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Are Lymphoblastoid Cell Lines Suitable to Study Increased SMN Gene Expression by Histone Deacetylase Inhibitors?*

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Aim: In this preliminary study, we aimed to analyze the effect of sodium butyrate (NB) and phenylbutyrate (PB), belonging to the class of histone deacetylase (HDAC) inhibitors, on lymphoblastoid cell lines established from spinal muscular atrophy (SMA) patients.

Materials and Methods: Lymphoblastoid cell lines were established from one type I and two type III SMA patients. Following treatment of the cell lines by HDAC inhibitors, the levels of survival motor neuron 2 (*SMN2*) gene full length (fl-*SMN2*) transcripts were assessed by both standard and real time RT-PCR. To determine the levels of SMN protein, Western blot analysis was performed.

Results: NB and PB, which are known to be effective in SMA fibroblasts, did not increase the levels of fl-*SMN2* transcripts or protein in lymphoblastoid cell lines.

Conclusions: Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines may not be suitable for studying the effect of at least some HDAC inhibitors on SMN2 gene expression.

Key Words: Spinal muscular atrophy, survival motor neuron gene, histone deacetylase inhibitors, treatment

Lenfoblastoid Hücre Hatları Histon Deasetilaz Inhibitörleri ile Arttırılan SMN Gen Ekspresyon Çalışmaları İçin Uygun mudur?

Amaç: Histon deasetilaz (HDAC) inhibitörleri sınıfına giren sodyum bütirat (NB) ve fenil bütirat (PB) adlı bileşiklerin spinal müsküler atrofi (SMA) hastalarından oluşturulan lenfoblastoid hücre hatları üzerindeki etkisini araştırmak

Yöntem ve Gereç: Bir SMA tip I ve iki SMA tip III hastasının lenfoblastoid hücre hatları oluşturuldu. HDAC inhibitör uygulamasını takiben, survival motor neuron 2 (*SMN2*) genine ait fl-*SMN2* transkript miktarı hem standart hem de real time RT-PCR yöntemi ile saptandı. SMN protein miktarındaki değişikliği incelemek amacıyla Western blot analizi gerçekleştirildi.

Bulgular: SMA fibroblastlarında etkili olduğu bilinen sodyum bütirat ve fenil bütiratın, lenfoblastoid hücre hatlarında fl-*SMN2* transkript ve protein miktarını arttırmadığı saptandı.

Sonuç: HDAC inhibitörlerinin *SMN2* gen ekspresyonu üzerindeki etkisinin araştırılmasında Epstein-Barr Virus (EBV) transformasyonu ile oluşturulan lenfoblastoid hücre hatlarının uygun olmadığı sonucuna varılmıştır.

Anahtar Sözcükler: Spinal müsküler atrofi, survival motor neuron geni, histon deasetilaz inhibitörleri, tedavi

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Introduction

Spinal muscular atrophy (SMA) is a childhood autosomal recessive disorder that is characterized by degeneration of alpha motor neurons in the anterior horn of the spinal cord. The disorder has an incidence of 1/6,000-1/10,000 live births (1). The *Survival Motor Neuron (SMN1)* gene, located in 5q13, is responsible for this condition. Homozygous absence of exon 7 of the *SMN1* gene can be detected in 95-98% of the patients (2,3). In the same region, a second gene (*SMN2*) is present, which is almost identical to *SMN1*. The main difference between the two genes is a C-to-T transition in exon 7 of *SMN2,* which disrupts a SF2/ASF dependent exonic splicing enhancer (ESE), causing exon skipping, and the prevalent production of transcripts lacking exon 7 (Δ7) and of truncated protein isoforms (4). The number of *SMN2* copies is highly variable in the population. The clinical phenotype can range from severe to mild (type I to type III); the severity of the disease is in part modulated by the number of *SMN2* genes, with higher copy number related to milder phenotypes (5).

