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Role of down-regulated neutral ceramidase during all-*trans* retinoic acid-induced neuronal differentiation in SH-SY5Y neuroblastoma cells

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Neutral ceramidase (NCDase) is considered to be a critical enzyme for controlling the turnover of ceramide, an important bioactive lipid, which determines cell's fate. All-*trans* retinoic acid (ATRA) has been reported to induce neuronal differentiation and cell-cycle arrest [Lopez-Carballo, Moreno, Masia, Perez, and Barettino (Activation of the phosphatidylinositol 3-kinase/Akt signalling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. *J Biol Chem* 2002:277: 25297–304.)]. In this study, we observed that ATRA-induced cellular ceramide accumulation, cell-growth arrest and differentiation accompanied with down-regulation of NCDase in SH-SY5Y cells, without a decrease in sphingosine or sphingosine 1-phosphate. We examined whether the down-regulation of NCDase was involved in the increase in ceramide and cell differentiation. ATRA was found to down-regulate mRNA, protein and the enzyme activity of NCDase. Interestingly, GATA-2 was also decreased with ATRA treatment, and experiments using its expression vector and siRNA and chromatin immunoprecipitation assay demonstrated GATA-2 acted as transcription-factor of NCDase gene expression. By establishing stable transfectants with decreased NCDase expression and activity, we clarified the significance of NCDase down-regulation for ATRA-induced neuronal differentiation. Those sub-clones showed both increased cellular ceramide and reduced cell growth as well as neuronal differentiation phenotypes. These results demonstrate that down-regulation of NCDase through ATRA-induced GATA-2 decrease plays an important role in induction of ceramide accumulation and neuronal differentiation in SH-SY5Y cells.

Keywords: Differentiation/Vitamin A/Regulation/Transcription/Sphingolipids.

Abbreviations: ACDase, acid ceramidase; ASMase, acid sphingomyelinase; ATRA, all-*trans* retinoic acid; CerS, ceramide synthase; ChIP, chromatin immunoprecipitation; d18:1-C16:0, *N*-palmitoyl-D-erythro-sphingosine; d18:1-C17:0, *N*-heptadecanoyl-D-erythro-sphingosine; d18:1-C18:0, *N*-stearoyl-D-erythro-sphingosine; d18:1-C22:0, *N*-behenoyl-D-erythro-sphingosine; d18:1-C24:0, *N*-lignoceroyl-D-erythro-sphingosine; d18:1-C24:1, *N*-nervonoyl-D-erythro-sphingosine; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCS, glucosylceramide synthase; HPTLC, high performance thin layer chromatography; LC–ESI–MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; NCDase, neutral ceramidase; NSMase, neutral sphingomyelinase; RT–PCR, reverse transcription polymerase chain reaction; S1P, sphingosine 1-phosphate; SCR, scramble; shRNA, small hairpin RNA; siRNA, small interfering RNA; SPT, serine palmitoyltransferase.

Ceramide is a bioactive lipid located in the hub of sphingolipid metabolism and plays an important role in intracellular signalling for regulation of apoptosis, cell senescence and cell differentiation (*1*, *2*). It is mainly produced via three major pathways (i) the *de novo* synthetic pathway, (ii) hydrolysis of sphingomyelin with sphingomyelinases (SMases) and (iii) the salvage pathway from sphingosine (*2*). Several stimuli, such as ultraviolet and ionizing radiation, ligation of death receptors, cytokines and chemotherapeutic agents, have been shown to activate those pathways, resulting in an increase of intracellular content of ceramide (*1*, *2*).

Voigt *et al*. (*3*) reported the effects of retinoic acid on proliferation, apoptosis, cytotoxicity, migration and invasion of various neuroblastoma cell lines and showed that the effect of retinoic acid on various markers is cell-type dependent. SH-SY5Y cells used in the current study are from a sub-clone of the SK-N-SH cell line, which shows a neuron-like phenotype with development of neurites induced by ATRA (*4*). An ATRA-induced increase of cellular ceramide followed by cessation of cell proliferation and induction of cell differentiation has been reported to be due to up-regulation of acid sphingomyelinase (ASMase) or neutral sphingomyelinase 2 (NSMase2) (*5*–*7*). However, the involvement of other sphingolipid metabolic enzymes remains to be determined.

Ceramidase (CDase), which hydrolyses ceramide to sphingosine and fatty acid, is one of the key enzymes regulating intracellular ceramide content, and has been classified into three groups, lysosomal acid (*8*, *9*), neutral (*10*–*13*) and alkaline (*14*–*16*). In contrast to lysosomal acidic CDase, the biological significance of neutral and alkaline CDase remains to be fully disclosed.

In the present study, we found that the increase of intracellular ceramide content in ATRA-treated SH-SY5Y cells is dependent on down-regulation of neutral ceramidase (NCDase) through a decrease of GATA-2 by ATRA. The direct involvement of GATA-2 as transcription-factor in NCDase transcription was demonstrated by chromatin immunoprecipitation (ChIP) assay. Furthermore, decreased NCDase expression and activity are responsible, at least in part, for ATRA-induced neuronal differentiation of SH-SY5Y cells.

