

Effect of over-expression of neuropathy target esterase on mammalian cell proliferation

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Abstract. Neuropathy target esterase (NTE), the human homologue of a protein required for brain development in *Drosophila*, is expressed primarily in neural cells but is also detected in non-neural cells. Although NTE has been proposed to play a role in neurite outgrowth and process elongation during neurodifferentiation, the function of NTE has not been defined in neural cells. In this study we have investigated the possible role of NTE in neuroblastoma cells and non-neural cells using an over-expression strategy. Over-expression of NTE in human neuroblastoma SH-SY5Y cells and monkey kidney COS7 cells led to an accumulation of NTE on the cytoplasmic surface of the endoplasmic reticulum and inhibition of cell proliferation. In particular, high levels of NTE arrested COS7 cells at G₂/M stage yet was not associated with arrest at a particular phase of the cell cycle in SH-SY5Y cells. Moreover, over-expression of NTE did not induce apoptosis in two kinds of cell lines as assessed by flow cytometry. These results suggest that the role of NTE over-expression in cell proliferation is associated with different mechanisms in different cells.

INTRODUCTION

Neuropathy target esterase (NTE) was identified over 30 years ago as the primary target of organophosphate compounds (OP) that cause a delayed paralysing syndrome with degeneration of nerve axons (Johnson 1974). Although the inhibition and subsequent ageing 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 pathology are not completely understood (Glynn 2003). As such, characterizing the molecular and cellular functions of NTE should elucidate the mechanism of OPIDN.

Neuropathy target esterase is a novel serine esterase protein that is highly conserved among species including insects, nematodes, yeast and bacteria (Lush *et al.* 1998). NTE is a polypeptide of 1, 327 amino acids and is anchored to the cytoplasmic face of endoplasmic reticulum (ER) through an amino-terminal transmembrane segment in non-neural cells and neurones (Li *et al.* 2003; Akassoglou *et al.* 2004). The NTE esterase domain (NEST) exists between amino acids

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727–1216 of human NTE and reacts with both ester substrates and covalent inhibitors in a manner very similar to NTE (Atkins & Glynn 2000). Within the NEST domain, serine⁹⁶⁶ and two aspartate residues, Asp⁹⁶⁰ and Asp¹⁰⁸⁶ are critical for NTE function. Previous observations indicated that NTE displayed potent lysophospholipase activity in the mouse brain. This activity has been further characterized as a novel phospholipase B responsible for converting phosphatidylcholine to glycerophosphocholine in mammalian cells and regulated phosphatidylcholine homeostasis in *Drosophila* (Quistad *et al.* 2003; Zaccheo *et al.* 2004; Muhlig-Versen *et al.* 2005). In mice, complete inactivation of the NTE gene resulted in embryonic lethality resulting from placental failure and impaired vasculogenesis (Winrow *et al.* 2003; Moser *et al.* 2004), whereas mice with a brain-specific deletion of NTE exhibited neurodegeneration. In adult *Drosophila*, loss of Swiss cheese/NTE activity causes neuronal and glial death (Akassoglou *et al.* 2004; Muhlig-Versen *et al.* 2005). Together, these data suggest that NTE is essential for embryonic and nervous development.

Cultured stable cell lines with neuronal properties are useful models for studying the functions of a particular gene in the nervous system. It has been proposed that non-cytotoxic biochemical disruptions through NTE phosphorylation and ageing may lead to the inhibition of neurite and process outgrowth in neuroblastoma cells (Li & Casida 1998). Although NTE activity is not required for neurite initiation or elongation per se of embryonic stem cells, it is essential for the optimal rate of neurite initiation (Li *et al.* 2005). However, little is known about the function of NTE in neural cells. The human neuroblastoma SH-SY5Y cell line exhibits higher activity of NTE than rat adrenal pheochromocytoma PC-12 cells, and also demonstrates a distinct neuronal phenotype when treated with retinoic acid (Nostrandt & Ehrlich 1992). Therefore we choose SH-SY5Y cells as an *in vitro* cell model to study the effects of NTE over-expression. Although NTE expression is primarily concentrated in the nervous system, significant amounts of NTE are also present in some non-neural tissues of the mouse, particularly in the kidney, liver and testicle (Winrow *et al.* 2003). Therefore, NTE may play a universal role in mammalian neural cells and non-neural cells. As such, we choose monkey kidney COS7 cells as an *in vitro* model for non-neural tissue. In order to elucidate the function of NTE in mammalian cells, an enhanced green fluorescent protein (EGFP)-tagged NTE was over-expressed in SH-SY5Y and COS7 cells and the effect on cell proliferation was analysed.