To date, no cure for SMA is available. Recently, efforts have been made to investigate the possibility of a pharmacological approach to SMA aimed at increasing the synthesis of full length *SMN2* (fl-*SMN2*) mRNA and protein. Different classes of compounds have been tested and it has been shown that histone deacetylase (HDAC) inhibitors are effective in increasing *SMN2* gene expression both *in vivo* and *in vitro*, although with variable results from patient to patient and in different cell types (6-12). Further studies on various cell types will provide useful information for the treatment of SMA.

Lymphoblastoid cell lines are immortal lines that can easily be obtained from blood lymphocytes and provide large amounts of biological materials such as RNA and protein (13). In this study, we investigated whether lymphoblastoid cell lines can be used as a tool for identifying therapeutic agents for SMA and analyzed the effect of sodium butyrate (NB) and phenylbutyrate (PB) on *SMN2* gene expression in lymphoblastoid cell lines.

Materials and Methods

Establishment of Lymphoblastoid Cell Lines

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines were established from one type I and two type III SMA patients. Patients were confirmed to have homozygous deletions of exons 7 and 8 of the *SMN1* gene. Informed consent was obtained from the families. Lymphocytes were isolated from heparinized peripheral blood by Ficoll-Histopaque separation (Biochrom, Germany). The buffy coat interface was collected and cell pellet was resuspended in 2 ml RPMI 1640 medium with 20% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and of an equal volume of EBV-containing medium. After O/N incubation, 500 ng of cyclosporin A were added per ml of culture medium (14). Flasks were incubated in a humidified 37°C, 5% CO_2 incubator.

This study was approved by the Research Ethics Committee of Hacettepe University, Faculty of Medicine (TBK 02/ 4-8).

Treatment of Lymphoblastoid Cell Lines with Sodium Butyrate (NB) and Phenylbutyrate (PB)

For transcript analysis, cell cultures from one SMA type I (Patient 1) and one SMA type III (Patient 2) patient were incubated with 0.05, 0.5 and 5 mM NB ($CH_3CH_2CH_2COONa$, Merck, Germany) for 4, 8, 24 and 32 h, and the amount of *SMN2* mRNA was determined by densitometric analysis (6).

Cell cultures from two type III patients (Patients 2 and 3) were incubated with 0.05, 0.5, 1, 2 and 5 mM PB ($C_6H_5(CH_2)_3COOH$, Fluka Chemika, Switzerland) for 8 h, and transcript analysis was performed by real time reverse transcriptase-polymerase chain reaction (RT-PCR) (7). These two cell lines were also treated with 2 mM PB for different time intervals (8, 16, 24 and 48 h) for protein analysis. The medium was changed every 24 h and in case of 48 h treatment, PB was added after 24 h.

Analysis of *SMN2* mRNA from Lymphoblastoid Cell Lines

Total RNA was extracted from treated and untreated cell lines using the RNeasy Mini Kit (Qiagen, Germany). Two µg of RNA were used for first strand cDNA synthesis by using the ImpromII RT System kit (Promega, Germany) according to the manufacturer's instructions.

For semiquantitative standard PCR, the following conditions were used: 22 cycles at 95 ºC for 15 s, 56 ºC for 30 s, 72 ºC for 45 s using primers within *SMN2* exon 6 (5′-CTCCCATATGTCCAGATTC-TCTTGATGATGC-3′ and exon 8 (5′-ACTGCCTCACCACCGTGCTGG-3′) (6). Densitometric measurements were carried out with gel

analyzer and Documentation System (Biodoc, Biometra, Germany). PCR products were visualized on a 4% agarose gel by ethidium bromide staining.

Real time RT-PCR was performed using the ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Italy) as described by Andreassi et al. (7). The relative amount of transcripts was calculated by using the threshold cycle (Ct) method, comparing SMN to two internal standards such as glyceraldehyde phosphate dehydrogenase (GAPDH, Applied Biosystems) and ß-actin (Applied Biosystems). The reactions were performed in triplicate and averaged. SMN Ct values were corrected for GAPDH and β-actin Ct values using the ΔΔCt method. The experiments were repeated twice and untreated lymphoblastoid cell lines were provided as a control. The statistical analysis was done with a non-parametric Kruskal-Wallis test (SPSS program 11.5). *P* values ≤0.05 were considered significant.