Materials and Methods

Materials

ATRA, puromycin and polybrene were purchased from Sigma-Aldrich (St. Louis, MO). *N*-palmitoyl-D-*erythro*-sphingosine (d18:1-C16:0), *N*-heptadecanoyl-D-*erythro*-sphingosine (d18:1-C17:0), *N*-stearoyl-D-*erythro*-sphingosine (d18:1-C18:0), *N*-behenoyl-D-*erythro*-sphingosine (d18:1-C22:0), *N*-lignoceroyl-D-*erythro*-sphingosine (d18:1-C24:0), *N*-nervonoyl-D-*erythro*-sphingosine (d18:1-C24:1), palmitoyl–CoA (C16:0-CoA), lignoceroyl–CoA (C24:0-CoA), nervonoyl-CoA (C24:1-CoA) and D-*erythro*-sphingosine (C17 base) (d17:1) were purchased from Avanti Polar Lipid (Albaster, AL). Anti-AP2α (C-18), anti-GATA2 (H-116), anti-GATA3 (HG3-31), anti-GATA4 (G-4), anti-β-actin (H-196) and rabbit normal IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while anti-MAP2 (4542) antibody were from Cell Signalling Technology (Beverly, MA, USA), and anti-NCDase (PRS4743) antibody from Sigma-Aldrich.

Cell proliferation and quantification of neurite outgrowth

A human neuroblastoma cell line, SH-SY5Y (American Type Culture Collection Rockville, MD), was cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% foetal bovine serum (FBS) with or without ATRA (10 μM). Cell proliferation was determined using a MTS assay according to the manufacture's protocol (Promega, Madison, WI, USA). Cells with neurites >50 μm were counted as neurite positive, as previously described (*17*).

Quantification of ceramide, sphingosine and sphingosine 1-phosphate using LC–ESI–MS/MS

For quantification of intracellular ceramide, SH-SY5Y cells were treated with or without 10 μM of ATRA for 24 h in medium containing 10% FBS. To measure intracellular and extracellular sphingosine and sphingosine 1-phosphate, the culture medium was changed to serum-free medium without ATRA after 8 h of incubation with or without ATRA treatment in FBS-containing medium, in order to reduce the contributions of sphingosine and S1P in FBS, after which incubation was continued for an additional 16 h. We chose this culture method because simultaneous ATRA addition to serum-free medium induces apoptosis of cultured cells. The method of sample preparation for ceramide quantification was performed as previously described (*18*). Briefly, lipids were extracted from SH-SY5Y cells using the Bligh and Dyer procedure with d18:1-C17:0 as an internal standard. The methods of sample preparation of sphingosine and sphingosine 1-phosphate (S1P) for quantification were performed as described previously, with d17:1 utilized as an internal standard (*19*). To measure the lipid contents, LC–ESI–MS/MS analysis was performed using a 320 LC–MSMS triple quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) (*18*, *20*).

RT–PCR

RNA was isolated using a RNeasy Mini kit (QIAGEN, Hidden, Germany), and first strand cDNA was prepared with 5 μg of RNA using a Super Script III First-Strand system (Invitrogen). Semi-quantitative RT–PCR (sqRT–PCR) was performed with Ampli Taq Gold 360 Master Mix (Applied Biosystems, Foster City, CA). The primers used are shown in Table 1. Quantitative RT–PCR (qRT–PCR) was performed in triplicate with TaqMan Gene Expression Assays (Applied Biosystems) for *NCDase* (Hs00184096_m1) and *GAPDH* (Hs99999905_m1).

Western blotting

Western blotting was performed using the antibodies described above, as described previously (*18*). Nuclear and cytosol fractions for western blotting were prepared using a CelLyticTM NuCLEAR^{TM} Extraction kit (Sigma-Aldrich) according to the manufacturer's instructions.

Transient over-expression and knockdown of GATA2

An expression vector of pcDNA3.1 GATA2 (*21*) was transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen). GATA2 (5′-CCUACACACAAGGUUGGAGTT-3′ and 5′-CUCC AACCUUGUGUGUAGGTT-3′) and scramble (SCR) siRNAs were obtained from Applied Biosystems, then transfected into SH-SY5Y cells using Lipofectamine RNAiMAX (Invitrogen). Effective over-expression or knockdown of GATA2 was confirmed by western blotting at 48 h after transfection.

ChIP assays

ChIP assays were performed as previously described (*17*). SH-SY5Y cells with or without ATRA treatment were cross-linked with formaldehyde. For immunoprecipitation, normal IgG and anti-GATA2 (H-116) antibody (2 μg each) were added to the lysate, and incubated at 4°C overnight with constant rotation. Immune complexes were extracted, then cross-linking was reversed by heating the elute at 65°C overnight. DNA was purified using a High Pure PCR Cleanup micro Kit (Roche, Mannheim, Germany). The NCDase promoter region was amplified by PCR using the following primer set: forward, 5′-GGTCTATGGAATGAGTGGTAAGTGC-3′; reverse, 5′-CAGGTACAGCAGAGATGGAAGAAG-3′.

Stable knockdown of NCDase

Lenti viral pLKO.1 plasmids containing shRNA sequences for NCDase (RHS4533-NM019893) and scrambled shRNA (RHS3979-97052336) were purchased from Open Biosystems (Huntsville, AL, USA). The packaging plasmid psPAX2 and envelope protein plasmid pMD2.G were obtained from Addgene (Cambridge, MA, USA). HEK293FT cells (Invitrogen) were grown in DMEM supplemented with 10% FBS. Cells at 80–90% confluence were used to produce a lentivirus. After replacing the medium, psPAX2 and pMD2.G along with either SCR or NCDase-pLKO1 were transfected into the cells using Lipofectamine 2000. Medium was changed after overnight incubation with DMEM containing 10% FBS. Culture media were collected at 36 and 60 h after transfection with the virus. Viral particles present in the supernatant were filtered using a 0. 45 -μm syringe filter to remove cellular

Table I. Primer sequence for semi-quantitative RT–PCR.