MATERIALS AND METHODS

Cells and chemicals

The human neuroblastoma SH-SY5Y cell line was purchased from Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). Monkey kidney COS7 cells were kindly provided by Prof Y.-L. Wang (State Key Laboratory of Reproductive Biology, Beijing, China). Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). Plasmid pEGFP-N3 and Living Colors monoclonal antibody (JL-8) were purchased from Clontech (Palo Alto, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. pNTE-EGFP was kindly provided by Dr Y. Li (MRC Toxicology Unit, UK). Monoclonal anti β -actin antibody (AC-15) and anti-mouse IgG (Fc-specific) peroxidase conjugate, paraoxon, propidium iodide (PI), RNase A were obtained from Sigma (St. Louis, MO, USA). Mipaflox and phenyl valerate were synthesized in our laboratory. The bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA). The transfection reagent Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Groningen, the Netherlands).

Cell culture

SH-SY5Y and COS7 cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 g/ml each ampicillin and streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The cells were maintained in the logarithmic phase of growth and sub-cultured at 3–4 days intervals.

DNA constructs and transfection

The expression constructs of pEGFP-N3 and pNTE-EGFP were transfected into human neuroblastoma SH-SY5Y cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western blotting analysis

The level of NTE protein in transfected cells was evaluated by Western blot analysis. Forty-eight hours after transfection, cells were washed two times with cold phosphate-buffered saline (PBS), harvested in lysis buffer (50 mM Tris pH 7.5, 0.3 M NaCl, 5 mM ethyleneglycotetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 0.5% NP40, 0.1 mM phenylmethylsulphonyl fluoride, and 10 µg/ml each of aprotinin, leupeptin and pepstatin), and then sonicated on ice. Lysates were clarified, and the protein concentrations were determined using the BCA assay. Lysates (40 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with a 4% stacking gel and 10% separating gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). Following transfer, membranes were washed in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) 2 × and blocked in 5% non-fat milk in TBST (TBS containing 0.1% Tween-20) at room temperature for at least 1 h. Membranes were incubated in TBST containing 1 : 1000 monoclonal anti-GFP antibody at 4 °C for overnight. Membranes were washed three times in TBST and then incubated with horseradish peroxidase-conjugated goat-antimouse IgG diluted 1 : 1000 in TBST at 37 °C for 1 h. Membranes were washed three times in TBST and then developed using standard ECL method.

Neuropathy target esterase assay

Forty-eight hours after transfection, the cells were harvested. The cell pellet was resuspended in TE buffer (50 mM Tris-HCl, 0.2 mM ethylenediaminetetraacetic acid, pH 8.0), homogenized with 20 passages through a 25-gauge hypodermic needle, and centrifuged at 100 × g at 4 °C for 2 min. In the supernatant fraction, NTE activity was determined by measuring phenyl valerate hydrolysis using the absorbance difference between samples exposed to 40 µM paraoxon and 40 µM paraoxon plus 50 µM mipafox, as previously described by Johnson (1977).

Confocal microscopy

Cells were mounted on glass slides in six-well cluster plates and transfected with the described constructs for 24 h in culture. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and imaged using confocal scanning microscopy with a TCS-4D laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

To investigate the effects of sustained NTE expression on cell morphology, cells were transfected with the described constructs and grown in selection media containing 500 µg/ml G418 for 3 weeks. Surviving SH-SY5Y cells were photographed directly. COS7 cells were seeded on glass slides in six-well cluster plates and cultured for 24 h before fluorescent images were acquired as described in previous discussions.

MTT reduction assay

Cell viability was studied using the MTT assay. At the end of each transfection experiment, cell medium containing 0.5 mg/ml MTT was added to each well and incubated at 37 °C in 95% air/5% CO₂ for 4–5 h. The insoluble formazan was dissolved in dimethyl sulfoxide, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm.

Cell cycle assay

Cell cycle analysis of COS7 cells before transfection and 48 h after transfection was performed using PI staining. In brief, 5×10^6 cells were harvested and washed twice with Ca²⁺/Mg²⁺-free PBS, fixed overnight in 75% cold ethanol, digested with RNase A and stained with PI (100 µg/ml). Data were obtained and analysed by flow cytometry using the CELLQUEST software on a FACScan (Becton Dickinson Company, Franklin Lakes, NJ, USA) using gates to exclude debris from the population to be studied.

Statistical analysis

All results are represented as the mean ± standard error of the mean (SEM) of at least three independent experiments unless stated otherwise. Significance was determined using one-way ANOVA (SPSS software). Difference between groups were considered significant at a value of $P < 0.05$.

RESULTS

Over-expression of NTE in mammalian cells

In order to evaluate the role of NTE in mammalian cells, SH-SY5Y and COS7 cells were transfected with either pNTE-EGFP or the control vector pEGFP-N3. At 48 h post-transfection, cells were analysed for NTE expression by Western blot analyses using the EGFP antibody. As shown in Fig. 1, cells transfected with the control vector expressed a protein of 27 kDa, which correlates to EGFP protein expression; however, cells transfected with pNTE-EGFP expressed a protein of 170 kDa corresponding to EGFP-tagged NTE protein (Fig. 1a). The expression levels of EGFP and NTE in COS7 cells were higher than that in SH-SY5Y cells, as a result of higher transfection efficiency. In COS7 cells, the transfection efficiency was about 80% compared to 50% efficiency in SH-SY5Y cells (data not shown).

