Cordycepin-mediated transcriptional regulation of human GD3 synthase (hST8Sia I) in human neuroblastoma SK-N-BE(2)-C cells

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In the present study, we firstly found that cordycepin elevated the gene expression of the human GD3 synthase (hST8Sia I) in human neuroblastoma SK-N-BE(2)-C cells. To elucidate the mechanism underlying the upregulation of hST8Sia I gene expression in cordycepin-treated SK-N-BE(2)-C cells, functional characterization of the promoter region of the hST8Sia I gene was performed. Analysis of promoter activity using varying lengths of 5'-flanking region showed a dramatic increase by cordycepin in the −1146 to −646 region, which contains putative binding sites for transcription factors c-Ets-1, CREB, AP-1, and NF-κB. Site-directed mutagenesis for these binding sites and chromatin immunoprecipitation assay revealed that the NF-κB binding site at −731 to −722 is essential for the cordycepin-induced expression of the hST8Sia I in SK-N-BE(2)-C cells. Moreover, the hST8Sia I expression induced by cordycepin was significantly repressed by pyrrolidinedithiocarbamate, an inhibitor of NF-κB. These results suggested that cordycepin induces upregulation of hST8Sia I gene expression through NF-κB activation in SK-N-BE(2)-C cells.

Keywords cordycepin; human GD3 synthase; SK-N-BE(2)-C cell; transcription factor

Received: July 25, 2013 Accepted: October 10, 2013

Introduction

Cordycepin (3'-deoxyadenosine) is the main component of the parasitic fungus Cordyceps militaris, an ingredient of traditional Chinese medicine and has been reported to have remarkable anti-cancer activity by several mechanisms including inhibition of cell proliferation [1,2], induction of apoptosis [3–5], and inhibition of cell migration and invasiveness [6,7]. Previous studies have also shown that cordycepin has various biological effects, such as anti-inflammation, inactive mRNA polyadenylation [8–10], and reinforcement of the immune system [11]. It has been reported that cordycepin inhibits 12-O-tetradecanoylphorbol-13-acetate-induced matrix metalloproteinase (MMP)-9 expression by suppressing AP-1 activation via mitogen-activated protein kinases signaling pathway in MCF-7 human breast cancer cells [12] and UVB-induced MMP expression by repressing the NF-κB pathway in human dermal fibroblasts [13]. It has also been reported that cordycepin suppresses tumor necrosis factor-α-induced invasion, migration, and MMP-9 expression by reducing the transcriptional activity of NF-κB and AP-1 in human bladder cancer cells [7].

Gangliosides, sialic acid-containing glycosphingolipids, mainly exist in the outer leaflets of vertebrate plasma membranes and play important roles in multiple biological processes, such as cell–cell interaction, adhesion, cell differentiation, growth control, oncogenic transformation, and receptor function [14,15]. Among the various gangliosides, GD3 is a structurally simple ganglioside that is markedly overexpressed in human melanoma and neuroblastoma cells, playing a key role in tumor progression [16,17]. GD3 is synthesized by GD3 synthase (ST8Sia I) known as CMP-NeuAc:GM3 α2,8-sialyltransferase and GD3 expression is generally regulated at the transcriptional level of ST8Sia I gene [17–20].

Although there have been a few reports on the effects of cordycepin on transcriptional regulation of MMP-9 gene in human cancer cells [12,13], the effect of cordycepin on the gene expression of human sialyltransferases responsible for ganglioside expression has not yet been studied.

Therefore, the present study was undertaken to investigate whether cordycepin regulates gene expression of human sialyltransferases related to ganglioside biosynthesis in human neuroblastoma cells. We have found for the first time that the mRNA expression of hST8Sia I was induced by cordycepin in human neuroblastoma SK-N-BE(2)-C cells. In this study,
furthermore, to investigate the molecular basis of hST8Sia I gene expression induced by cordycepin, the promoter region to direct upregulation of reporter gene transcription in response to cordycepin was functionally characterized.