Analysis of SMN Protein Levels

Protein samples were extracted from treated and untreated cell lines by standard blending buffer. Western blot analysis was performed as described by Andreassi et al. (7). Membranes were probed with anti-SMN (dilution 1/5000, Transduction Laboratories, Italy), primary antibody 1 h at room temperature. After washing, membranes were incubated with the anti-mouse horseradish peroxidase-conjugated secondary antibody (dilution 1/2000) for 1 h at room temperature and visualized using chemiluminescence (ECL, Amersham, UK). Membranes were reprobed with the anti-actin polyclonal primary antibody (dilution 1/2000, Sigma-Aldrich, Italy), and anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution 1/2000, Amersham, UK).

The intensity of Western blot bands was measured by densitometry (Scion Image Software, Maryland, USA). SMN/ß-actin density ratios were normalized to untreated samples, which were used as a control. For each sample, three different Western blots were performed.

Results

Cell lines from one type I (Patient 1) and one type III (Patient 2) patient were treated with NB and the amount of fl-*SMN2* transcripts was determined by densitometric analysis. The results of fl-*SMN2*, Δ7 *SMN2* and FL/Δ7 are shown in Table 1. No increase in transcript levels was found, suggesting that EBV-transformed cell lines may not be responsive to NB. Since NB did not increase fl-*SMN2* transcript levels, real time RT-PCR and Western blot analysis were not performed.

PB, which has a longer half-life *in vivo* compared to NB, has been reported to be more effective in increasing fl-*SMN2* transcripts in SMA type III than type I fibroblasts (7). Therefore, we treated two SMA type III lymphoblastoid cell lines with PB. The effect of different concentrations and treatment durations of PB on fl-*SMN2* transcripts are shown in Figure 1. Only slight variations in fl-*SMN2* transcript levels, which were not statistically significant, were found ($P > 0.05$). To determine whether PB has an effect on SMN protein level, we performed Western blot analysis. No increase in SMN protein levels was observed in lymphoblastoid cell lines compared to untreated controls. The effect of PB on SMN protein level is shown in Figure 2. Thus, neither NB nor PB treatment modified the levels of fl-*SMN2* transcripts or protein in lymphoblastoid cell lines.

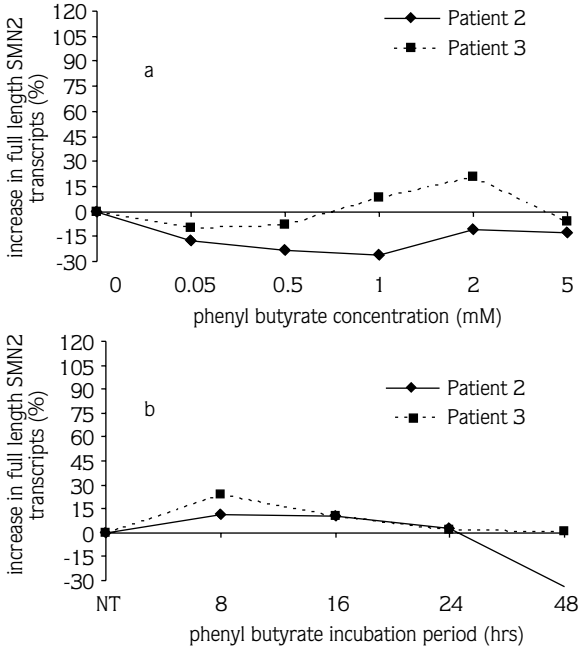

Figure 1. The effect of phenylbutyrate on full length *SMN2* transcripts as determined by real time PCR.
a. Effect of different concentrations of phenylbutyrate.
b. Effect of different treatment durations of phenylbutyrate.

