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Inhibition of neuropathy target esterase expressing by antisense RNA does not affect neural differentiation in human neuroblastoma (SK-N-SH) cell line

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Abstract

Neuropathy target esterase (NTE) is phosphorylated and aged by oraganophosphorus compounds (OP) that induce delayed neuropathy in human and some animals. NTE has been proposed to play a role in neurite outgrowth and process elongation during neural differentiation. However, to date, there is no direct evidence of the relevance of NTE in neural differentiation under physiological conditions. In this study we have investigated a possible role for NTE in the all-*trans* retinoic acid (ATRA)-induced differentiation of neuroblastoma cells by antisense RNA. A NTE antisense RNA construct was generated and then transfected into human neuroblastoma SK-N-SH cells. A positive cell clone that can stably express NTE antisense RNA was obtained by G418 selection and then identified by western blotting. NTE activity was depressed in the transfected cells with only about 50% activity of the enzyme in the control cells. ATRA-induced differentiation of the neuroblastoma cells with lowered NTE activity revealed that inhibition of NTE expression does not affect neural differentiation in SK-N-SH cells. The result suggested that organophosphates may inhibit neural differentiation by initially acting on other targets other than NTE. (Mol Cell Biochem **272**: 47–54, 2005)

Key words: antisense RNA, human neuroblastoma cell, neural differentiation, neuropathy target esterase

Introduction

Neuropathy target esterase (NTE) was identified over 30 years ago as the primary site of action of those oraganophosphorus compounds (OP) that cause a delayed paralyzing syndrome with degeneration of nerve axons [1]. Although the inhibition and subsequent aging of NTE has been proposed to be an initiating event in OP-induced delayed neuropathy (OPIDN), the events that occur between NTE inhibition and the appearance of clinical effects are not completely understood [2]. Elucidation of the molecular and cellular functions of NTE is a priority in understanding the pathogenesis of OPIDN.

NTE is a polypeptide of 1327 amino acids, and is anchored to the cytoplasmic face of endoplasmic reticulum (ER) by an amino-terminal transmembrane segment in mammalian cells

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and neurons [3, 4]. The NTE esterase domain (NEST), which comprises residues 727–1216 of human NTE, reacts with an ester substrate and covalent inhibitors in a manner very similar to NTE [5]. NTE is a novel serine esterase protein that is highly conserved among various species including insects, nematodes, yeast, and bacteria [6]. Recently, NTE was identified as a novel phospholipase B that is responsible for converting phosphatidylcholine to glycerophosphocholine and also has potent lysophospholipase activity [7, 8]. Complete inactivation of mouse NTE gene resulted in embryonic lethality, which resulted from placental failure and impaired vasculogenesis [9, 10], indicating NTE is essential for embryonic development.

NTE is mainly concentrated in nervous system, although it is present in some non-neural tissues, such as kidney, liver, and testicle of the mouse [9], so it may play some special part in neuron. It has been suggested that NTE may play an important role in neural development, possibly via involving in a signaling pathway between neurons and glial cells [11]. Indeed, brain-specific deletion of NTE resulted in neurodegeneration, which was characterized by vacuolation and neuronal loss [4].

Potential models for studying morphological and biochemical damage from neurotoxic OP include cultured cells with neuronal properties and particularly stable cell lines. OP that induces delayed neurotoxicity in animal models can inhibit outgrowth of axon-like processes in several differentiated cell lines, such as human neuroblastoma SH-SY5Y cells [12], mouse neuroblastoma N2a cells [13–16], rat adrenal pheochromocytoma PC-12 cells, and brain glial tumor C6 cells [17–19]. As such, the inhibitory effect of neuropathic OPs on neurite outgrowth in cell cultures has been a basis screening for delayed neurotoxicity *in vitro* [17]. It has been proposed that the role of NTE in cytotoxicity is in both neural function and the maintenance of neural integrity, suggesting that noncytotoxic biochemical disruption of NTE phosphorylation and aging may lead to inhibition of neurite and process outgrowth [19].

