Brain & Development 38 (2016) 738–745

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Staurosporine allows dystrophin expression by skipping of nonsense-encoding exon

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Received 5 December 2015; received in revised form 13 March 2016; accepted 17 March 2016

Abstract

Background: Antisense oligonucleotides that induce exon skipping have been nominated as the most plausible treatment method for dystrophin expression in dystrophin-deficient Duchenne muscular dystrophy. Considering this therapeutic efficiency, small chemical compounds that can enable exon skipping have been highly awaited. In our previous report, a small chemical kinase inhibitor, TG003, was shown to enhance dystrophin expression by enhancing exon skipping.

Purpose: Staurosporine (STS), a small chemical broad kinase inhibitor, was examined for enhanced skipping of a nonsense-encoding dystrophin exon.

Methods: STS was added to culture medium of HeLa cells transfected with minigenes expressing wild-type or mutated exon 31 with c.4303G > T (p.Glu1435X), and the resulting mRNAs were analyzed by RT-PCR amplification. Dystrophin mRNA and protein were analyzed in muscle cells treated with STS by RT-PCR and western blotting, respectively.

Results: STS did not alter splicing of the wild-type minigene. In the mutated minigene, STS increased the exon 31-skipped product. A combination of STS and TG003 did not significantly increase the exon 31-skipped product. STS enhanced skipping of exon 4 of the CDC-like kinase 1 gene, whereas TG003 suppressed it. Two STS analogs with selective kinase inhibitory activity did not enhance the mutated exon 31 skipping. When immortalized muscle cells with c.4303G > T in the dystrophin gene were treated with STS, skipping of the mutated exon 31 and dystrophin expression was enhanced.

Conclusions: STS, a broad kinase inhibitor, was shown to enhance skipping of the mutated exon 31 and dystrophin expression, but selective kinase inhibitors did not.

Keywords: Exon skipping; Chemical; Staurosporine; Nonsense mutation; Dystrophin

1. Introduction

Splicing is a process that removes introns from pre-mRNA during the joining of exons to form mature mRNA, and proceeds without error under the control

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http://dx.doi.org/10.1016/j.braindev.2016.03.003
0387-7604/

of a strict splicing regulatory system. Splicing regulatory elements, such as consensus sequences at splice donor and acceptor sites, and exonic splicing enhancer sequences, promote proper splicing. Recently, modulation of splicing has attracted considerable attention as a target for disease treatment strategies [1–3]. The most remarkable achievements have been obtained in the treatment of Duchenne muscular dystrophy (DMD; MIM#310200) [4–6].

DMD is the most common inherited myopathy, and is characterized by progressive muscle wasting, succumbing to cardiac or respiratory failure in one's twenties, and dystrophin deficiency caused by mutations in the dystrophin gene on the X-chromosome. Becker muscular dystrophy (BMD; MIM#310376) is an adult-onset slowly progressive muscle-wasting disease caused by mutations in the dystrophin gene. The current major therapeutic approach for DMD is to convert DMD to BMD phenotypes by inducing exon skipping during dystrophin pre-mRNA splicing using antisense oligonucleotides (AOs) [1,7–10]. By exon skipping, out-of-frame mutations are corrected in-frame, thereby restoring dystrophin expression. Recently, two clinical trials were conducted that employed different AOs to induce dystrophin exon 51 skipping [4,5]. The results of these trials convinced us to conduct a further extended trial [11].

Considering the therapeutic cost and convenience, small chemical compounds that can enable exon skipping have been highly awaited [12–14]. Regulation of dystrophin pre-mRNA splicing is considered extraordinarily complex, because the dystrophin gene contains seven alternative promoters, 79 exons, huge introns, and more than ten cryptic exons [15,16]. Therefore, it has been considered extremely difficult to modulate the splicing of dystrophin pre-mRNA with small chemical compounds. However, single nucleotide changes within exon sequences were reported to cause dystrophin pre-mRNA splicing errors [17–19]. These findings suggested the possibility that dystrophin pre-mRNA splicing could be modulated by small chemical compounds. Accordingly, we found that TG003, a small chemical kinase inhibitor, enhanced skipping of dystrophin exon 31 with a nonsense mutation (c.4303G > T) in a mutation-specific and dose-dependent manner, and enhanced dystrophin expression [14].

Staurosporine (STS) is a chemical isolated from *Streptomyces staurosporeus* [20] that exhibits strong inhibitory effects against a variety of kinases [21]. Owing to its high levels of cross-reactivity, STS has not been used as a therapeutic agent. Recently, alterations in splicing with STS have been identified in pre-mRNAs such as those for caspase-2, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Bcl-x [22,23]. Taking these findings into consideration, it was highly suspected that STS would modulate splicing of the mutated dystrophin exon 31.

In this study, we examined the activity of STS to modulate splicing of the mutated exon 31 of dystrophin using a minigene splicing system. As a result, STS was shown to enhance dystrophin expression in immortalized muscle cells by inducing exon 31 skipping. STS, a broad kinase inhibitor, was further shown to enhance skipping of the mutated exon 31 and dystrophin expression.

2. Materials and methods

2.1. Cells

HeLa cells were obtained from American Tissue Culture Collection (Manassas, VA) and cultured as described previously [14]. Immortalized muscle cells (DMD 4kDp) were established from biopsied muscle samples of a patient with the c.4303G > T mutation as described previously [24]. A stock of the cell line (passage 21) was prepared and stored in liquid nitrogen. Prior to use, the cells were thawed in a 37 °C water bath and cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 20% fetal bovine serum (Gibco by Life Technologies, New York, NY), 2% Ultroser™ G serum substitute (Pall Corp., New York, NY), and 1% antibiotic–antimycotic (Gibco by Life Technologies) at 37 °C in a 5% CO_2 humidified incubator for 1 day. The medium was then changed to Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, and test chemicals were added into the medium.

2.2. Minigene splicing analysis

Pre-constructed minigenes with insertion of wild-type exon 31 (H492-dys Ex31w) or mutated exon 31 with a single nucleotide change (c.4303G > T, p.Glu1435X) (H492-dys Ex31m) [14] were used in this study. The constructed minigenes were transfected into HeLa cells or immortalized muscle cells for splicing assays. Splicing of the minigenes was allowed to proceed for 24 h in the presence of a variety of chemicals. Total RNA from HeLa cells or immortalized muscle cells was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics, Basel, Switzerland). The extracted RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA), RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen Corp.), Random Hexamers (Invitrogen Corp.), and dNTP Mixture (Takara Bio Inc., Shiga, Japan) as described previously [14]. The synthesized cDNA was used as a template for PCR amplification as described previously [25]. The PCR products were separated and semiquantitatively measured according to the peak areas by high-resolution capillary electrophoresis with an Agilent 2100 Bioanalyzer and a DNA1000 LabChip Kit (Agilent Technologies, Menlo Park, CA). The