abstract **Objective** To investigate the effects of sodium butyrate on neuroblastoma cell differentiation and the numbers of cytoskelental protein. **Methods** Neuroblastoma(N2a) cell was treated with sodium butyrate. Morphological change was observed by microscope. Cell viability was assayed by MTT. The expression of high molecular neurofilament was detected by western blot and immunocytochemical procedure. **Results** Compared to the control, N2a cell induced by sodium butyrate extended axon or dendrite-like, longer processes and showed slower proliferation rate. In differentiating cells, the total content of high molecular neurofilament (NF-H) located in cytoplasm and neurites was comparable to control, but phosphporylated NF-H was increased in differentiating cells and translocated from axon hillock to neurites. The effect of sodium butyrate was time and dose dependent. **Conclusions** Sodium butyrate can induce N2a cell to differentiate and increased phosphporylation of NF-H is involved in neurite outgrowth.

Key Words sodium butyrate; differentiation; neuroblastoma; high molecular neurofilament

Induction of cell differentiation is one of the promising therapeutic methods for treating patients with malignant tumors. Basic and clinical studies of differentiation-inducing therapy have been conducted mainly with retinoic acid, which has been shown to be effective for the treatment of promyelocytic leukemia and squamous cell carcinoma of the skin. Sodium butyrate (SB), a much more effective differentiation-inducing agent than retinoic acid [1,2], has been demonstrated to inhibit cellular proliferation in a number of cell lines [1-4]. As a four-carbon short-chain fatty acid produced by intestinal flora, sodium butyrate is generally employed in colon cancer research[5-7] and may account for the close relationship between the intake of fiber-rich food and the decreased incidence of colon cancer.

Neuroblastoma is one of the most commonly malignant solid tumors with weak prognosis in childhood and usually serves as a model of differentiation-inducing treatment due to its biochemical property of spontaneous differentiation and regression. Pathologically, part of neuroblastoma cells spontaneously differentiated to mature neurons at some extent. Neurofolament, the neuron-specific intermediate filaments and considered as a marker for neuron, are major cytoskeleton components of neurons. Neurite outgrowth depends on cytoskeleton proteins elongation. Neurofilament proteins, particularly NF-H, are most extensively phosphorylated in large myelinated axons under normal conditions [8]. The phosphorylation of NFs has been shown to associate with alterations in the physical and chemical behavior of NFs[8]. Here, the present study is to investigate the effects of sodium butyrate on neuroblastoma cell morphology and phosphorylation of NF-H.

MATERIAL AND METHODS

Cell culture

Mouse neuroblastoma N2a cells (obtained from America Type Culture Collection) were cultured in Dulbecco´ modified Eagle´s medium (DMEM) plus 10% fetal bovine serum, containing 100IU/ml penicillin and 100IU/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37℃. The differentiation of N2a was induced by addition of sodium butyrate (Sigma, USA).

Cell viability assay

N2a cells were plated in 96-well plates at a concentration of 2.5×10^3 cells/well in 100μl of media and treated with 1mmol/L and 2mmol/L sodium butyrate for 1, 2, 3, 4 days, respectively. Cell viability was measured by treating N2a with 3-[4,

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5-dimethyhlthizaol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT, Sigma, USA) at a final concentration of 5 mg/ml for 4 h. Then, cell medium was replaced with 100μl dimethyl sulfoxide (DMSO) and the optical density of each well at 570 nm was determined using a microtitre plate reader. According to the results of MTT, cells treated with 2mM SB for 4 days were further analyzed the expression of NF-H.

Western blot

Cultures were rinsed twice in ice-cold PBS, homogenized in RIPA buffer containing 50mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxychlolate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 100μg/ml phenylmethysulfonyl fluoride, 1 μg/ml Aprotinin, spun at 12,000g for 20 min at 4℃. The supernatants were removed and denatured immediately by adding sample buffer. Protein concentration was determined with BCA Protein Assay Reagent (PIERCE, USA). Equal amounts of protein (20μg) were electrophoresed in 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membranes. The membranes were blocked with 3%BSA in TBS for 1h at room temperature, then consecutively incubated with primary antibodies (2D2 and 7H11, 1: 1000, Santa cruz), secondary ALP-labeled antibodies and developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium (BCIP/NBT).

Immunocytochemistry

Cultures were fixed for 7min with ice-cold acetone. They were then permeabilized in 0.5% Triton X-100 in PBS and rinsed twice with PBS. Endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for 10min and non-specific binding sites were blocked with goat serum for 10min at room temperature. The cells were exposed to each one of the following primary antibodies overnight at 4℃ diluted in 3% BSA/TBS: 2D2(1:500), a monoclonal antibody to total NF-H, or 7H11(1:500, Santa cruz), a monoclonal antibody to phosphorylated NF-H. Finally, the cultures were rinsed in PBS and incubated with secondary HRP-labeled antibody for 1 h at 37℃. Diaminobenzidine (500 mg/ml) was used as substrate.

Statistical analysis

Statistical analysis was evaluated by t-test with SPSS software. P values less than 0.05 were considered statistically significant.