SH-SY5Y cell, the third cloned subline of human neuroblastoma SK-N-SH cell, has been widely used as in vitro cell model to test the inhibitory effects of different OPs on neural differentiation and it has been shown that NTE activity is higher in differentiated SH-SY5Y cells than in undifferentiated cells [12, 20]. Additionally, NTE activity in SK-H-SH cells is higher than that in PC-12 cells and SK-H-SH cell maintains the properties of neuroblastoma cells, which indicates that SK-N-SH is a good in vitro cell model for study of the mechanism of neurotoxicity [21]. Therefore, the SK-N-SH cell lines, chosen as the model for this study, extend processes following ATRA treatment [20, 21]. The role of NTE in neural differentiation in SK-N-SH cells was investigated by antisense RNA strategy to inhibit its expression.

Materials and methods

Materials

The human neuroblastoma SK-N-SH cell line was purchased from the Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Cell culture reagents were obtained from Gibco (Grand Island, NY, U.S.A.). Human NTE-specific antibodies against a peptide corresponding to amino acids 37–48 (LPQEPPGSATDA) were generated by Shanghai Casarray Co. (Shanghai, China). Mipafox and phenyl valerate (PV) were synthesized in our laboratory as described by Johnson [22]. The characteristics of mipafox were identified by nuclear magnetic resonance (data not shown). The boiling point of PV is 70–72 °C at 0.2 mm Hg; the melting point of mipafox is 60–62 °C. All-*trans* retinoic acid (ATRA) was obtained from Sigma (St. Louis, MO, U.S.A.). Transfection reagent lipofectamine 2000 were purchased from Invitrogen (Groningen, The Netherlands).

Cell culture

SK-N-SH cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and subcultured at 3–4 day intervals.

DNA constructs

To construct a NTE antisense RNA expression vector, a forward primer (NTEaF), GCGAATTCCCACACTCGGAACTCACCAAC, was designed to make use of an internal E*co*R I site. Polymerase chain reaction (PCR) was used to amplify a 1.2-kb DNA fragment, using NTEaF and reverse primer (NTEaR) including B*am*H I site, TTGGATCCGTCCGCTGGCAGATTGTTG. Total volume for the PCR is 50 μl, containing 50 ng DNA of D16 (a plasmid containing full length NTE cDNA), 1× buffer, 0.4 μM forward and reverse primer respectively, 1.6 mM Mg^{2+}, 0.15 mM dNTP, and 2.5 U Pfu DNA polymerase. Amplification procedure is 94 °C for 3 min to pre-denature, then 94 °C for 45 sec; 60 °C for 1 min, and 72 °C for 1min to amplify production for 20 cycles. PCR production was cloned into the B*am*H I and E*co*R I sites of the vector pCDNA3.1(+) to make pCDNA-aNTE; the construct, verified by DNA sequencing, contained the protein-coding sequence from 2324 to 3445 of NTE cDNA.

Stable expression cell clone

pCDNA-aNTE construct and vector (pCDNA3.1(+)) were transfected into human neuroblastoma SK-N-SH cells with lipofectamine 2000 and selected with 500 μg/mL G418 for 3 weeks. The positive cell clones were identified with western blotting and NTE activity was assayed. Stable clones were maintained in DMEM mediun containing 200 μg/mL G418.

Immunoblotting

NTE expression levels in pCDNA-aNTE transfected cell lines were evaluated by preparing cell extracts. The cells were washed twice with cold PBS, harvested in lysis buffer (50 mM Tris pH 7.5, 0.3 M NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and a 10 μg/ml final concentration of each of aprotinin, leupeptin and pepstatin), and then sonicated on ice. Lysates were clarified, and the protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Lysates (40 μg) were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose and probed with primary antibody. After incubating with horseradish peroxidase-conjugated secondary antibody, the immunoblots were developed with using standard enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, U.S.A.).

Northern blot

Total RNA was obtained from PBS-washed cell monolayers with Trizol RNA (15–20 μg/lane) and was then denatured, electrophoresed through 1% agarose-formaldehyde gels, and transferred to Hybond ECL cellulose membranes (Amersham Biosciences) (Little Chalfont, Buckinghamshire, England). DNA probes were labeled with (α-32P) dCTP (Amersham Biosciences, specific activity 3000 Ci/mmol) by random prime labeling (Prime-a-Gene Labeling System, Promega) (Mdison, WI, U.S.A.). Hybridization was carried out overnight at 65 °C according to Church and Gilbert [23]. Filters were washed with 0.5 × SSC, 0.1% SDS, first at room temperature for 10 min and then at 65 °C for 30 min. The membranes were exposed to X-ray films at −70 °C with intensifying screens.