Table 1. The effect of sodium butyrate on full length (FL) SMN2 RNA, Δ7 SMN2 RNA and FL/Δ7 as determined by densitometric analysis.

SMA lymphoblastoid cell lines			Sodium butyrate concentration (mM)			
			0	0.05	0.5	5
Patient 1	4 h	FL	13	10	13	11
		Δ7	12	10	13	15
		FL/Δ7	1.1	1	1	0.7
	8 h	FL	20	17	15	16
		Δ7	14	14	13	15
		FL/Δ7	1.4	1.2	1.2	1.1
	24 h	FL	16	21	15	13
		Δ7	14	16	13	12
		FL/Δ7	1.1	1.3	1.2	1.1
	32 h	FL	22	23	24	22
		Δ7	22	23	25	22
		FL/Δ7	1	1	1	1
Patient 2	4 h	FL	60	62	66	63
		Δ7	66	67	69	69
		FL/Δ7	0.9	0.9	1	0.9
	8 h	FL	14	15	17	15
		Δ7	13	13	14	14
		FL/Δ7	1.1	1.2	1.2	1.1
	24 h	FL	15	14	15	13
		Δ7	14	13	13	12
		FL/Δ7	1.1	1.1	1.2	1.1
	32 h	FL	30	27	25	14
		Δ7	23	21	19	13
		FL/Δ7	1.3	1.3	1.3	1.1

FL: full length

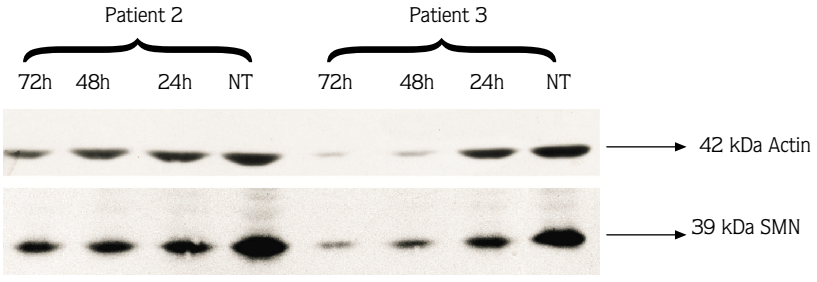

Figure 2. The effect of 2 mM phenyl butyrate on SMN protein as determined by Western blot analysis.
NT: Non–treated.

Discussion

Currently, the most promising therapeutic strategy is to increase fl-*SMN2* gene expression by HDAC inhibitors. These compounds may directly activate the *SMN2* promoter or alter the *SMN2* splicing pattern (15). HDAC inhibitors are known to affect the expression of 2-5% of genes through the hyperacetylation of histones, which alters the chromatin structure of DNA and makes it accessible to the transcriptional machinery. In addition to their effect on transcription, HDAC inhibitors possess diverse biological activities (16). Well- known HDAC inhibitors such as NB and PB are approved by the Food and Drug Administration for application in the treatment of various disorders (17,18). A few studies showed that these compounds enhanced growth inhibition, morphological changes, apoptosis, cytokine production, and oncogene and HLA expressions in lymphoblastoid cell lines (19-22).

In this study, we hypothesized whether SMA lymphoblastoid cell lines would be suitable in increasing SMN expression after NB and PB treatment. We demonstrated that treatment of lymphoblastoid cell lines did not result in an increase in fl-*SMN2* transcripts or protein. Although we analyzed a few cell lines, our data suggest that lymphoblastoid cell lines may not be a suitable *in vitro* model for studying the effect of the HDAC inhibitors on SMN expression. Barth et al. (23) demonstrated that EBV nuclear antigen 2, which is essential for viral transformation of lymphocytes, binds to SMN protein. The interaction between these two proteins may explain why fl-*SMN2* expression is not increased in lymphoblastoid cell lines after HDAC inhibitor treatment. The explanation for our findings remains elusive, although it can be hypothesized that EBV transformation modifies the responsiveness of cell cultures at least to some HDAC inhibitors.

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