Next, COS7 or SH-SY5Y cells were transfected with either control vector (pEGFP-N3) or NTE expressing construct (pNTE-EGFP) and the activity of NTE was analysed. COS7 and SH-SY5Y cells transfected with the control vector displayed minimal NTE activity; however, the activity of NTE in both COS7 and SH-SY5Y cells was increased when transfected with pNTE-EGFP compared to that of cells expressing EGFP alone (Fig. 1b). In particular, COS7 cells transfected with pNTE-EGFP demonstrated a 20-fold increase in NTE activity compared to cells transfected with EGFP. In contrast, SH-SY5Y cells transfected with pNTE-EGFP demonstrated only a 2.5-fold increase in NTE activity when compared to cells transfected with EGFP. This phenomenon may be the result of lower transfection efficiency of SH-SY5Y cells than that of COS7 cells. Indeed, the endogenous NTE activity in COS7 is approximately 30% of that exhibited in SH-SY5Y cells.

Distribution of EGFP tagged NTE protein constructs in transfected mammalian cells

Because the NTE protein is tagged with EGFP, the distribution of NTE can be analysed directly using microscopy. As shown in Fig. 2, SH-SY5Y cells transfected with pEGFP-N3 alone demonstrated fluorescence that was distributed throughout the cell (Fig. 2a). However, in SH-SY5Y

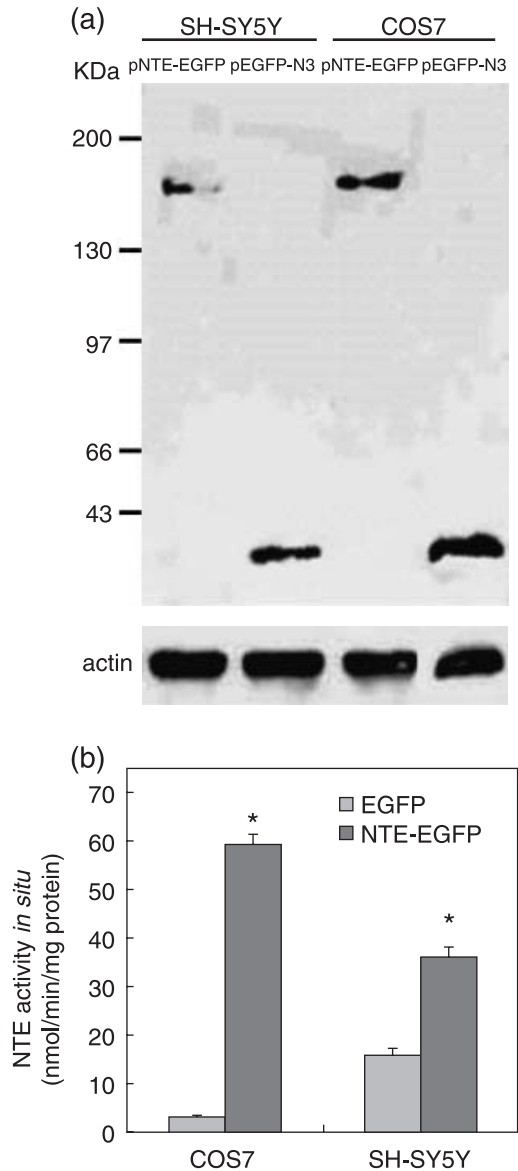


Figure 1. Expression of NTE-EGFP constructs in the transfected COS7 and SH-SY5Y cells. SH-SY5Y cells and COS7 cells were transfected with either control vector pEGFP-N3 or NTE tagged with EGFP-expressing vector pNTE-EGFP. After 48 h post-transfection, the cells were harvested. NTE activity in the cells was determined and whole cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. (a) Western blotting analysis of the expression of NTE tagged with EGFP. Migration of molecular weight standard proteins is indicated to the left of the figure. (b) Quantitative analysis of NTE activity. Data represents NTE activity in the transfected COS7 and SH-SY5Y cells. NTE activity was significantly elevated in NTE-EGFP transfected cells ($n = 4$, $*P < 0.01$).