Gene	Forward	Reverse
SPT1	TTACAGGCATCCCGTAGTGA	AACCTCCAATAGAAGCAAGTG
SPT2	GGAGGGATCTATTGTTCGTC	GAGCATCAAAGGCACTACTG
SPT3	TGCAGCCAAGTATGATGA	GCAGATGCACGATGGAAC
CerS1	ACAATGTGGGCATCCTTGTG	CTCAGTGGCTTCTCGGCTTT
CerS2	CATCGTCTTCGCCATTGTTT	TTGTTATTGAGGATGGGGTG
CerS3	TTTAGGAGTCGGCGGAATCAAG	GGTGTTACAGGTCTGCGTCCAT
CerS4	TCGGTCCTGTACCACGAGTCA	GCCCGTTGAAGAAGTAGTAGCC
CerS5	ATTTATTGCCAAACCCTGTGC	GAACCAAGGTGACGACCAGAG
CerS6	GGCTCCCGCACAATGTCACC	AGCAATGCCTCGTATTCCAC
ASMase	AAGCCCTGCGCACCCTCAGAA	CCTGAAGCTCCCCCACCAGCC
NSMase	ACTTTGATAACTGCTCCTCTGAC	TTCGTGTCCAGCAGAGTACC
ACDase	GATATTGGCCCCAGCCTACTTT	ACCCTGCTTAGCATCGAGTTCA
NCDase	TCCTGGGAAAGCCATCTGAA	TGCGTGCGGTCCATAAAT
GCS	CAAGCTCCCAGGTGTCTCTCTTC	GATTAATGCCAACTTTTTTACCAC
GAPDH	AAGGTCATCCATGACAAC	CACCCTGTTGCTGTAGCCA

debris. SH-SY5Y cells at 60% confluence were infected overnight with 4 ml of viral supernatant and polybrene (6 μg/ml) in DMEM containing 10% FBS. Medium was changed to fresh DMEM containing 10% FBS and puromycin (2 μg/ml) the next day. Cells that survived for >1 week in the presence of puromycin were used as bulk clones. Five different shRNAs against NCDase were tried for transfction. After examining the suppression of NCDase mRNA in each clone (Fig. 5A), suitable bulk clones 1 and 5 were used for further studies. They were described thereafter just as clone 1 and 5 in the text, respectively.

Sphingolipid metabolic enzyme activity

CDase activities were determined as described previously, with minor modifications (*10*, *13*). Acetate buffer (100 mM; pH 5.0) with 0.15% sodium cholate and 0.01% Triton X-100 for acid CDase (ACDase), 100 mM Tris–HCl (pH 7.5) with 0.2% taurodeoxycholic acid for NCDase, and 50 mM Tris–HCl (pH 8.0) with 5 mM $CaCl_2$ for alkaline CDase were used as buffer solutions. CDase activities were analysed using 50 μg of protein from the cell homogenate as an enzyme source. Enzyme reactions were carried out at 37°C in 50 μl of each buffer containing the enzyme source and 36 μM ceramide composed of *N*-[oleoyl-9,10-^{3}H]-D-sphingosine (18.5 μCi/mmol) (GE Healthcare) which was adjusted to 0.2 μCi with cold *N*-oleoyl-D-sphingosine (Avanti Polar Lipid). After 30 min, the reaction was terminated by adding 50 μl of 1 N H_2SO_4 and 200 μl of $CHCl_3$:MeOH (2:1). ^{3}H-labelled lipids recovered from the organic phase were separated by HPTLC and radioactivity from the band of ^{3}H-labelled oleic acid was measured using a BAS 2000 image analyser. The activities of SPT (*22*), CerS (*23*), SMase (*6*) and GCS (*24*) enzymes were determined as described previously, with minor modifications (see Supplementary Methods section).

Statistical analysis

Measurements were repeated three times and the results are expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test.

Results

Ceramide accumulation in SH-SY5Y cells with ATRA treatment

SH-SY5Y cells were treated with ATRA (final concentration: 10 μM) or the vehicle for 24 h. The amounts and species of ceramide were analysed by LC–ESI–MS/MS as described previously (*18*). The levels of d18:1-C16:0, d18:1-C18:0, d18:1-C24:1 and d18:1-C24:0 in ATRA-treated SH-SY5Y cells were increased by 1.9-, 1.8-, 4.2- and 1.7-fold, respectively, as compared to the control cells (Fig. 1A). In contrast, d18:1-C22:0 did not show any significant difference by ATRA treatment. Cellular sphingosine and S1P levels in SH-SY5Y cells treated with ATRA or the vehicle were also measured by LC–ESI–MS/MS. ATRA slightly decreased cellular sphingosine and S1P levels, though the differences were not significant (Fig. 1B and C). We also attempted to measure secreted S1P, however, that in culture medium could not be detected, probably due to the limit of our LC–ESI–MS/MS assay or negligible amounts (data not shown).