Neuropathy target esterase and acetylcholinesterase activity assay

Cellular NTE activity was determined in homogenates of control (vector-transfected) and SH/aNTE cells by the absorbance difference for PV hydrolysis between samples exposed to paraoxon and paraoxon plus mipafox, previously described by Johnson [22]. AChE activity was assayed as described by Gorun *et al*. [24].

MTT reduction assay

Cell viability was studied by the MTT assay. At the end of each experiment, cell medium containing 0.5 mg/ml MTT was added to each well and incubated at 37 °C in 95% air/5%CO_2 for 4–5 h. The insoluble formazan formed was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm.

Differentiation of SK-N-SH clones

To initiate ATRA-mediated differentiation, cells were grown in the DMEM medium containing 20 μM ATRA for one week in the dark with replacement of the conditioned medium every 48 h. To assess the effect of lowered-expression of NTE, cells were photographed in an inverted phase contrast microscope (model: DMRBE, Leica). Differentiated cells were quantified by counting at least 500 cells in randomly chosen fields. Cells were considered to be differentiated if they had at least one process longer than the cell body, which could be regarded a neurite [25]. The results are expressed as percentage of the cells differentiated. The length of the longest neurite was measured in at least 100 cells in randomly chosen fields (40 × magnification). At least three independent experiments were conducted with each clone and the results are expressed as mean ± S.E.

Results

Generation of NTE antisense RNA construct

The PCR production is about 1.2 kilobases, which is consistent with estimated length from NTE cDNA (Fig. 1). The PCR production was then cloned into the B*am*H I and E*co*R I sites of the vector pCDNA3.1(+) to make pCDNA-aNTE, in which the orientation of NTE cDNA was reversed (Fig. 2). As shown in Fig. 2, pCDNA-aNTE cut with B*am*H I and E*co*R I included 2 bands, one is equal to the band of pCDNA3.1(+) cut with B*am*H I, the other is consistent with extraction of PCR production.

Inhibition of NTE expression in SK-N-SH cells

To evaluate the role of NTE in neural differentiation, human neuroblastoma SK-N-SH cells were transfected with pCDNA-aNTE and selected with G418 for 3 weeks. Cell

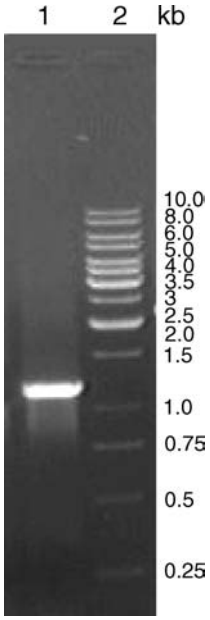

Fig. 1. PCR amplification of antisense NTE (aNTE). Lane 1: PCR fragment; lane 2: Marker.

clone that stably expresses NTE antisense RNA was identified by western blotting and activity assay and the positive cell clones were named SH/aNTE. As shown in Fig. 3A, the expression of NTE was virtually eliminated in SH/aNTE cells, a weak NTE band could be detected by the blotting even when the blots were overexposed. Basal *in situ* NTE activity was assayed by the absorbance difference for phenyl valerate hydrolysis between samples exposed to paraoxon and paraoxon plus mipafox. The basal NTE activity in SH/aNTE cells was significantly decreased *in situ* compared to the vector control cells; SH/aNTE abolished about 50% of NTE activity (Fig. 3B). As a control, we measured AChE activity. As shown in Fig. 3C, AChE activity did not show a significant reduction between control and SH/aNTE (Fig. 3C). In addition, the mRNA level of NTE in SH/aNTE cells was monitored with northern blots. The results showed that NTE mRNA in SH/aNTE cells was almost abolished compared to the vector control and wild type cell (Fig. 3D). These results domonstrated that the pCDNA-aNTE construct efficiently and specifically inhibited the expression of NTE.