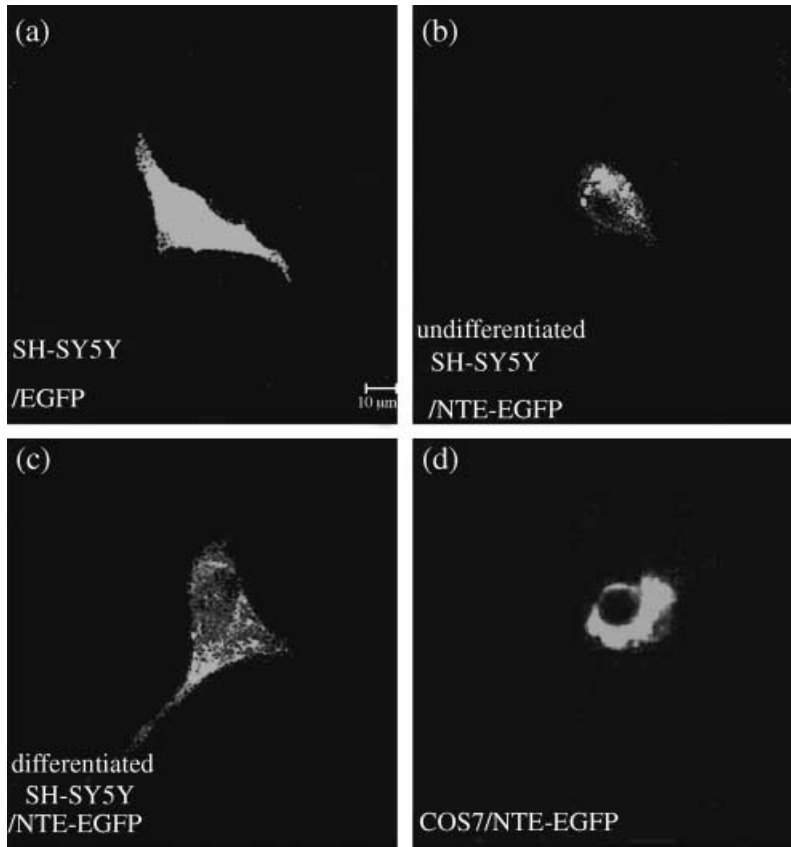


Figure 2. Distribution of EGFP-tagged NTE protein construct in transfected SH-SY5Y and COS7 cells. SH-SY5Y cells were transfected with pEGFP-N3 vector (a) or pNTE-EGFP (b and c) and visualized by confocal microscopy as described in Materials and Methods section. The expression of EGFP was distributed in whole cells (a); however, the marked abnormal distribution of NTE anchored on the endoplasmic reticulum surface in undifferentiated (b) and differentiated (c) cells expressing high levels of NTE-EGFP and weak fluorescence was detected in the neurite of the differentiated cell (c). High levels of NTE-EGFP protein are still expressed in COS7 cells after selection with G418 for 3 weeks (d), which suggested that high levels of NTE expression did not affect cell adhesion.

cells transfected with pNTE-EGFP, intense fluorescence was observed in the juxtannuclear area of both undifferentiated and differentiated cells (Fig. 2b,c), although fluorescence was weaker in the neurite of the differentiated cell (Fig. 2c). Transfectants of COS7 cells exhibited the same distribution pattern NTE expression. Interestingly, this distribution pattern was not altered even after COS7 was selected with G418 and NTE expression was sustained for 3 weeks (Fig. 2d).

High levels of NTE inhibit the proliferation of mammalian cells

To analyse the function of NTE in SH-SY5Y cells, we attempted to generate stable cell lines that would over-express NTE. However, although we were successful in generating stable SH-SY5Y cells that expressed EGFP alone (Fig. 3), our attempts to generate cell lines that over-expressed NTE were unsuccessful. These results suggested that high levels of NTE in human neuroblastoma cells may inhibit cellular proliferation. Indeed, this phenomenon was also observed in the non-neuronal COS7 cells (data not shown). Although we were unable to clonally expand

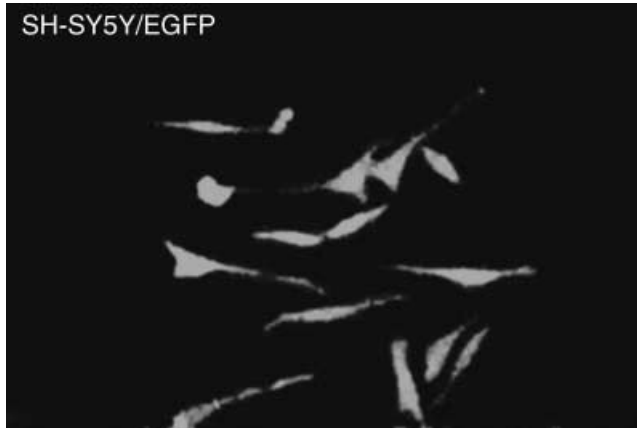


Figure 3. Photomicrograph of SH-SY5Y cells with stable expression of EGFP. SH-SY5Y cells were transfected with pEGFP-N3 vector and then selected with G418 for 3 weeks. Several stable expressing EGFP cell clones were obtained and visualized by confocal microscopy as described in Materials and Methods section.

cells that stably expressed NTE-EGFP, transfected cells did attach to glass slides and could be analysed by fluorescent microscopy even after sustained 3-week expression, suggesting that high levels of NTE did not inhibit cell attachment.