Decreased NCDase expression through down-regulation of GATA2 with ATRA treatment

We examined the effects of ATRA on SPT, CerS and GCS expression levels, which are considered to be key enzymes for regulation of intracellular ceramide content. Neither the activities of those enzymes nor mRNA expression were changed with ATRA treatment (Fig. 2A and B). It was previously reported that ATRA-induced ceramide accumulation via activation of SMase in NB4 cells (*6*), thus, we also determined the mRNA expression and activity of SMase in SH-SY5Y cells. However, no significant changes in ASMase and NSMase were observed after ATRA treatment (Fig. 2A and B). Finally, we found that ATRA decreased the mRNA expression, protein and activity of NCDase (Fig. 2A–C). In contrast, ACDase activity and mRNA level were not changed by ATRA, and the activity of alkaline CDase was not detected in SH-SY5Y cells (data not shown).

Recent analyses have revealed an important region for endogenous NCDase transcription including AP-1-, NF-Y-, AP-2-, Oct-1- and GATA-binding motifs (*25*, *26*). Several transcription factors have been suggested to be involved in NCDase transcription (*26*, *27*). However, the transcriptional mechanism of ATRA-induced suppression of NCDase has not been reported. Thus, we searched for several putative transcription factors responsible for the ATRA-induced transcription of NCDase. The protein levels of the candidates, AP-2α, GATA-2, GATA-3 and GATA-4 were examined (Fig. 3A). ATRA treatment decreased GATA-2 expression as compared with untreated

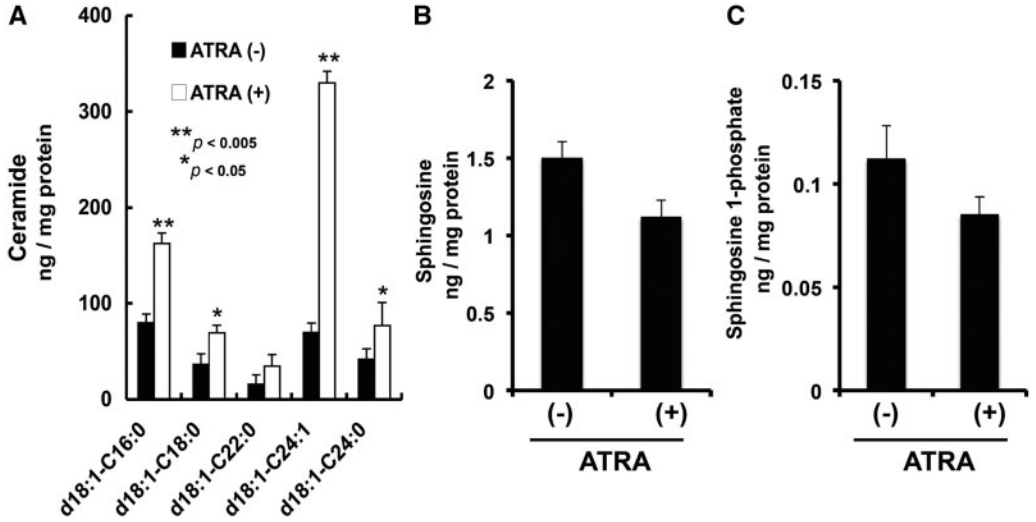

Fig. 1 Ceramide, sphingosine and S1P levels analysed by LC–ESI–MS/MS. (A) The amount of each ceramide species was analysed using LC–ESI–MS/MS at 24 h after addition of 10 μM ATRA as described in 'Materials and Methods' section. Solid column: untreated control cells. Open column: cells treated with ATRA for 24 h. Values are shown as the mean ± SD. (B) The amount of cellular sphingosine was analysed by LC–ESI–MS/MS after culturing cells, as described in 'Materials and Methods' section. Values are shown as the mean ± SD from three different experiments. (**C**) The amount of cellular S1P was analysed by LC–ESI–MS/MS, as described in 'Materials and Methods' section. The culture condition was the same as that for sphingosine measurement. Values are shown as the mean ± SD from three different experiments.

