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Research Report

Reduction of neuropathy target esterase does not affect neuronal differentiation, but moderate expression induces neuronal differentiation in human neuroblastoma (SK-N-SH) cell line

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Abstract

Neuropathy target esterase (NTE) is inhibited and aged by organophosphorus compounds that induce delayed neuropathy in human and some sensitive animals. NTE has been proposed to play a role in neurite outgrowth and process elongation during neurodifferentiation. However, to date, there is no direct evidence of the relevance of NTE in neurodifferentiation under physiological conditions. In this study, we have investigated a possible role for NTE in the all-*trans* retinoic acid-induced differentiation of neuroblastoma cells. The functional inactivation of NTE by RNA interference indicated that reduction of NTE does not affect process outgrowth or differentiation of the cells, although moderate expression of NTE by expression of the NTE esterase domain accelerates the elongation of neurite processes. Mipafox, a neurotoxic organophosphate, was shown to block process outgrowth and differentiation in cells that have lowered NTE activity due to RNA interference, suggesting that mipafox may interact with other molecules to exert its effect in this context.

Theme: Disorders of the nervous system
Topic: Neurotoxicity, cell differentiation and migration

Keywords: Neuropathy target esterase; Neural differentiation; Human neuroblastoma cell; RNA interference; Organophosphate

1. Introduction

Neuropathy target esterase (NTE) was identified over 30 years ago as the primary site of action of organophosphorus compounds (OP) that cause a delayed paralyzing syndrome with degeneration of nerve axons [14]. 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 [10]. 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 by an amino terminal transmembrane segment in mammalian cells and neurons [1,18]. 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 [2]. NTE is a novel serine esterase protein that is highly conserved among various species including insects, nematodes, yeast and bacteria [20]. Recently, NTE was identified in mammalian cells as a novel phospholipase B responsible for converting phosphatidylcholine to glycerophosphocholine and also has potent lysophospholipase activity in mouse [26,33]. In mice, complete inactivation of the NTE gene resulted in embryonic lethality due to placental failure and impaired vasculogenesis [21,31]. Additionally, brain-specific deletion of

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NTE resulted in neurodegeneration, thus indicating that NTE is essential for embryonic and nervous development [1].

Cultured cells with neuronal properties and stable cell lines are potential models for studying morphological and biochemical damage from neurotoxic OPs that induce delayed neurotoxicity in animal models can inhibit outgrowth of axon-like processes in several differentiated cell lines, such as human neuroblastoma SH-SY5Y cells [13], mouse neuroblastoma N2a cells, rat adrenal pheochromocytoma PC-12 cells and brain glial tumor C6 cells [6–8,12,17,28,29]. As such, the inhibitory effect of neuropathic OP on neurite outgrowth in cell cultures has provided a standard in the screening for delayed neurotoxicity in vitro [12]. NTE is a preferred candidate target since two direct-acting NTE inhibitors, mipafox and 2-octyl-4*H*-1,3,2-benzodioxophosphorin 2-oxide, selectively inhibit cell process outgrowth and NTE is more sensitive than acetylcholinesterase (AChE) to the active agents [17]. Therefore, it has been proposed that NTE's role in cytotoxicity is both in neural function and the maintenance of neural integrity. As such, noncytotoxic biochemical disruption of NTE phosphorylation and aging may lead to inhibition of neurite and process outgrowth [17]. Chlorpyrifos, a metabolic precursor of NTE inhibitor, inhibits outgrowth of axons and also decreases the activity of NTE in co-differentiation mouse neuroblastoma N2a cells [28]. Human neuroblastoma (SH-SY5Y) cells have been widely used as an in vitro cell model to test the inhibitory effects of different OPs on neural differentiation and previous reports have shown that the NTE activity of SH-SY5Y cells is higher in differentiated cells than in undifferentiated cells [13,23]. These results suggest that NTE may play a certain role in differentiation of neuroblastoma cells.

SK-N-SH cells are a good in vitro cell model for studying the mechanism of neurotoxicity since they extend processes following retinoic acid treatment, maintain the properties of neuroblastoma cells and have higher NTE activity than PC-12 cells [22]. Therefore, SK-N-SH was chosen as cell model in this study. RNA interference (RNAi) has been a new effective way to inhibit the expression of gene in mammalian cells; pSUPER vector was used as a stable system to produce short interfering RNAs (siRNA) against NTE in mammalian cells [4,30,32]. The present investigation was designed to study the role of NTE in all-*trans* retinoic acid-induced neurodifferentiation by genetic methods. In addition, the relation between OP and its target was verified in this paper.

2. Materials and methods

2.1. Materials

The NTE cDNA clone D16 and the construct pNTE-GFP were kindly provided by Dr. Paul Glynn and Dr. Yong Li (The MRC Toxicology Unit, University of Leicester, UK). pSUPER and pcDNA3.1(+) vectors were purchased from Oligoengine (Seattle, Washington) and Invitrogen (Groningen, The Netherlands), respectively. The human neuroblastoma SK-N-SH cell line was purchased from the Cell Center of Chinese Academy of Medical Sciences. Cell culture reagents were obtained from Gibco (Grand Island, NY, USA). Human NTE-specific antibodies against a peptide corresponding to amino acids 37–48 (LPQEPPGSATDA) were generated by Shanghai Casarray Co. LTD (Shanghai, China). Paraoxon, all-*trans* retinoic acid (ATRA), monoclonal anti-HA antibody (clone HA-7), monoclonal anti-neurofilament 200 antibody (clone N52), anti-mouse IgG (Fc specific) peroxidase conjugate antibody and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Horse fluorescein anti-mouse IgG (H + L) was purchased from Vector laboratories (Burlingame, CA, USA). Mipafox and phenyl valerate were synthesized in our laboratory. Transfection reagent lipofectamine 2000 was purchased from Invitrogen (Groningen, The Netherlands).

2.2. 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- to 4-day intervals.

2.3. DNA constructs

To construct a stable RNA interference expression vector of human NTE to be expressed in mammalian cells, a pSUPER neo vector was generated by cloning a *Spe*I- and *Xho*I-digested fragment from pSUPER, which contains the H1 RNA Polymerase III promoter and the multiple cloning site, into the compatible in the pcDNA3.1(+) vector. Next, NTEiF 5′-GATCCCC**CGTCCGGGTGCTGGGCCAC**TTCAAGAGA**GTGGCCCAGCACCCGGACG**TTTTTGGAAA-3′ and NTEiR 3′-GGG**GCAGGCCCACGACCCGGTG**AAGTTCTCT-**CACCGGGTCGTGGGCCTGC**AAAAACCTTTTCGA-5′ were annealed to form a double-strand DNA (NTEi) with *Hin*dIII and *Bgl*II sites at the 5′ and 3′ ends, respectively. NTEi was ligated into the *Hin*dIII and *Bgl*II sites of the pSUPER neo vector. The construct was verified by DNA sequencing and was shown to produce siRNA targeted against nucleotides 430–448 of NTE mRNA in mammalian cells.

To construct an expression vector for human NEST, PCR was used to add a *Bam*HI site and the influenza hemagglutinin (HA) tag in frame at the 5′ end with the following primers: NESTF 5′-TTGGATCCGCCATGGGCTACCCA-TACGATGTTCCAGATTACGCTACCCGCCTTATC-CACCTACTG-3′ and NESTR 5′-GCGAATTCTTACC-