Materials and Methods

Cell cultures
The human neuroblastoma cell line SK-N-BE(2)-C, obtained from American Type Culture Collection (Manassas, USA) was maintained at 37°C in a 5% CO2 incubator and cultured in Dulbecco’s modified Eagle’s medium (WelGENE Co., Daegu, Korea) containing 1 mM sodium pyruvate and 1× MEM non-essential amino acids, supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, USA). The human neuroblastoma cell line SK-N-BE(2)-C, obtained from American Type Culture Collection (Manassas, USA) was maintained at 37°C in a 5% CO2 incubator and cultured in Dulbecco’s modified Eagle’s medium (WelGENE Co., Daegu, Korea) containing 1 mM sodium pyruvate and 1× MEM non-essential amino acids, supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, USA). For the treatment of cordycepin (Sigma, St Louis, USA), cells were starved in serum-free medium for 12 h. The starved cells were induced by 150 µM cordycepin for various time periods.

Cell viability assay
Cell viability assay was performed as described previously [19–21]. The amount of formazan salt was determined by measuring the optical density at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, USA). Cell viability was quantified as a percentage compared with the control.

Reverse transcription-polymerase chain reaction
Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen; Carlsbad, USA) and first-strand cDNA was synthesized using RNA to EcoDry™ Premix (Oligo dT) kit (Clontech 639543; Clontech, Mountain View, USA). The synthesized cDNA was amplified by polymerase chain reaction (PCR) with specific hST8Sia I and β-actin primers, as described previously [18–21]. PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

Quantitative real-time PCR analysis
Total cellular RNAs and single-stranded cDNAs were prepared from cells as described above.

Real-time quantitative reverse transcription-PCR was performed using a CFX96™ real-time system with SYBR Premix (Bio-Rad). The primers were: hST8Sia I (D26360.1) sense, 5’-CAGAGCCATCTTGGAGGTTTA-3’; antisense, 5’-CTTTCCAATGCCTACCGAAAG-3’ and human β-actin (NM_001101.3) sense, 5’-ACCCACTCCTCCACC TTTGAC-3’; antisense, 5’-CCCTTGCTGCTAGCGAAATT CG-3’. The transcript copy number of the hST8Sia I gene was normalized to the β-actin transcript copy number for each sample. Relative quantitation was performed using CFX Manager v2.1 software (Bio-Rad). Real-time PCR amplification of the hST8Sia I and β-actin genes was carried out for 50 cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 15 s.

Transfection and luciferase assay
The luciferase reporter plasmids used herein, namely pGL3-2646/-646 and its derivatives (pGL3-1146/-646 to pGL3-2246/-646) with base substitutions at the CREB, AP-1, c-Ets-1, NF-κB binding sites, have been described elsewhere [18–21]. Transient transfection and luciferase assays were performed as previously described [20,21]. SK-N-BE(2)-C cells were transiently co-transfected with 0.5 µg of the indicated reporter plasmid and 50 ng of the control Renilla luciferase vector pRL-TK (Promega, Madison, USA), using 1 µl Lipofectamine 2000 (Invitrogen). After incubation for 3 h, transfection medium was replaced by normal medium without cordycepin and cultured for 18 h. Then, the medium was changed to serum-free medium and incubated for 12 h. After being cultured for an additional 6 h in serum-free medium containing 150 µM cordycepin, cells were collected and treated with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, and a GloMax™ 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to Renilla luciferase activity and expressed as a fold induction over the empty pGL3-Basic vector, used as a negative control. Independent triplicate experiments were performed for each plasmid.

Western blot analysis
Whole-cell pellets were solubilized with a RIPA buffer (Pierce, Rockford, USA) containing protease inhibitor mix (GE Healthcare, Piscataway, USA). Protein concentrations were measured using the Bradford assay. Aliquots of cellular proteins (20 µg per lane) were electrophoresed on 10% sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare). The membrane was blocked in 5% skim milk for 1 h and then incubated with specific antibody (H-76) for hST8Sia I (Santa Cruz Biotechnology, Santa Cruz, USA) for 12 h. The signals of bound antibody were visualized using the ECL chemiluminescence system (GE Healthcare) with horseradish peroxidase-complexed anti-rabbit and anti-mouse IgG antibody. Equal loading was confirmed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Milford, USA).