In order to investigate the effect of over-expression of NTE on cell proliferation, COS7 and SH-SY5Y cells were transiently transfected with either pEGFP-N3 or pNTE-EGFP and the cell viability was examined using the MTT assay. As shown in Fig. 4, COS7 and SH-SY5Y cells transfected with pNTE-EGFP was markedly reduced in cell number (curves of COS7/NTE-EGFP and SH/NTE-EGFP) compared to that transfected with pEGFP-N3 (curves of COS7/EGFP and SH/EGFP). Interestingly, the effect of NTE-EGFP expression on cell growth is more pronounced in COS7 cells than in SH-SY5Y cells after 48 h transfection; the reason for which is that the difference of MTT reduction between COS7/EGFP and COS7/NTE-EGFP is more than that between SH/EGFP and SH/NTE-EGFP. This is consistent with the above result that showed a higher NTE activity between COS7/NTE-EGFP and COS7/EGFP than between SH/NTE-EGFP and SH/EGFP (see Fig. 1b). These results indicate that over-expression of NTE in mammalian cells may inhibit cell proliferation in a manner depending on the levels of NTE activity.

Effect of over-expression of NTE on cell cycle in mammalian cells

To confirm the ability of NTE to inhibit cellular proliferation, we further analysed cell cycle profiles using FACS analysis. COS7 and SH-SY5Y cells were left untransfected, or transfected with either control vector (pEGFP-N3) or NTE expressing construct (pNTE-EGFP) and analysed at 48 h post-transfection. As shown in Fig. 5, untransfected cells and cells transfected with the control vector exhibited a similar cell cycle profile, suggesting that EGFP alone did not adversely affect the cell cycle (Fig. 5a–d). However, COS7 cells transfected with NTE-EGFP demonstrated an increase in the number of cells in G_2/M phase, from 11% to 32.3%, with a corresponding decrease in the number of cells in the G_1 phase, from 55.5% to 32.8%, when compared to cells transfected with control vector (Fig. 5c,e). Additionally, there was little change in the S fraction between cell populations. Together, these data indicate that over-expression of NTE in COS7 cells induces cell cycle arrest, specifically at the G_2/M phase. In contrast, over-expression of NTE in SH-SY5Y cells did not alter the cell cycle profile (Fig. 5d,f), suggesting that NTE does

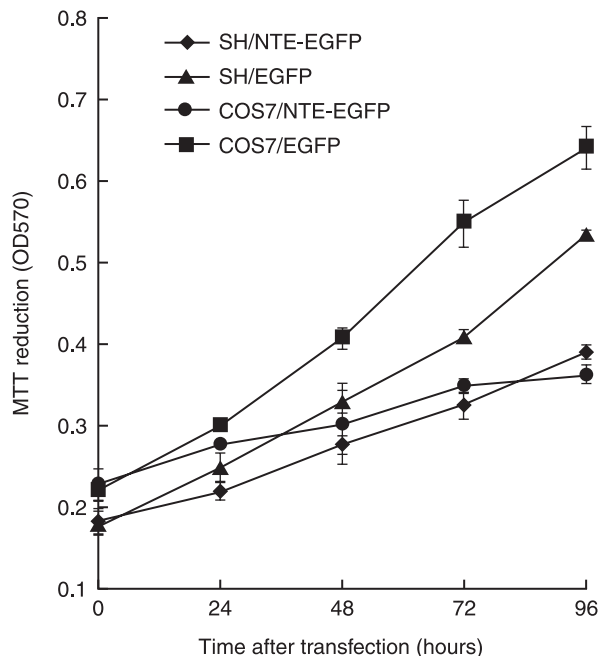


Figure 4. Effect of NTE expression on COS7 and SH-SY5Y cell viability. COS7 and SH-SY5Y cells were transiently transfected with pEGFP-N3 (vector control) or pNTE-EGFP construct. Cell viability was determined at various times using the MTT assay in SH-SY5Y cells expressing EGFP (SH/EGFP) or NTE tagged with EGFP (SH/NTE-EGFP) or COS7 cells expressing EGFP (COS7/EGFP) or NTE tagged with EGFP (COS7/NTE-EGFP). Data are presented as mean \pm SEM of three independent experiments.

not arrest cells in a particular phase in SH-SY5Y cells. Moreover, because there was no significant increase in the sub-G₁ population of NTE transfectants in either COS7 or SH-SY5Y cells, these data suggest that high levels of NTE did not cause cells to undergo apoptosis.

DISCUSSION

NTE was identified originally in adult vertebrate neural tissue as a protein targeted by organophosphates (OP), which cause a syndrome, OPIDN, of axonal degeneration (Johnson 1974). Elucidation of the role played by NTE in OPIDN and neural development is of obvious neurobiological interest. However, NTE is present not only in neurones but also in a variety of non-neural tissues including intestine, placenta, lymphocytes, kidney, liver and testicle (Williams 1983; Winrow *et al.* 2003). Furthermore, the existence of a putative homologue in *Saccharomyces cerevisiae* (Lush *et al.* 1998) suggests that NTE may be involved in a fundamental process common to cells from yeast to mammal.