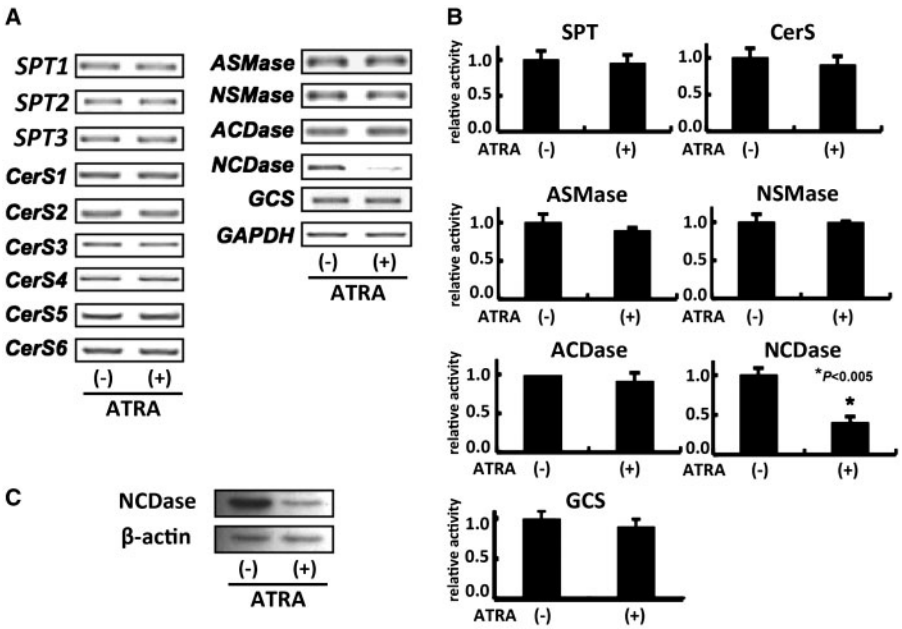

Fig. 2 Effects of ATRA on NCDase mRNA, protein and enzyme activity. (A) mRNA levels of various sphingolipid metabolic enzymes were examined using semi-quantitative RT–PCR at 12 h after addition of 10 μM ATRA. (B) Enzyme activities at 24 h after addition of 10 μM ATRA were determined as described in 'Materials and Methods' and Supplementary Methods section. CerS activity was determined using C24:1-CoA as a substrate and is presented as representative of CerS activity. Similar results were observed using C16:0-CoA and C24:0-CoA (data not shown). (**C**) NCDase protein levels were analysed by western blotting, with β-Actin used as the internal control.

control cells in the nuclear fraction and did not affect the level and localization of other GATA factors (Fig. 3A).

ATRA significantly inhibited the expression of luciferase promoter vectors including the control vector with our experimental conditions (data not shown), thus we could not directly identify the 5′-region responsible for ATRA-induced inhibition. To confirm the direct involvement of GATA-2 in the regulation of NCDase transcription, the effects of transient over-expression by pcDNA3.1-GATA2 and NCDase knockdown by siRNA-GATA2 were examined 48 h after transfection (Fig. 3B). The level of NCDase mRNA was significantly increased by 1.6-fold in GATA2 over-expressed cells (Fig. 3C, left), while that expression was decreased significantly by 0.5-fold in GATA2-silenced cells, (Fig. 3C, right).

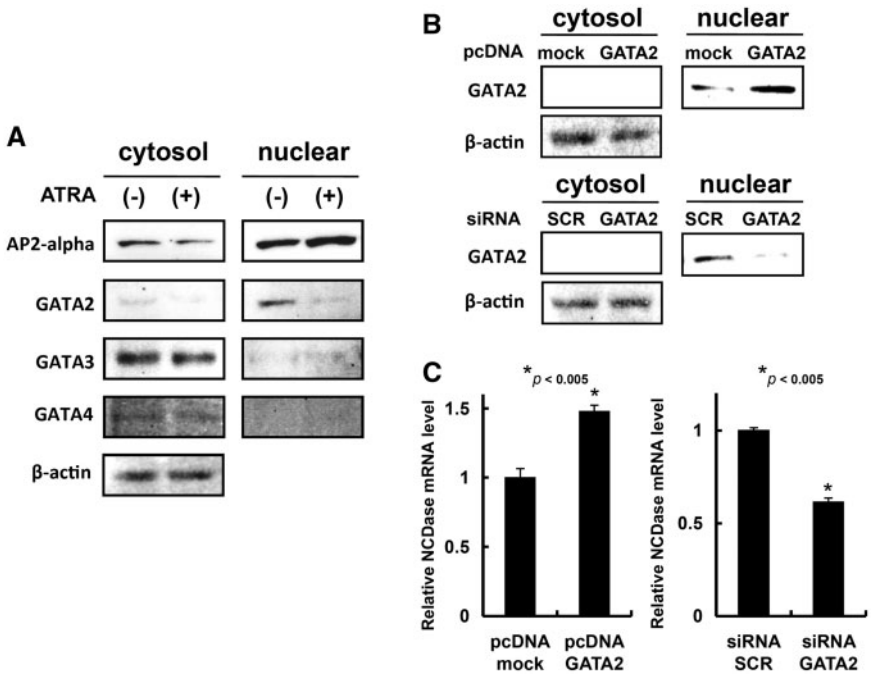

Fig. 3 ATRA-induced down-regulation of NCDase *via* GATA-2 suppression. (A) The protein levels of AP-2α, GATA-2, GATA-3 and GATA-4 were determined by western blotting 24 h after the treatment of 10 μM ATRA. Cytosol and nuclear fraction were separately examined. (B) Transient over-expression (upper) and knockdown of GATA2 (lower) were performed as described in 'Materials and Methods' section. GATA-2 protein level was analysed in both cytosol and nuclear fractions at 48 h after each transfection. (**C**) The mRNA level of NCDase was examined using quantitative RT–PCR 48 h after transfection. The left side shows results of GATA-2 over-expression experiment, while the right shows those of siRNA experiment. Values are shown as the mean ± SD.

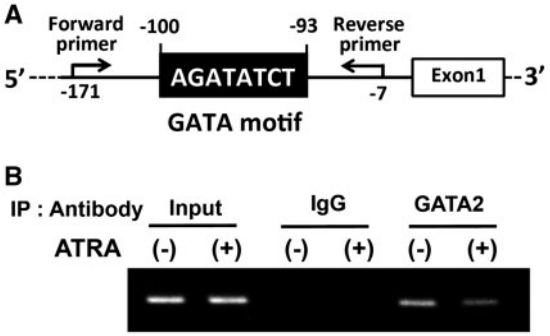

Fig. 4 Direct binding of GATA-2 to the human NCDase promoter region. (A) The GATA-binding motif is located between −100 and −93 bp from the first exon of NCDase. (B) ChIP assays were performed using SH-SY5Y cells with or without ATRA treatment for 24 h, as described in 'Materials and Methods' section. Normal IgG and the anti-GATA2 antibody were used for immunoprecipitation. The expected PCR product was 164 bp, which covered the GATA-binding motif illustrated in Fig. 4A.

