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A novel evaluation method of survival motor neuron protein as a biomarker of spinal muscular atrophy by imaging flow cytometry

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ABSTRACT

Spinal muscular atrophy (SMA) is caused by mutations within the survival motor neuron 1 (*SMN1*) gene. These mutations result in the reduction of survival motor neuron (SMN) protein expression and SMN complex in spinal motor neurons and other tissues. SMN protein has been used as a therapeutic biomarker in recent SMA clinical studies using enzyme-linked immunosorbent assay (ELISA). Here, we investigated whether imaging flow cytometry can be a viable source of quantitative information on the SMN protein. Using a FlowSight imaging flow cytometer (Merck-Millipore, Germany), we demonstrated that imaging flow cytometry could successfully identify different expression patterns and subcellular localization of SMN protein in healthy human fibroblasts and SMA patient-derived fibroblasts. In addition, we could also evaluate the therapeutic effects of SMN protein expression by valproic acid treatment of SMA patient-derived cells *in vitro*. Therefore, we suggest that imaging flow cytometry technology has the potential for identifying SMN protein expression level and pattern as an evaluation tool of clinical studies.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by mutations of the survival motor neuron 1(*SMN1*) gene, leading to progressive limb and trunk muscle weakness associated with muscle atrophy [1,2]. SMN protein is ubiquitously expressed in mammalian tissues; it plays a critical role in RNA metabolism, participating in small ribonucleoproteins (sRNPs) biogenesis and in pre-mRNA splicing [2]. Two *SMN* genes translate SMN proteins: a telomeric copy (*SMN1*) and an inverted centromeric copy (*SMN2*). The *SMN2* gene is present in all patients, but is not able to compensate for *SMN1* gene defects completely, resulting in low levels of the full-length SMN protein in order to have a single point mutation in exon7 of the *SMN2* gene [3,4]. In SMA clinical specimens, reduction of SMN has been assessed by several methods. Typically, immunocytochemistry and Western blotting have been used on primary dermal fibroblasts and leukocyte cell lines, leading to a correlation between healthy human controls and SMA patients in preclinical studies [5,6]. However, this method is not sufficiently reliable for clinical and diagnostic use. Recently, enzyme-linked immunosorbent assay (ELISA) has also been used in preclinical and clinical studies of SMA [7–10]; nevertheless, SMN protein levels in human peripheral-blood mononuclear cells (PBMC) were not correlated between healthy controls, carriers, and SMA phenotypic severity in clinical trials [9,10]. These methods should be optimized to allow detection of SMN protein in human cells for SMA clinical studies.

In this study, we focus on the imaging flow cytometry technique as a new assay method of SMN protein evaluation. Usually, standard flow cytometry cannot be used to assess the localization of molecules within specific cellular compartments. However, imaging flow cytometry can evaluate intact proteins, using a digital microscope system, and immunological technologies [11,12]. SMN proteins are localized intracellularly throughout the cytoplasm and nucleus, as a multi-protein complex. Specifically, SMN proteins form SMN complexes in the nucleus, where they accumulate in structures called Gemini of Cajal bodies (Gems) that play an essential role in the assembly of spliceosomal snRNPs and biogenesis during mRNA processing [13,14]. The predicted outcome of decreased snRNPs assembly is an alteration in gene splicing, containing minor introns due to reduced snRNPs levels [2,15]. In SMA-derived cells, gems formation is clearly decreased compared to that of healthy controls [16].

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At present, there is no effective treatment for SMA. Some therapeutic approaches are recently under investigation; therapies aim at increasing the amount of full-length SMN protein levels produced by *SMN2* promoter activation, while reducing *SMN2* exon7 alternative splicing, using small molecules, and antisense oligonucleotides [6,17–20]. Therefore, a new method to accurately measure SMN protein levels is needed, to assess disease severity and response to treatment.

The aim of this study was to evaluate SMN protein expression and to qualitatively assess its cellular localization using imaging flow cytometry. We therefore explored the applicability of this new technology for evaluating SMN protein as a biomarker in SMA clinical trials.

2. Materials and methods

2.1. Materials

Human fetal dermal fibroblasts (from healthy controls) were obtained from Cell Applications, Inc., SMA patient-derived dermal fibroblasts were obtained from skin biopsies of SMA patients. The patient having SMA type I was a 7-month-old female who had not acquired head control with *SMN1* deletion and two copies of *SMN2*, as assessed by molecular diagnoses. Ethical approval for tissue collection was granted by the Institutional Review Board of Tokyo Women's Medical University, Japan. For immunocytochemical analyses, we used a mouse monoclonal FITC-conjugated anti-SMN, (clone 2B1, Merck Millipore, Germany), and a mouse monoclonal anti-SMN antibody (BD Transduction Laboratories, San Diego, USA).

