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Role of Neu4L sialidase and its substrate ganglioside GD3 in neuronal apoptosis induced by catechol metabolites

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Abstract **Mammalian sialidases are key enzymes in the degradation of glycoconjugates. Neu4L sialidase is localized to mitochondria and specifically expressed in brain. To elucidate the pathophysiological roles of Neu4L in the nervous system, we investigated the possible involvement of Neu4L in the apoptotic neurodegeneration under the existence of catechol metabolites generated by tyrosinase. We demonstrated that: (i) the expression level of Neu4L was dramatically decreased prior to apoptosis; (ii) the apoptotic phenotype was characterized by cytochrome *c* release into cytosol concomitant with the trafficking of ganglioside GD3 to mitochondria; and (iii) the inhibitor of glucosylceramide synthase partially recovered cell viability. Neu4L and its substrate GD3 may act as key molecules in the mitochondrial apoptotic pathway in neuronal cells.**

Keywords: Sialidase; Neu4L; Ganglioside GD3; Mitochondria; Apoptosis; Neuronal death

1. Introduction

Mammalian cells have effective glycosylation machinery and specific pattern of expression of gangliosides is determined by the balance of their biosynthesis and degradation [1–3]. Sialidases constitute a family of enzymes that catalyze the removal of sialic acid residues from glycoconjugates. In addition to being a key enzyme in the degradation of glycoconjugates, mammalian sialidase has been considered to play crucial roles in various biological phenomena, such as cell proliferation, differentiation, cell death, signal transduction, and cell surface interactions [4–14]. Previous studies provided evidence for four types of sialidase that differ in subcellular localization and enzymatic properties [15–17]. They are classified as intralysosomal sialidase (Neu1), cytosolic sialidase (Neu2), and membrane-associated sialidases (Neu3 and Neu4). Neu3 is localized mainly in caveolae microdomains of plasma membrane [18,19], whereas Neu4 is recovered predominantly in the mitochondrial/lysosomal membrane fractions [20]. These mammalian sialidases have been cloned and characterized so far and very recently, murine and human Neu4 cDNA has been cloned [20–23].

The Neu4 cDNA encodes two isoforms. These isoforms preferentially hydrolyze gangliosides as well as glycoproteins and oligosaccharides. Although the isoforms cannot be distinguishable by substrate specificity, they exhibited different subcellular localization. The long form (Neur4L) possesses an N-terminal 12 amino-acid sequence predicted to be a mitochondrial sorting signal and is distributed in the mitochondrial inner and outer membranes [20]. Interestingly, in contrast to the short form (Neu4S) which is ubiquitously expressed, Neu4L is specifically expressed in brain [20]. Although the physiological roles of Neu4L in brain still remain elusive, its substrate specificity and subcellular localization suggest that it may be implicated in the mitochondrial apoptotic pathway in neuronal cells [20,24–26].

We previously established SH-SY5Y cell lines expressing human tyrosinase under the transcriptional control of exogenous inducer [27,28]. Overexpression of tyrosinase in cultured neurons resulted in: (1) increased intracellular dopamine content; (2) induction of oxidase activity not only for DOPA but also for dopamine; and (3) increased intracellular reactive oxygen species [28]. Furthermore, co-expression of α-synuclein in these cells facilitated apoptotic cell death accompanied by significant reduction of mitochondrial membrane potential [27]. In the present study, using this cellular model, we investigated the possible involvement of Neu4L and its substrate ganglioside GD3 in the apoptotic cell death under the existence of catechol-oxidized metabolites generated by tyrosinase. We demonstrate that overexpression of tyrosinase causes apoptotic neurodegeneration in SH-SY5Y cells. The apoptotic phenotype is characterized by cytochrome *c* release into neuronal cytosol concomitant with the trafficking of GD3 to mitochondria. Furthermore, the expression level of Neu4L is dramatically decreased in the early course of apoptosis. Our data provide evidence that Neu4L and its substrate GD3 may be implicated in the mitochondrial apoptotic cascades in neuronal cell death.

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Abbreviations: DMEM, Dulbecco's modified eagle's medium; D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; Ab, antibody; DA, dopamine; Apaf-1, apoptosis protease activating factor-1; AIF, apoptosis inducing factor

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2. Materials and methods

2.1. Cell culture

In a previous study, we established SH-SY5Y neuroblastoma cell line (TR5TY6 clone) expressing human tyrosinase under the transcriptional control of the T-REx™ Tetracycline-regulated mammalian expression system (Invitrogen) [28]. In this cell line, addition of tetracycline (Sigma, 1 μg/ml) to the culture media, resulting in a depression of the cytomegalovirus promoter activity, promotes expression of the tyrosinase encoded by pcDNA4/TO/tyrosinase. Cells were maintained in Dulbecco's modified eagle's medium (DMEM; Invitrogen/GIBCO) containing 7 μg/ml Blasticidin S (Invitrogen) and 300 μg/ml Zeocin (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen/GIBCO) and 2 mM L-glutamine (Invitrogen/GIBCO) at 37 °C under humidified 5% CO_2/air.