ChIP assays

We performed ChIP assays to examine the direct effect of GATA-2 on NCDase expression. Since the 5′-promoter of NCDase contains a single GATA site located between −100 and −93 bp from the first exon (Fig. 4A), the region containing this GATA site was amplified after immunoprecipitation with the anti-GATA2 antibody. Figure 4B shows a PCR band observed in an anti-GATA-2 antibody-immunoprecipitated sample that was significantly reduced by ATRA, while but no such band was seen in the control IgG sample. These results indicate that GATA-2 functions as a transcription factor with the *NCDase* promoter region, while ATRA decreases that function by reducing the level of nuclear GATA-2 (Fig. 3A).

Increases in intracellular ceramide content, growth arrest and cell differentiation caused by stable knockdown of NCDase

Several studies have reported that ATRA modulates the expression of several differentiation markers in various cell lines (*28*, *29*). To evaluate the relationship between ceramide increase and neuronal differentiation, five different shRNAs against NCDase were used for transfection and cell selection was performed with puromycin using lentivirus shRNA system. By the qRT–PCR, it was confirmed that NCDase mRNA levels of sub-clones 1 and 5 were suppressed ∼80% by shRNA, thus they were used in further studies (Fig. 5A). NCDase activity of sub-clones 1 and 5 was decreased to ∼0.4-fold (Fig. 5B) and sub-clones 1 and 5 exhibited reduced NCDase expression as compared to the SCR shRNA-transfected cells (Fig. 5C). In addition, the intracellular ceramide contents of both clones were higher, as the level of d18:1-C16:0, d18:1-C18:0, d18:1-C22:0, d18:1-C24:1 and d18:1-C24:0 were increased by 3.0-, 3.0-, 2.8-, 3.3- and 2.5-fold, respectively, as compared to the SCR-shRNA clone. In contrast, d18:1-C20:0 was not detected in either clone (Fig. 5D).

We also investigated changes in cell morphology of SH-SY5Y sub-clone 1 and 5, which showed increased

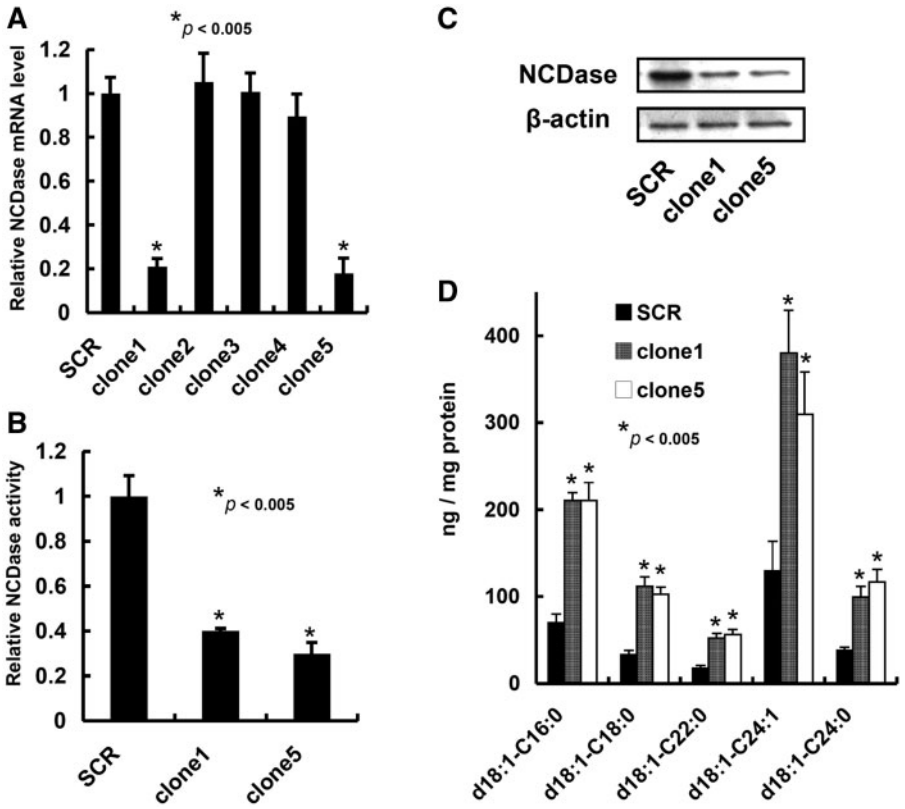

Fig. 5 Ceramide increase by stable knockdown of NCDase. (A) SH-SY5Y cells with stably reduced NCDase expression (clones 1 and 5) were selected in the presence of puromycin (2 μg/ml), then their NCDase mRNA levels. The mean ± SD was illustrated. (B) NCDase enzyme activities of clones 1 and 5 were examined. The mean ± SD was shown. (**C**) NCDase protein levels in clones 1, 5 as well as that of scrambled shRNA (SCR) treated cells were shown. (D) The amounts and species of ceramides in NCDase knockdown cells (clones 1 and 5) and control cells (SCR) were determined by LC–ESI–MS/MS, as described in 'Materials and Methods' section. Values are shown as the mean ± SD.

intracellular ceramide content by NCDase shRNA. The ratio of neurite positive cells was determined and used as an index of cell differentiation. In cells with stably inhibited NCDase, neurite positive cells were increased to 46.2% in clone 1 and 48.8% in clone 5 as compared to 9.0% in the scramble control cells (SCR) (Fig. 6A and B).