NTE is not required for neural differentiation in human neuroblastoma cell

Transfection with the empty pCDNA3.1(+) vector and pCDNA-aNTE vector did not result in any morphological alterations. Furthermore, we observed no detectable differences in growth rates between experitmental and control cells (Table 1). In order to evaluate a role for NTE in ATRA-mediated neurodifferentiation of SK-N-SH cells, these different cell lines were treated with ATRA for one week and their morphology was evaluated. ATRA significantly increased neurite outgrowth in the vector-transfected cells (compare Fig. 4A and B). In contrast, ATRA treatment resulted in the same effect in SH/aNTE cells (compare Fig. 4C and D). The effect of ATRA on neuronal differentiation of SH/aNTE was quantified. As shown in Table 2, the percent and average length of differentiated cells in SH/aNTE were not significantly different to those of control cells. These findings

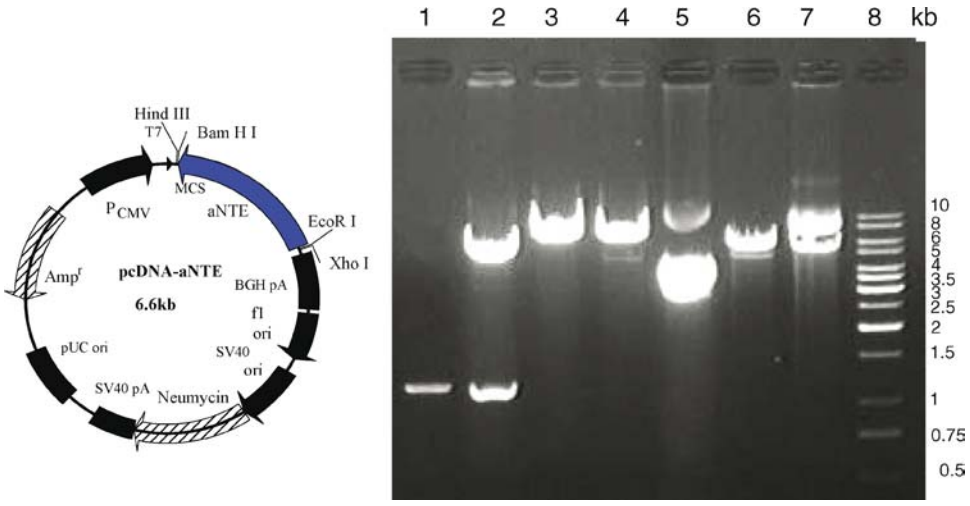

Fig. 2. Generation of NTE antisense RNA construct. *Left*: the map of pCDNA-aNTE. *Right*: Identification of pCDNA-aNTE with restriction endonuclease. Lane 1: extraction of PCR production; lane 2: pCDNA-aNTE cut with E*co*R I and B*am*H I; lane 3: pCDNA-aNTE cut with B*am*H I; lane 4: pCDNA-aNTE cut with E*co*R I; lane 5: pCDNA-aNTE; lane 6: pCDNA3.1(+) cut with E*co*R I; lane 7: pCDNA3.1(+); lane 8: 1 kb ladder.

Table 1. MTT reduction of control and lower NTE activity cells at different times

	12 h	24 h	36 h	48 h	72 h
SH/pCDNA	0.438 ± 0.002	0.632 ± 0.031	0.862 ± 0.051	1.028 ± 0.084	1.644 ± 0.071
SH/aNTE	0.416 ± 0.001	0.573 ± 0.022	0.761 ± 0.041	0.987 ± 0.061	1.738 ± 0.082

Note. Data shown are the mean absorbance from four separate culture wells grown on at least three separate occasions.