Previously, it has been proposed that non-cytotoxic biochemical disruptions through NTE phosphorylation and ageing may lead to the inhibition of neurite and process outgrowth in neuroblastoma cells. This suggests that NTE may contribute to neuroblastoma development by regulating neuronal differentiation (Li & Casida 1998). However, in this study, we show that the over-expression of NTE in human neuroblastoma SH-SY5Y cells does not alter their phenotype.

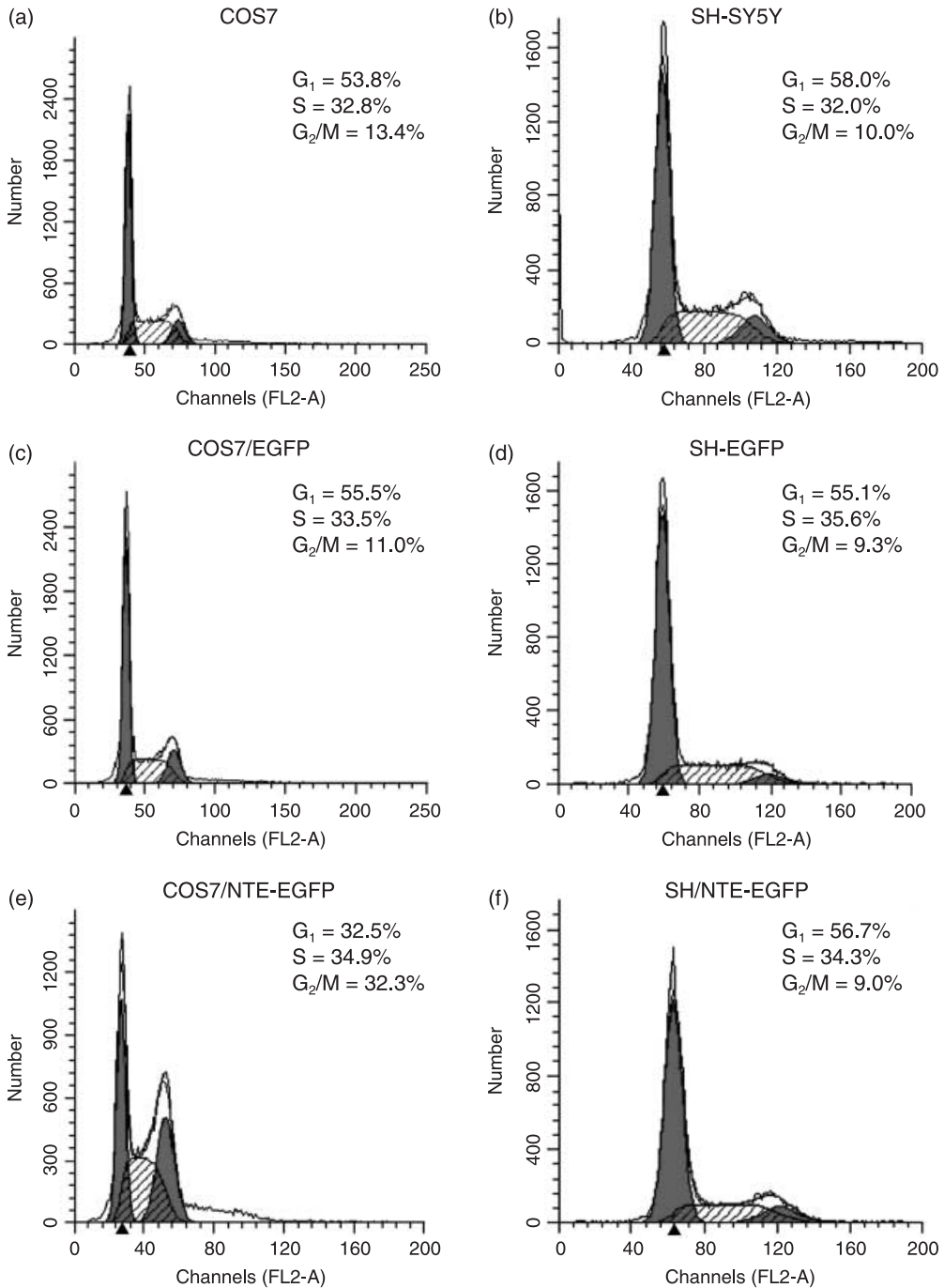


Figure 5. Effect of over-expression of NTE on cell cycle in mammalian cells. COS7 (a, c and e) and SH-SY5Y (b, d and f) cells were left untransfected (a and b), or transfected with either control vector pEGFP-N3 (c and d) or the pNTE-EGFP construct (e and f). At 48 h post-transfection, cells were collected and analysed for DNA content by propidium iodide staining and flow cytometry. DNA histograms of COS7 and SH-SY5Y transfected with no vector, pEGFP-N3 or pNTE-EGFP are shown. Percent of cell population at different stages in the cells is shown in corresponding DNA histogram.