In order to confirm that SH-SY5Y cells were differentiated by the expression of shRNA against NCDase, the amount of microtubule associated protein 2 (MAP2) (*30*), a biomarker for differentiated neuronal cells, was examined by western blotting and found to be increased in NCDase-suppressed clone 5 cells (Fig. 6C). Interestingly, the cell growth of clone 5 was suppressed by ~50% as compared to the SCR group (Fig. 6D) with a similar result observed for clone 1 (data not shown).

Discussion

Recent studies have revealed that ceramide and its metabolizing enzymes are involved in several important cellular events (*1*). Thus, the mechanism regulating intracellular ceramide levels has been extensively studied. The amount of intracellular ceramides is regulated at a constant level by a network of multiple sphingolipid enzymes. Various stimuli tend to increase ceramide level through their activation (*31–35*), among which the sphingolipid metabolic enzymes, SMase, SPT, CerS and GCS are considered to be particularly important to control intracellular ceramide levels.

In the present study, we found that NCDase plays an important role as a modulator of intracellular ceramide level in ATRA-treated SH-SY5Y cells, because NCDase mRNA, protein and enzyme activity in ATRA-treated SH-SY5Y cells were remarkably reduced (Fig. 2). In mammals, NCDase is present in the plasma membrane as a type II integral membrane protein, with its catalytic domain facing the extracellular space (*36*). Ceramide present on the cell surface is hydrolysed by NCDase to produce sphingosine, which is an important material to produce sphingosine-1-phosphate. Mouse NCDase is ubiquitously expressed in several organs, though a relatively high expression has only been confirmed in the small intestine, kidney, liver and lung (*37*). In contrast to lysosomal acidic CDase, the physiological significance of NCDase has not been fully recognized (*38*).

Ceramide-metabolic enzymes other than NCDase did not show significant changes following ATRA treatment of SH-SY5Y cells (Fig. 2). It is unlikely that enzymes other than NCDase play a major role in ATRA-induced ceramide accumulation. However, down-regulation of ceramide kinase by ATRA has recently been reported (*29*) and this change may play a role in the neuronal differentiation in SH-SY5Y cells

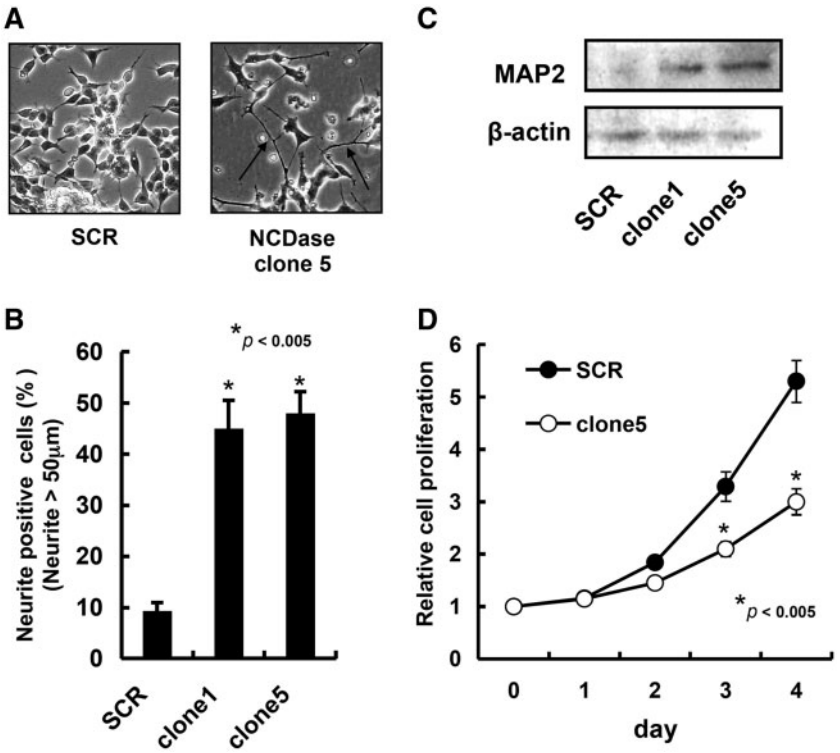

Fig. 6 Characterization of NCDase knockdown cells. (A) The morphology of NCDase-suppressed clone 5 and control (SCR) cells were observed under an inverted microscope. Notably, NCDase-suppressed clone 5 showed neuron-like characteristics associated with differentiation (arrows). (B) The percentage of neurite-positive cells was determined as described in 'Materials and Methods' section. (C) MAP2 protein levels of control (SCR) cells, clones 1 and 5 were analysed using western blotting. (D) Cell proliferation was examined sequentially by the MTS assay as described in 'Materials and Methods' section. Cells were cultured in triplicate and values are shown as the mean ± SD.

through a decrease in the bioactive lipid, ceramide-1-phosphate, rather than ceramide accumulation. It would be interesting to examine the collaborative role of suppressed NCDase and ceramide kinase in the induction of neuronal differentiation by ATRA in a future study.