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChiP) assay was performed using the ChiP kit (Upstate Biotechnology, New York, USA) following the manufacturer’s protocol. Immunoprecipitation using 4 µg of NF-κB (Santa Cruz Biotechnology, New York, USA) was solubilized with a RIPA buffer (Pierce, Rockford, USA) containing protease inhibitor mix (GE Healthcare, Piscataway, USA). Protein concentrations were measured using the Bradford assay. Aliquots of cellular proteins (20 µg per lane) were electrophoresed on 10% sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare). The membrane was blocked in 5% skim milk for 1 h and then incubated with specific antibody (H-76) for hST8Sia I (Santa Cruz Biotechnology, Santa Cruz, USA) for 12 h. The signals of bound antibody were visualized using the ECL chemiluminescence system (GE Healthcare) with horseradish peroxidase-complexed anti-rabbit and anti-mouse IgG antibody. Equal loading was confirmed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Milford, USA).
Flow cytometry analysis
SK-N-(BE)2-C cells were detached with trypsin/ethylenediaminetetraacetic acid and washed twice with cold Dulbecco’s phosphate-buffered saline. Cells were incubated for 1 h at 4°C with a GD3 monoclonal antibody (mouse IgM, Kappa-chain, clone: GMR19; Seigakagu, Tokyo, Japan) diluted 1 : 100 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing with PBS, cells were incubated for 1 h at 4°C with secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG/M/A mix antibody (Sigma) diluted 1 : 100 in PBS containing 1% BSA. Staining without the primary anti-GD3 antibody served as a negative control. A total of 1 × 10⁶ labeled cells were analyzed using a Beckman-Coulter Cytomics FC500 flow cytometer and CXP software (Beckman-Coulter, Miami, USA).

Results

Effect of cordycepin on cell proliferation
Before the investigation into the regulatory effect of cordycepin on hST8Sia I expression, we first examined the cytotoxicity of cordycepin in SK-N-BE(2)-C cells using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. Relative cell viability was determined by the amount of MTT converted into formazan salt. SK-N-BE(2)-C cells were treated with cordycepin with various concentrations for 24 h. As shown in Fig. 1, cell viability at 150 µM was about 67%, but cordycepin at the concentration of more than 200 µM significantly inhibited the cell proliferation (<50%).

Figure 1. Effect of cordycepin on viability of SK-N-BE(2)-C cells

Effect of cordycepin on hST8Sia I expression in SK-N-BE(2)-C cells
Initial experiments were designed to determine whether cordycepin modulates the expression of human sialyltransferase genes responsible for the ganglioside biosynthesis in SK-N-BE(2)-C cells. After cells were treated with varying doses of cordycepin for varying periods of time, we investigated the specific expressions of sialyltransferase genes including ST3Gal II, ST3Gal V, ST8Sia I, and ST8Sia V by reverse transcription-PCR (RT-PCR) and did not find marked changes of gene expression patterns, except for hST8Sia I (data not shown). RT-PCR and quantitative real-time PCR results showed that mRNA levels of hST8Sia I were markedly increased at 150 µM cordycepin treatment and then continued in a dose-dependent manner (Fig. 2A,B). In addition, the induction of hST8Sia I mRNA was remarkably increased at 150 µM cordycepin treatment for 3 h and then continued for 24 h after cordycepin treatment (Fig. 2C,D). These results clearly showed that the expression of hST8Sia I was induced by cordycepin.

To investigate whether an increase of hST8Sia I mRNA levels by cordycepin treatment leads to induction of increased protein levels of hST8Sia I, we performed western blot analysis using the specific antibody for hST8Sia I. As shown in Fig. 2E, protein levels of hST8Sia I were markedly increased at 150 µM cordycepin treatment for 3 h and then continued for 24 h after cordycepin treatment. These results indicated that the expression of hST8Sia I is induced by cordycepin at both transcriptional and translational levels.

Effect of cordycepin on ganglioside GD3 expression in SK-N-BE(2)-C cells
To investigate whether the induction of hST8Sia I expression by cordycepin increases the cellular expression level of ganglioside GD3 synthesized by hST8Sia I, we performed ganglioside GD3 analysis by flow cytometry (fluorescence-activated cell sorting (FACS)) using GD3 monoclonal antibody and FITC-conjugated anti-mouse IgG/M/A mixture as secondary antibody. As shown in Fig. 3, FACS analysis clearly demonstrated the shift in GD3 binding, indicating that the levels of cellular GD3 were significantly increased in cordycepin-treated SK-N-BE(2)-C cells, compared with cordycepin-untreated cells.