2.2. Cell viability assay and detection of DNA fragmentation

Cells (0.5×10^5 cells/cm^2), seeded in 96-well plates, were incubated overnight and, thereafter, cultured in DMEM with or without tetracycline for indicated periods. In some experiments, cells were treated for 12 h with an inhibitor of glucosylceramide synthase, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP; Sigma) prior to the tyrosinase induction. Cell survival rates were evaluated using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as described elsewhere. Assessment of apoptosis by DNA fragmentation were conducted using whole cell lysates (1×10^6) according to the method described elsewhere [29].

2.3. RT-PCR analyses of mammalian sialidases

Total RNA was extracted from cultured cells using the RNA-Bee™ reagent (TEL-TEST, Inc.) according to the manufacturer's protocol. From 5 μg total RNA, cDNA was synthesized using 200 U murine leukemia virus-reverse transcriptase (Superscript™ III RT; Invitrogen) and oligo(dT)$_{12–18}$ primers in a final volume of 20 μL. The cDNAs were used as a template for PCR amplification. The following primer pairs were used: Neu1-F (5′-TGAGAACGACTTCGGTCTGGTG-3′), and Neu1-R (5′-CCAGGAAACACCATCATCCTTG-3′) for Neu1 (403-bp), Neu3-F (5′-GACTGGTCATCCCTGCGTAT-3′), and Neu3-R (5′-GAGCCATGATTCTGACGGTGTT-3′) for Neu3 (469-bp), Neu4-F (5′-CCGTCTTCCTCTTCTTCATCGC-3′), and Neu4-R (5′-CATTGCAGTAGAGGAAGCTGCC-3′) for Neu4 (411-bp), and Neu4L-F (5′-CCACCCATGATGAGCTCTGCAG-3′), and Neu4L-R (5′-GCGATGAAGAAGAGGAAGACGG-3′) for Neu4L (447-bp). PCRs were conducted under the conditions described previously [20]. To normalize sample variations, the expression of β-actin was measured as a loading control. Gel photo was scanned and densitometric analysis was performed using the Scion Image software, version 4.03 (Scion Corporation).

2.4. Subcellular fractionation

Collected cells (5×10^7) were homogenized in 0.8 ml of ice-cold fractionation buffer mix using Dounce tissue grinder (50 strokes in each preparation). Cell lysates were separated into mitochondrial and cytosolic fractions by using ApoAlert™ Cell Fractionation Kit (Clontech) according to the manufacturer's specifications. Antibodies (Abs) to β-tubulin and Cox 4 were used to confirm the successful separation of mitochondrial and cytosolic fractions. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.5. Western blot analysis

SDS–PAGE and Western immunoblot analyses were performed according to established technique [30]. In brief, 10 μg of total protein from cell lysates were electrophoresed on a 13% SDS–PAGE gel and transferred to a PVDF membrane (Immobilon-P; Millipore). The membranes were incubated with anti-cytochrome *c* Ab (1:100), anti-Cox 4 Ab (1:250, as parts of ApoAlert kit described above), and anti-β-tubulin Ab (1:500; Sigma) followed by horseradish peroxidase-conjugated secondary Ab (1:1000; Amersham Biosciences) and developed with the ECL plus detection system (Amersham Biosciences). Immunoreactive bands were visualized with a LAS-3000 luminescent image analyzer (Fuji Photo Film). Densitometric analysis was performed as described previously.

2.6. Immunocytochemistry

Immunocytochemical studies were conducted as previously described [31]. The following primary antibodies were used: mouse monoclonal anti-ganglioside GD3 (1:500; abcam), mouse monoclonal anti-tyrosinase (1:2000; NeoMarkers), and rat polyclonal