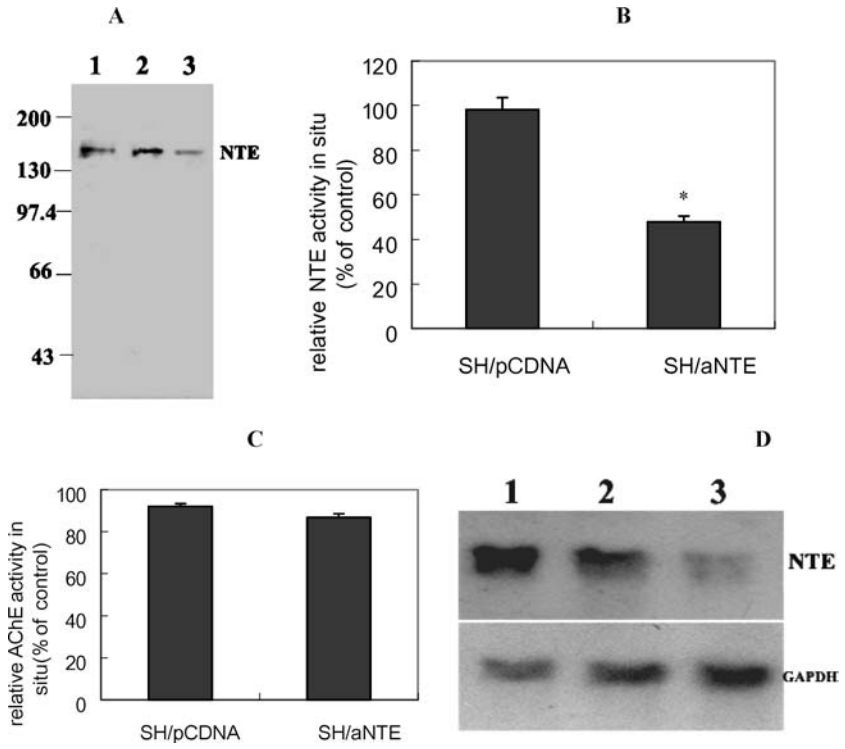

Fig. 3. Inhibition of NTE in SK-N-SH cells. A: Representative immunoblots of NTE expression levels in human neuroblastoma SK-N-SH cells stably transfected with wild-type NTE (lane 1), pCDNA (vector control) (lane 2) and with pCDNA-aNTE (lane 3). B: Quantitative analysis of basal *in situ* NTE activity. Data are presented as a percentage of *in situ* NTE activity in wild-type cells. Basal *in situ* NTE activity was significantly decreased in SH/aNTE cells ($n = 3$, $p < 0.05$). C: Quantitative analysis of basal *in situ* AChE activity. Data are presented as a percentage of *in situ* AChE activity in control cells. Basal *in situ* AChE activity was not significantly decreased in SH/aNTE cells ($n = 3$, $p > 0.05$). D: Representative northern blots of NTE and GAPDH mRNA expression levels in human neuroblastoma SK-N-SH cells stably transfected with wild-type NTE (lane 1), pCDNA (vector control) (lane 2) and with pCDNA-aNTE (lane 3), expression level of GAPDH mRNA as loading control.

reveal that inhibition of NTE expression did not affect ATRA-induced neuronal differentiation in SK-N-SH cells.

Discussion

Neuropathy target esterase is concentrated in the nervous system in mammalian animals and anchored to the cytoplasmic face of ER [3, 4], As such, it may play an important role in the nerve development since mutation of Swiss cheese protein, its homologous protein in *Drosophila*, resulted in an age-dependent neurodegeneration in flies [11]. Indeed, Brain-specific deletion of neuropathy target esterase led to neurodegeneration in mouse. Moreover, absence of NTE resulted in disruption of ER, vacuolation of nerve cell bodies, and abnormal reticular aggregates [4].

SK-N-SH cells, a human neuroblastoma cell line, withdraw from the cell cycle and express a distinct neuronal phenotype when treated with ATRA. SK-H-SH cells are used extensively as a cell culture model of neurodifferentiation. Therefore, SK-N-SH cells provide a useful model for studying changes in signal transducing proteins, cytoskeletal proteins, neural protein and expression and activity of neural proteins during the process of differentiation into neuronal phenotype. NTE, the main target protein in OPIDN, was also considered to be the preferred candidate target for inhibitory