Analysis of transcriptional activity of hST8Sia I promoter by cordycepin in SK-N-BE(2)-C cells
Because the levels of hST8Sia I mRNA were significantly increased in SK-N-BE(2)-C cells stimulated with cordycepin (Fig. 2A), the transcriptional activity of the hST8Sia I promoter using luciferase reporter gene assay system has been checked in order to analyze whether the transcriptional activity of hST8Sia I is regulated in cordycepin-induced conditions.
SK-N-BE(2)-C cells. As shown in Fig. 4A, cells harboring the pGL3-1146/-646 construct showed a marked increase in luciferase activity after cordycepin treatment, about 2.5-fold higher than untreated cells. On the contrary, cordycepin stimulation did not induce the significant increase of the luciferase activity in cells expressing other promoter constructs and the pGL3-basic (negative control). These results clearly suggested that the region containing nucleotides 21146 to 2646 functions as the cordycepin-inducible promoter of hST8Sia I in SK-N-BE(2)-C cells.

Identification of cordycepin-responsive element in nucleotide −1146 to −646 region of hST8Sia I promoter

Our previous studies have demonstrated that the region from −1146 to −646 contains putative binding sites for transcription factors such as c-Ets-1, AP-1, CREB, and NF-κB binding sites [18–21]. To determine whether these binding sites contributed to cordycepin-induced expression of hST8Sia I in SK-N-BE(2)-C cells, four mutants (pGL3-1146/-646mtCREB, mtAP-1, mtNF-κB, and mtc-Ets-1) were...
used, which contained exactly the same construct as wild-type pGL3-1146/-646 except that the combined nucleotides within these binding sites had been changed [18–21]. Luciferase constructs with substituted mutations (Fig. 4B) were transfected into SK-N-BE(2)-C cells and luciferase assays were performed. The luciferase activity of each construct was

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**Figure 4. Analysis of hST8Sia I promoter activity in SK-N-BE(2)-C cells stimulated by cordycepin**

The schematic diagrams represent DNA constructs (A) containing various lengths of the wild-type hST8Sia I promoter, or constructs (B) with mutants c-Ets-1, AP-1, CREB, and NF-κB sequences in the 5'-flanking region, upstream of a luciferase reporter gene; the transcription start site is designated +1. The pGL3-basic construct, which did not contain a promoter or an enhancer, was used as a negative control. Each construct was transfected into SK-N-BE(2)-C cells, with pRL-TK co-transfected as an internal control. The transfected cells were incubated in the presence (solid bar) or absence (open bar) of 150 μM cordycepin for 12 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the Renilla luciferase activity derived from pRL-TK. The values were represented as the mean ± SD of three independent experiments with triplicate measurements. (C) PCR amplification in the −1146 and −646 region of the hST8Sia I promoter on immunoprecipitated chromatin obtained from SK-N-BE(2)-C cells treated with or without cordycepin. The input (10-fold diluted) represents the positive control. (D) Cells were incubated with 20 μM of NF-κB inhibitor, PDTC, for 1 h, and then co-incubated with 150 μM cordycepin for 24 h. hST8Sia I mRNA was detected by RT-PCR. β-Actin was used as an internal control.
Transcriptional activation of hST8Sia I gene by cordycepin

compared with that of pGL3-basic or wild-type (pGL3–1146/-646) used as negative or positive control, respectively. In cordycepin-treated cells, pGL3–1146/-646mtNF-κB of four constructed mutations remarkably decreased transcriptional activity to more than 3 fold of pGL3–1146/-646wt, whereas the activities of the pGL3–1146/-646mtCREB, mtAP-1, and mtc-Ets-1 constructs were not reduced (Fig. 4B). These results indicated that this NF-κB site is indispensable for the cordycepin-induced expression of hST8Sia I.