ATRA has also been reported to influence multiple transcription factors including GATA (*29*, *39*, *40*). However, the number of reports analyzing NCDase transcriptional regulation was scarce (*25–27*). Our findings showed that ATRA decreases NCDase expression at both mRNA and protein levels (Fig. 2) and that GATA-2 regulates NCDase expression (Fig. 3). It has also been reported that ATRA decreased GATA-2 expression in the differentiation process of acute leukaemia cells (*40*). A single GATA motif is present ~100 bp upstream of exon 1 of NCDase, which may be the binding site of GATA-2 protein in SH-SY5Y cells. Our ChIP assay results (Fig. 4B) directly demonstrated a decrease in GATA-2 binding to this promoter region by ATRA.

In neuronal cells, the importance of GATA-2 for their differentiation and migration has been reported (*41–43*). However, GATA-2 expression is transitory and down-regulated before terminal differentiation, though it is retained in some differentiated cells (*44*), suggesting that the roles of GATA-2 in neuronal cells are both varied and subtype dependent. Our preliminary experiments with transfected siRNA against GATA-2 in SH-SY5Y cells showed that transient GATA-2 inhibition was not sufficient to induce neuronal differentiation (data not shown). Further analysis is needed to evaluate the roles of GATA-2 in our differentiation model.

The process of differentiation started by ATRA in SH-SY5Y cells may be very complex and propagated by various downstream signalling pathways. Thus, it is important to clarify whether ATRA-induced increase of cellular ceramide plays a significant role as an inducer of cell differentiation or not. The role of ceramide in neuronal differentiation has long been disputed. Using the mouse neuroblastoma cell line, NB2a, it was found that ATRA increased cellular ceramide level by up-regulation of neutral shingomyelinase, while exogenous ceramide-induced neurite outgrowth (*45*), with similar observations reported in experiments with other neuronal cells (*46*). Ceramide signalling downstream of the p75 neurotrophin receptor has also been reported to mediate NGF-induced outgrowth of neurons downstream of the p75 neurotrophin receptor (*47*). However, other reports have described opposite results and showed the inhibitory effect of ceramide on neurite formation (*48–50*). Thus, it is thought that the role of ceramide in neurite formation is dependent on cell-type and stimulus. The present LC–MS/MS data demonstrated that ATRA induced a significant increase in ceramide, while it slightly decreased intracellular sphingosine and S1P (Fig. 1), suggesting that ceramide accumulation is the most significant factor for ATRA-induced SH-SY5Y

cell differentiation. It is rather unlikely that the metabolic pathway downstream of ceramide contributes to the differentiation process. Our failure to detect extracellular S1P was probably due to the small amount of S1P secreted. To measure secreted S1P in culture medium, cells must be cultured in serum-free medium. SH-SY5Y cells were unable to maintain cell viability with ATRA treatment in the present serum-free culture condition for a long period. The significance of secreted S1P in the medium of ATRA-treated SH-SY5Y cells remains to be determined.

We established sub-clones (clone 1 and 5) with stably reduced NCDase activity using lentivilus shRNA system (Fig. 5A and C). As expected, ceramide was markedly increased in those cells (Fig. 5D). Those clones clearly showed reduced cell proliferation as well as a differentiation phenotype, such as neurite extension and MAP2 expression (Fig. 6). It has been reported that NCDase down-regulation by gemcitabine suppressed cell growth (*51*), which is compatible with the present findings. In addition, since the amounts of sphingomyelin and ceramide-monohexoside were not changed by ATRA (data not shown), there is likely no participation by sphingomyelinase and ceramide-monohexoside synthase in ATRA-induced ceramide accumulation in SH-SY5Y cells. We examined NCDase mRNA levels in a ATRA-treated rat pheochromocytoma cell line, PC12 and a mouse neuroblastoma cell line, Neuro2A, both of which are differentiated by ATRA (*52*, *53*), and found no decrease in NCDase mRNA. Thus, it remains to be determined whether the decrease in NCDase is a general phenomenon associated with neuronal differentiation of human cell lines induced by ATRA.

The MAP family includes MAP1, MAP2 and tau, which are primarily expressed in neurons and regulate microtubule dynamics. MAP2 expression is considered to be a hallmark of neuronal differentiation (*54*), and it has been reported that the MAP2 promoter is activated by the neurogenic bHLH factor, NeuroD, while it is repressed by the bHLH repressor, HES1, in melanoma cells (*55*). Interestingly, it has been reported that ATRA reduced differentiation inhibitory transcription factors, ID1, ID3 and ID3 but increased NeuroD1 and NeuroD6 in SH-SY5Y cells (*56*). Thus, it is possible that ceramide can modulate MAP2 gene expression either directly or indirectly through modulation of NeuroD/ID (or HES1). Nevertheless, the significance of MAP in neuronal cells remains to be disclosed fully, and is an interesting subject for future analysis.

Taken together, our findings show that ATRA suppresses NCDase expression in SH-SY5Y cells, resulting in increased intracellular ceramide. In addition, down-regulation of NCDase-induced differentiation markers that are characteristics of neuronal cells and suppressed cell growth, indicating that the NCDase/ceramide pathway involved, at least in part, in the process of ATRA-induced differentiation. These are the first results to present a unique function of NCDase in differentiation of ATRA-treated SH-SY5Y cells.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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