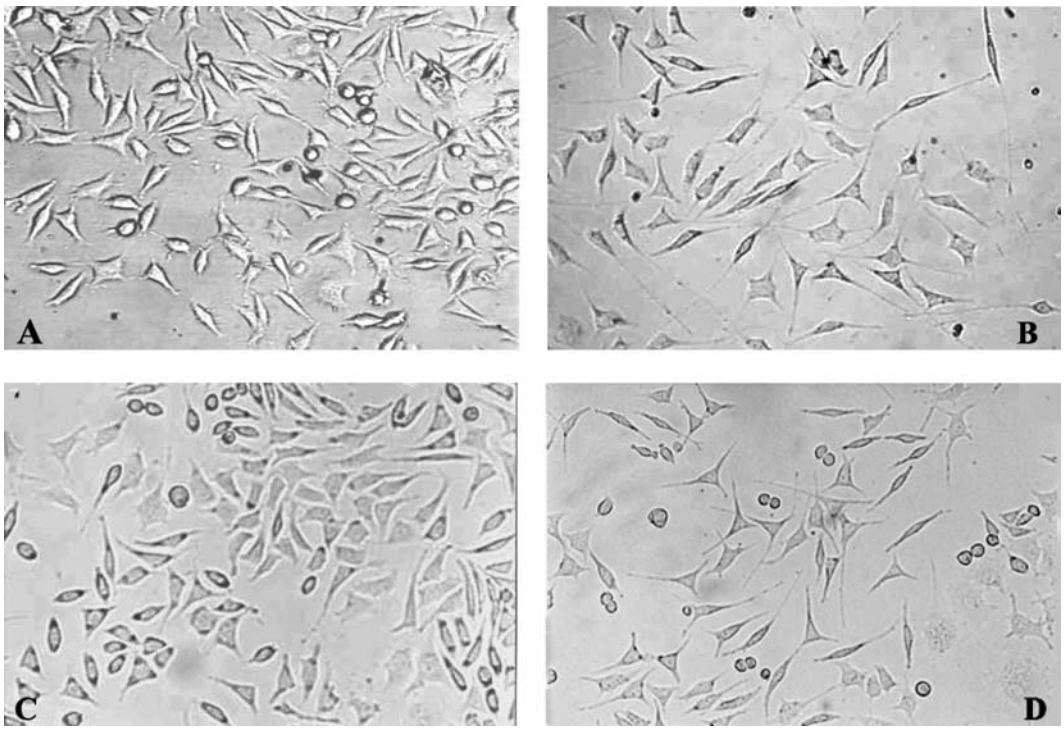

Fig. 4. All-trans retinoic acid (ATRA)-induced neuronal differentiation in SH/aNTE cells. To initiate ATRA-mediated differentiation, cells were grown in DMEM medium containing 20 μM ATRA. The medium was replaced with fresh condition medium once every 48 h. The cells were photographed on day 7 of culture in an inverted phase contrast microscope. At least three independent experiments were conducted with each clone and the photographs given are representative of these experiments, undifferentiated control cell (A), differentiated control cell (B), undifferentiated SH/aNTE cell (C), and differentiated SH/aNTE cell (D).

Table 2. Effect of NTE expression in different levels on the differentiation of human neuroblastoma SK-N-SH cells

	Average neurite length (mm)	Differential cells (% of total cells)
Control	114.4 ± 7.0	33.9 ± 1.3
SH/aNTE	108.6 ± 3.2	26.6 ± 1.7

Note. NTE lowered expression doesn't affect ATRA-induced neuronal differentiation in stable SH/aNTE cells. Percent of cells differentiated were counted in five randomly chosen fields (20 × magnification) from each dish and the data represent mean ± S.E. from three independent experiments. The length of neurite on each cell (40 × magnification) was measured in at least 100 cells per treatment. Results given are the mean ± S.E. from three independent experiments.

neuronal differentiation by OPs [19]. Therefore, the role of NTE in the neural differentiation was studied in SK-N-SH cell model.