2.2. Cell culture and valproic acid treatment

Human dermal fibroblasts (from healthy controls) and type I SMA patient-derived dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) including 1.0 g/L glucose and supplemented with 20% fetal bovine serum (FBS) without antibiotics. These fibroblasts were cultured in six-well plates for 24 h and then treated with the histone deacetylase inhibitor, valproic acid (VPA: 0, 0.1, 1, 10 mM) diluted in PBS for 24 h at 37 °C with 5% CO_2.

2.3. Immunocytochemical staining

After cells were cultured for 48 h, 1.5×10^6 cells were rinsed twice with cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and then rinsed three times for 5 min with PBS. The cells were then treated with 0.2% TritonX-100 in PBS for 10 min at room temperature. The cells were incubated in blocking buffer (10% normal goat serum in PBS) for 60 min at room temperature. Following blocking, the cells were incubated with a mouse monoclonal anti-SMN antibody (1:100, BD) at room temperature for 60 min and then cells were then visualized using an Alexa Fluor 488-conjugated goat anti-mouse (1:400, Molecular Probes) for 60 min at room temperature. The cells were treated with Hoechst 33342 (0.5 μg/mL) to stain the cell nuclei for 5 min at room temperature. Image photographs were taken using a Leica fluorescent microscope system.

2.4. Quantitative RT-PCR analysis

Cells were cultured for 24 h after VPA treatment, and total RNA was isolated using the RNeasy kit (QIAGEN Sciences, USA) according to the manufacturer's instructions. For reverse transcription reactions, 500 ng of total RNA was used with PrimeScripts RT Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Aliquots of cDNA were mixed with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) each containing 400 nM primers. Quantitative PCR was performed on Thermal Cycler Dice Real Time Systems (Takara Bio Inc., Shiga, Japan). Primers used in this paper were as follows: glyceraldehyde-3-phasphate dehydrogenase (*GAPDH*): 5′-GCACCGTCAAGGCTGAGAAC-3′ for forward and 5′-TGGTGAAGACGCCAGTGGA-3′ for reverse; *SMN2*, 5′-AACCTG TGTTGTGGTTTACACTGGA-3′ for forward and 5′-CAGATTTGGGCTT GATGTTATCTGA-3′ for reverse. All samples were assayed in duplicate.

2.5. Western blotting

Cells were cultured for 24 h VPA untreatment or treatment and then washed twice with PBS. Cells were homogenized on ice in protein lysis buffer (ER4, Enzo Life Sciences, Farmingdale, NY). After incubation on ice for 15 min, the samples were centrifuged at 10,000 rpm for 10 min at 4 °C. Sample protein concentrations were determined by the BCA method (Pierce, Rockford, IL). The amount of total protein was adjusted to equal levels between samples with SDS sample buffer, and the samples (10 μg of protein) were subjected to electrophoresis on 10% SDS polyacrylamide gels. Proteins were transferred to a PVDF membrane (Millipore Corp., Billerica, MA) and treated with blocking buffer (5% skim milk in 0.1% Tween20 in TBS) for 1 h at room temperature. After blocking, the membrane was treated with monoclonal anti-SMN antibody (1:2000, BD) diluted in blocking buffer for 1 h at room temperature and then incubated with an HRP-conjugated anti-mouse IgG antibody (1:2000, DAKO) for 1 h at room temperature. The membrane was treated with a substrate (ECL plus substrate kit). To test for equal amounts of loaded protein, membranes were stripped and incubated with monoclonal anti-α-tubulin antibody (1:5000, Sigma) as described above. Proteins were visualized using a luminescent image analyzer ImageQuant LAS-1000 (Fuji Photo Film, Tokyo, Japan). All samples were assayed in duplicate.

2.6. ELISA

Cells were cultured for 24 h after VPA treatment, and then washed twice with PBS. Cells were homogenized in protein lysis buffer on ice as described above. Aliquots of protein extracts were diluted with lysis buffer. SMN ELISA kit (Enzo Life Sciences, Farmingdale, NY) was carried out according to the manufacturer's instructions. All samples were assayed in duplicate.

2.7. Immunostaining for the imaging flow cytometry

SMA patient-derived fibroblasts were rinsed twice with PBS and trypsinized. Cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min on ice. After they were washed three times with PBS, we permeabilized the cells using chilled BD Phosflow Parm buffer II for 30 min on ice. Cells were washed with Stain buffer (BD) and counted; 10 μl FITC-conjugated human SMN antibody (Millipore) or normal mouse Ig (Sigma) was added in 1×10^6 cells/90 μl and incubated at room temperature for 45–60 min. After incubation, and a single wash with PBS, the cells were treated with Hoechst 33342 (5 μg/mL) in PBS for 5 min at room temperature.

2.8. Imaging flow cytometry analysis

Samples were analyzed on a FlowSight imaging flow cytometer (Merck-Millipore, German). Data from a minimum of 10,000 cells (counts) were acquired, utilizing the 405, 488, and 785-nm lasers to calculate cell granularity, at a 20× magnification, using INSPIRE