RESULTS

Cell morphology

Untreated cells presented short spindle with short neurites, while SB-treated cell showed neuron-like pattern of longer processes. More significantly, most differentiating cell extended one long and robust axon-like process and several short dendrite-like ones, some of which was branched. Some processes cross-linked with each other to be reticular formation. The cell morphology changed in a time dependant manner.

Effect of SB on cell proliferation

In control group the values of OD570 were 0.113±0.015, 0.132±0.009, 0.147±0.011 and 0.169± 0.013 at 1, 2, 3, 4 days, respectively. However, those were lower in SB-treated cells. At the time points used, the values of OD570 were significantly reduced in 1 mM SB-treated group at 3 and 4 days (0.125±0.013, $p<0.05$/0.137±0.014, $p<0.01$), and at 2, 3 and 4 days(0.103±0.016, $p<0.05$/0.109± 0.011, $p<0.01$/0.114±0.010, $p<0.01$) in 2mM SB group. The obtained data suggested that SB induced a marked growth inhibition. The inhibitory effect on proliferation was increased in a time and dosage dependent manner.

Western blot results of NF-H expression

According to the results of MTT, cells treated with 2 mM SB were analyzed the expression of NF-H. The cellular levels of NF-H and its phosphorylation state were further studied by probing western blots of cell extracts with antibodies to total and phosphorylated NF-H. Both the total NF-H and phosphorylated NF-H expressed in control and SB treated cells. In differentiating cells, total NF-H was comparable to control, but phosphorylated NF-H was increased.

Localization of NF-H distribution

Neurofilaments are synthesized in the cell body and transported to the axon in which they are phosphorylated. The distribution of total and phosphorylated NF-H was also investigated by immunocytochemistry with the same panel antibodies. The staining pattern of total and phosphorylated NF-H was different. The staining of total NF-H was distributed in cytoplasm and neurites. However, phos-

phorylated NF-H was translocated from axon hillock to neutites in the presence of SB.

DISCUSSION

Members of the short-chain fatty acids have been identified as cell differentiation-inducing agents. Among those agents, butyrate seems to have the most profound effect on growth inhibition and differentiation in various kinds of cancer cells, including human colon cancer cells and human glioma cells. The molecular mechanisms underlying such effects maybe include[9–11]: specifically inhibiting histone deacetylase (HDAC), directly inhibiting DNA synthesis and arresting cell at G0/G1 phase, disrupting mitochondrial membrane potential and inducing apoptosis. In present study, N2a cell induced to differentiate by SB presented neuron-like pattern (extension of long processes) and showed slower proliferation rate, indicating SB had promoting effect on N2a cell differentiation and inhibitory effect on cell proliferation.

Neurofilaments are intermediate filaments confined solely to the nervous system. In a mature mammalian neuron, NFs are composed of three protein subunits: NF-L (low), NF-M (medium), NF-H (high), with molecular weight of 70, 160 and 200KDa, respectively[12]. The expression of NF-H, however, occurs much later than NF-M and NF-L. Developmentally, NF-H only exists in mature neurites, but not in immature neurites. Induced to differentiation in the presence of SB, N2a cell extended long processes and presented neuron-like pattern. To explore cytoskeleton alterations constituting the framework of developing neurites, we also investigated the total NF-H and its phosphorylated state. NF-proteins, particularly, the NF-M and NF-H are highly phosporylated in most adult neurons [8]. The data from western blot demonstrated that, with the treatment of SB to induce differentiation, the content of total NF-H in N2a cell was comparable to control, but phosphorylated NF-H in which was obviously increased. Differentiation was accompanied by the increase of phosphorylated NF-H level. Morphological, antigenic and biochemical experiments have shown that NF-H and NF-M not only contribute to the neurofilament central core but also make up the side-arm or cross-bridge connections between filaments and provide an anchor for other constituents of the cytoplasm[13,14]. Phosphorylation of this protein was involved in regulating cytoskeleton neuronal organization, providing stability to the axonal structure, and determining nerve fiber caliber. Therefore, increased phosphorylation of NF-H might be part of the possible mechanism underlying neurite extension in differentiating N2a cell.

Actually, following synthesis in the cell body, neurofilaments are assembled and transported to the axon where they are extensively phosphorylated [15], which suggeste this post-translational modification is developmentally and topographically regulated. The staining profile of total NF-H and phosphorylated NF-H observed in immunocytochemistry was different. The total NF-H located both in cytoplasma and in neurites; whereas the most intense staining of phosphorylated NF-H was observed in the long processes. Enhanced phosphorylation of NF can slow the velocity of axonal transport and make it accumulated in perikaryon [16]. However, in this study, the lesion was not observed.

In conclusion, SB is effective to inhibit cell proliferation and promote differentiation in N2a cell. Phosphorylation of NF-H was involved in neurite extension. This study provided more information about intracellular mechanism of neurite outgrowth in differentiation. As a widely researched anti-tumor agent, the inducing-differentiation of SB in vitro is promising, but its metabolic feature limits clinical and in vivo research. Therefore, it is urgent to elucidate the mechanism of SB and explore the practicable and effective reagent.

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