Previous experiments in COS7 cells and primary cultures of hippocampal neurones demonstrated that NTE colocalized with calnexin, an ER marker (Li *et al.* 2003; Akassoglou *et al.* 2004). Herein, we showed that NTE tagged with EGFP was expressed and aggregated in the juxtannuclear area in SH-SY5Y cells. This abnormal distribution of calnexin may result from ER tubular structures that aggregated in the cytoplasm to form irregular clusters by interaction of NTE. Additionally, our results revealed that high levels of NTE in COS7 have no effect on cell attachment.

In COS7 and SH-SY5Y cells, high levels of NTE significantly inhibited cellular proliferation without altering cell adhesion, which suggests that NTE is involved in cell division and proliferation in both neural and non-neural cells. Human neuroblastoma SH-SY5Y cells expressing moderate levels of NTE induced neuronal differentiation, suggesting an increase of NTE may inhibit cell division and promote cell differentiation in part (Chang *et al.* 2005). Phosphatidylcholine is the major membrane lipid in eukaryotic cells and the homeostasis between synthesis and turnover of phosphatidylcholine is required for cell division and creation of new membranes (Jackowski 1994; McMaster & Bell 1994). Moreover, the disturbances in phosphatidylcholine metabolism have been associated with cell growth arrest (Cui & Houweling 2002). NTE exhibited potent lysophospholipase activity in mouse brain (Quistad *et al.* 2003), and was further characterized as a novel phospholipase B that was responsible for converting phosphatidylcholine to glycerophosphocholine in mammalian cells (Zaccheo *et al.* 2004), indicating a basic role for NTE in mammalian cells is to modulate the levels of phosphatidylcholine. Thus, the increase in NTE activity expressing NTE-EGFP protein may correlate with increased turnover of phosphatidylcholine and increase levels of glycerophosphocholine. It has been reported that the relatively modest degree (approximately 2-fold) of increased glycerophosphocholine production in HeLa cells over-expressing NTE compared with that in cells over-expressing NTE S966A mutant may, in part, reflect the inhibition of normal cell division in HeLa cells (Zaccheo *et al.* 2004). As such, higher levels of NTE may disrupt the phosphatidylcholine homeostasis, thus resulting in inhibition of cell proliferation.

Furthermore, cell cycle assays showed that high levels of NTE blocked the cell cycle progression at the G₂/M phase in COS7 cells over-expressing NTE protein. In contrast, inhibition of SH-SY5Y cell proliferation was not associated with arrest at a particular phase of the cell cycle as assessed by flow cytometry. These results suggested that high levels of NTE may inhibit cell proliferation in different kinds of cells by different mechanisms. The formation of membrane phosphatidylcholine is coordinated with the cell cycle (Jackowski 1994; Manguikian & Barbour 2004). Previous studies showed that general membrane phospholipid homeostasis was achieved by balancing the opposing activities of CTP-phosphocholine cytidylyltransferase (CCT) and calcium-independent phospholipase A2 (iPLA2) in some cell lines (Baburina & Jackowski 1999; Chiu & Jackowski 2001; Manguikian & Barbour 2004). However, it was reported that over-expression of NTE increases glycerophosphocholine production, which is offset by reduced choline uptake and incorporation into CDP-choline, suggesting a homeostatic coupling with CCT (Zaccheo *et al.* 2004). The levels of NTE activities in COS7 and SH-SY5Y cell lines differ (Fig. 1b). Previous other studies also indicated the levels of NTE differed among other different cell lines and suggested that it is possible that the homeostatic deacylation of phosphatidylcholine to glycerophosphocholine is mediated predominantly either by NTE or iPLA2 (with or without a distinct lysophospholipase) depending on the mammalian cell type (Glynn 2005). Therefore, the different effect of over-expression of NTE on cell cycle in COS7 and SH-SY5Y cells may be relative to the levels of NTE and its different role in the regulation of cell cycle. Clearly, further work is required to evaluate the relative contributions of NTE, iPLA2 and lysophospholipases to glycerophosphocholine production and their role in cell cycle in mammalian cells.

In summary, over-expression of NTE in human neuroblastoma cells led to an accumulation of NTE on the cytoplasmic surface of ER and inhibition of cell proliferation. Additionally, sustained high levels of NTE expression does not change the cellular morphology or induce apoptosis. Moreover, although high levels of NTE arrested COS7 cells at the G₂/M phase; inhibition of SH-SY5Y cell proliferation was not associated with arrest at a particular phase of the cell cycle. These results provide the first evidence that over-expression of NTE plays a role in cell proliferative response with different mechanisms in different cells.