Based on these results, we performed ChiP assay to confirm the binding of NF-κB to this site of hST8Sia I promoter in SK-N-BE(2)-C cells. An amplification of the hST8Sia I promoter regions was obtained in the presence of NF-κB specific antibody and IgG. As shown in Fig. 4C, only NF-κB has the specific amplification and DNA–protein complex was observed in SK-N-BE(2)-C cells untreated with cordycepin to regulate the expression of hST8Sia I gene. There was no detectable binding in a control assay with cordycepin non-treatment or IgG. Moreover, the cordycepin-induced expression of hST8Sia I was significantly repressed by pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB (Fig. 4D). These results indicated that hST8Sia I gene expression was modulated by the interaction between NF-κB and NF-κB elements at nucleotide positions −731 and −722.

Discussion

In previous studies, we have shown that valproic acid, a simple branched-chain fatty acid, induced transcriptional activation of hST8Sia I in SK-N-BE(2)-C [20], whereas triptolide, a diterpenoid triepoxide, downregulated hST8Sia I gene expression in SK-MEL-2 human melanoma cells [21]. In the present study, we also demonstrated for the first time that cordycepin upregulated hST8Sia I expression in human neuroblastoma cells. Moreover, cordycepin elicited a significant dose-dependent increase of hST8Sia I mRNA level: we detected a markedly induced hST8Sia I mRNA signal at 150 μM treatment of cordycepin, and the signal increased up to the 250 μM. In addition, as evidenced by western blot analysis, protein levels of hST8Sia I was also increased after 3 h of stimulation with 150 μM cordycepin and then continued for 24 h. This increase was coincident with the time-dependent increase in hST8Sia I mRNA level, suggesting that cordycepin-induced upregulation of hST8Sia I at both transcriptional and translational levels. As a downstream consequence of this cordycepin treatment, the ganglioside GD3 levels in SK-N-BE(2)-C cells were remarkably increased, as demonstrated by FACS analysis using GD3 monoclonal antibody, and our results suggested that this GD3 induction occurred in a close temporal relation to the cordycepin-induced increase of hST8Sia I gene expression. We also identified that the promoter region of the hST8Sia I gene contains cordycepin-responsive element(s), which supported the idea that the induction of hST8Sia I gene expression would lead to an active hST8Sia I production directing GD3 formation eventually in response to cordycepin.

In this study, we also clarified a part of the transcriptional regulation mechanism that underlies the induction of hST8Sia I gene expression in response to cordycepin. In order to investigate cordycepin-responsive elements involved in the enhanced expression of the hST8Sia I gene in SK-N-BE(2)-C cells, we firstly tried to identify the region within the hST8Sia I promoter that was crucial for cordycepin-induced gene expression. We isolated the region between −1146 and −646 as the core promoter for transcriptional activation of hST8Sia I in cordycepin-induced SK-N-BE(2)-C cells. Our previous studies showed four kinds of transcription factor binding sites (c-Ets-1, AP-1, CREB, and NF-κB) in this region [18–21]. We have demonstrated that only the NF-κB binding site at positions −731 to −722 in this region contributes to hST8Sia I promoter activity in Fas-induced Jurkat T cells [18], human melanoma SK-MEL-2 cells [19], triptolide-induced SK-MEL-2 cells [20], and valproic acid-stimulated SK-N-BE(2)-C cells [21]. In agreement with these findings, our present site-directed mutagenesis and ChiP analysis demonstrated that binding to this NF-κB element mediated cordycepin-dependent upregulation of hST8Sia I gene expression. And NF-κB is a crucial transcription factor that controls the expression of numerous genes involved in immune and inflammatory responses, proliferation, apoptosis, and oncogenesis [22,23].

Although cordycepin inhibits MMP expression by repressing the NF-κB pathway in human dermal fibroblasts [13] and human bladder cancer cells [7], NF-κB-mediated gene expression by cordycepin stimulation in human neuroblastoma cells have been not reported. Therefore, it is important to elucidate which signaling pathways in response to cordycepin are upstream of this NF-κB-mediated expression of the hST8Sia I gene. Further study is required to clarify the precise mechanisms involved in the cordycepin-mediated activation of NF-κB leading to a transcriptional upregulation of hST8Sia I gene SK-N-BE(2)-C cells.

Funding

This work was supported by a grant from the R&D Program of MOTIE/KIAT (Establishment of Infra Structure for Anti-aging Industry Support).

References