Compounds acting by an antisense mechanism include antisense oligodeoxynucleotides, antisense RNA, and ribozymes. Using expression vectors can continuously synthesize antisense RNA inside the cell [26]. In the stable expressing antisense RNA cells, the inhibition was due to the expression of the exogenous target antisense fragment integrated in cell chromosomes, which bound to endogenous target mRNA via base-pairing leading to accelerated targeted mRNA degradation, incorrect pre-mature splicing, mRNA translation handicap, and mRNA traffic disorder [27]. The levels of mRNA and protein of NTE in SH/aNTE cells were decreased significantly compared with that in the vector control cells. The SH/aNTE cell has less NTE activity. In SK-N-SH cells, deletion of neuropathy target esterase with antisense RNA did not alter the morphology nor affect the neural differentiation, which indicated that NTE is not required for the process. Although deletion of NTE in mouse resulted in embryonic lethality at embryonic day 9 postcoitum (E9), E8 embryo cells without any NTE activity after one week of culture in vitro survived and gave rise to different cell types with different cell morphologies including some highly differentiated cells, such as beating cardiac myocytes and cells resembling neurons with long branched neurite-like processes [10]. Taken together with our results, a number of different cells can survive and differentiate in the absence of NTE activity.

NTE was identified as a novel phospholipase B responsible for degrading phosphatidylcholine to glycerophosphocholine

and also has potent lysophospholipase activity [7, 8]. In SH/aNTE cells with lower NTE activity level, the catalytic rate from phosphatidylcholine to glycerophosphocholine may be inhibited and led to increase of phosphatidylcholine. However, in mammalian cells, the increasing level of phosphatidylcholine could be abolished by phospholipase D hydrolysis or inhibition of its synthesis with decreasing the activity of CTP-phosphocholine cytidylyltransferase (CCT), which controls the rate-limiting step from cytidene diphosphocholine to phosphatidylcholine [28]. In yeast lacking YML059c that is a protein with sequence homology to NTE, phosphatidylcholine synthesis is restricted at the rate-limiting step mediated by CCT [7]. The levels of phosphatidylcholine and glycerophosphocholine in SK-N-SH and SH/aNTE cells need to be assayed further.

NTE was thought to be a preferred candidate target of OPs. The two direct-acting NTE inhibitors, mipafox and 2-octyl-4H-1,3, 2-benzodioxophosphorin 2-oxide(OBDPO), selectively inhibited cell process outgrowth and NTE was more sensitive than acetylcholinesterase (AChE) to the active agents [19]. Therefore it has been proposed that NTE may be the initial target of organophosphate during its inhibitory effect for neural differentiation. There was a higher NTE activity in differentiated than undifferentiated SH-SY5Y cells and the inhibitory effect on axon outgrowth of tri-ortho-cresyl phosphate (TOCP) in N2a cells was enhanced in the presence of a microsomal activation system which converted TOCP into its active metabolite that is more potent to inhibit NTE activity [15, 20]. However, the outgrowth of axons in N2a cells after exposure to chlorpyrifos, a common organophosphorus insecticide, could be inhibited by distinct mechanisms except inhibition of NTE [16]. A direct link between NTE activity and neurodifferentiation has not yet been demonstrated. Our results indicated that SH/aNTE cells could be induced to differentiation by ATRA, which suggested that organophosphates may inhibit neural differentiation by initially acted on certain targets other than NTE. Although OPIDN has been long thought to be initiated by NTE phosphorylation (more than 70% inhibition) with the subsequent modification (aging) of this phosphorylated enzyme, it was found recently that diisopropyl phosphorofluoridate (DFP), an organophosphate that induces OPIDN, bound other sites than NTE in cytosol of hen spinal cord, suggesting that DFP binding sites in the initiation of OPIDN may be different from the active sites of NTE [29, 30]. In fact, there are various forms of NTE in different fractions of hen brain and sciatic nerves [31, 32] and there are lots of potential targets for OP in cells [33]. For example, some OPs inhibit lysophospholipase. NTE has also potent lysophospholipase activity and constitutes only 29% of the total lysophospholipase in mouse brain [34]. The target and mechanism of inhibitory neural differentiation by OP remain to be elucidated.

In summary, noncytotoxic biochemical disruptions from NTE activity with genetic method do not lead to inhibition of neurite and process outgrowth. As such, inhibition of NTE expression does not affect neural differentiation in human neuroblastoma cell. These findings suggest that organophosphate may interact with certain molecules, other than NTE, to exert its inhibitory effect on neural differentiation in neuroblastoma cell.

Acknowledgments

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