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REFERENCES

- Akassoglou K, Malester B, Xu J, Tessarollo L, Rosenbluth J, Chao MV (2004) Brain-specific deletion of neuropathy target esterase/Swiss cheese results in neurodegeneration. *Proc. Natl. Acad. Sci. USA* **101**, 5075–5080.
- Atkins J, Glynn P (2000) Membrane association of and critical residues in the catalytic domain of human neuropathy target esterase. *J. Biol. Chem.* **275**, 24477–24483.
- Baburina I, Jackowski S (1999) Cellular responses to excess phospholipid. *J. Biol. Chem.* **274**, 9400–9408.
- Chang PA, Chen R., Wu YJ (2005) Reduction of neuropathy target esterase does not affect neuronal differentiation, but moderate expression induces neuronal differentiation in human neuroblastoma (SK-N-SH) cell line. *Mol. Brain Res.* **141**, 30–38.
- Chiu CH, Jackowski S (2001) Role of calcium-independent phospholipases (iPLA2) in phosphatidylcholine metabolism. *Biochem. Biophys. Res. Commun.* **287**, 600–606.
- Cui Z, Houweling M (2002) Phosphatidylcholine and cell death. *Biochim. Biophys. Acta.* **1585**, 87–96.
- Glynn P (2003) NTE: one target protein for different toxic syndromes with distinct mechanisms? *Bioessays* **25**, 742–745.
- Glynn P (2005) Neuropathy target esterase and phospholipid deacylation. *Biochim. Biophys. Acta* **1736**, 87–93.
- Jackowski S (1994) Coordination of membrane phospholipid synthesis with the cell cycle. *J. Biol. Chem.* **269**, 3858–3867.
- Johnson MK (1974) The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. *J. Neurochem.* **23**, 785–789.
- Johnson MK (1977) Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* **37**, 113–115.
- Li WW, Casida JE (1998) Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cell. *Toxicol. Lett.* **98**, 139–146.
- Li Y, Dinsdale D, Glynn P (2003) Protein domains, catalytic activity, and subcellular distribution of neuropathy target esterase in Mammalian cells. *J. Biol. Chem.* **278**, 8820–8825.
- Li Z, Szurek PF, Jiang C, Pao A, Bundy B, Le WD, Bradley A, Yu YE (2005) Neuronal differentiation of NTE-deficient embryonic stem cells. *Biochem. Biophys. Res. Commun.* **330**, 1103–1109.
- Lush MJ, Li Y, Read DJ, Willis AC, Glynn P (1998) Neuropathy target esterase and a homologous Drosophila neurodegeneration-associated mutant protein contain a novel domain conserved from bacteria to man. *Biochem. J.* **332**, 1–4.
- Manguikian AD, Barbour SE (2004) Cell cycle dependence of group VIA calcium-independent phospholipase A2 Activity. *J. Biol. Chem.* **279**, 52881–52892.
- McMaster CR, Bell RM (1994) Phosphatidylcholine biosynthesis via the CDP-choline pathway in *Saccharomyces cerevisiae*. Multiple mechanisms of regulation. *J. Biol. Chem.* **269**, 14776–14783.

- Moser M, Li Y, Vaupel K, Kretzschmar D, Kluge R., Glynn P, Buettner R. (2004) Placental failure and impaired vasculogenesis result in embryonic lethality for neuropathy target esterase-deficient mice. *Mol. Cell. Biol.* **24**, 1667–1679.
- Muhlig-Versen M, da Cruz AB, Tschape JA, Moser M, Buttner R, Athenstaedt K, Glynn P, Kretzschmar D (2005) Loss of Swiss cheese/neuropathy target esterase activity causes disruption of phosphatidylcholine homeostasis and neuronal and glial death in adult *Drosophila*. *J. Neurosci.* **25**, 2865–2873.
- Nostrandt AC, Ehrich M (1992) Development of a model cell culture system in which to study early effects of neuropathy-inducing organophosphorus esters. *Toxicol. Lett.* **60**, 107–114.
- Quistad GB, Barlow C, Winrow CJ, Sparks SE, Casida JE (2003) Evidence that mouse brain neuropathy target esterase is a lysophospholipase. *Proc. Natl. Acad. Sci. USA* **100**, 7983–7987.
- Williams DG (1983) Intramolecular group transfer is a characteristic of neurotoxic esterase and is independent of the tissue source of the enzyme. *Biochem. J.* **209**, 817–829.
- Winrow CJ, Hemming ML, Allen DM, Quistad GB, Casida JE, Barlow C (2003) Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity. *Nat. Genet.* **33**, 477–485.
- Zaccheo O, Dinsdale D, Meacock PA, Glynn P (2004) Neuropathy target esterase and its yeast homologue degrade phosphatidylcholine to glycerophosphocholine in living cells. *J. Biol. Chem.* **279**, 24024–24033.