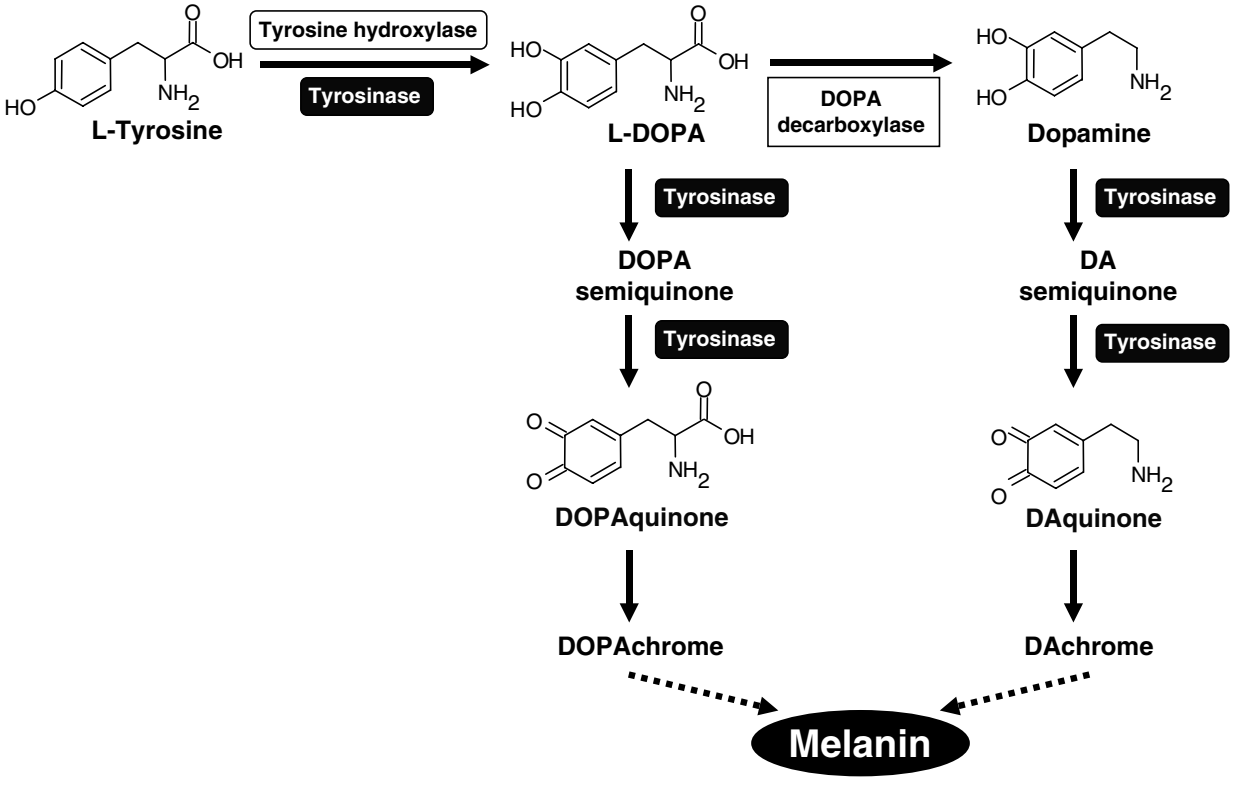

Fig. 1. Generation of cytotoxic catechol *o*-quinones during the enzymatic action of tyrosinase.

anti-cytochrome *c* (1:500; Santa Cruz Biotechnology). Positive immunostainings were detected after treatment with AlexaFluor 488 and AlexaFluor 647-conjugated secondary Abs (1:5000; Molecular Probes). For the staining of mitochondria, cells were incubated with MitoTracker Green (Molecular Probes) prior to fixation. Fluorescent images were analyzed with a FV300 confocal laser scanning microscope system (Olympus) equipped with HeNe-Green (543 nm), HeNe-Red (633 nm), and Ar (488 nm) laser units. In double labeling experiments, images were collected using a single excitation for each wavelength separately, and then were merged by a Fluoview image acquisition software (version 4.3; Olympus).

2.7. High-performance thin-layer chromatography

Cells (10^7) were mechanically harvested and washed with PBS and lyophilized. The glycolipids were extracted in sequence with 5 ml of chloroform/methanol (C/M) (1:1, v/v), 2.5 ml of C/M (2:1, v/v), and 2.5 ml of C/M (1:2, v/v), and then evaporated to dryness. After desalting by dialysis, the glycolipids were again lyophilized and dissolved in a small volume of C/M (2:1, v/v) and one-tenth of the amount was chromatographed on high-performance thin-layer chromatographic plate (Baker) in C/M/0.2% $CaCl_2$ (60:40:9, v/v/v). Glycolipids were visualized with the orcinol-H_2SO_4.

2.8. Statistical analysis

The data from the MTT assay were statistically analyzed using multivariate analysis of variance (ANOVA) using the Dr SPSS II statistical software for Windows (SPSS Japanese Inc.). Each treatment and corresponding control were carried out in triplicate. Eight wells per group were used and the data were reported as means ± S.E.

3. Results and discussion

3.1. Catechol-oxidized metabolites generated by tyrosinase induce mitochondria-mediated apoptosis in SH-SY5Y cells

Although glycoconjugates including gangliosides are major constituents of vertebrate brain and much evidence has accu-

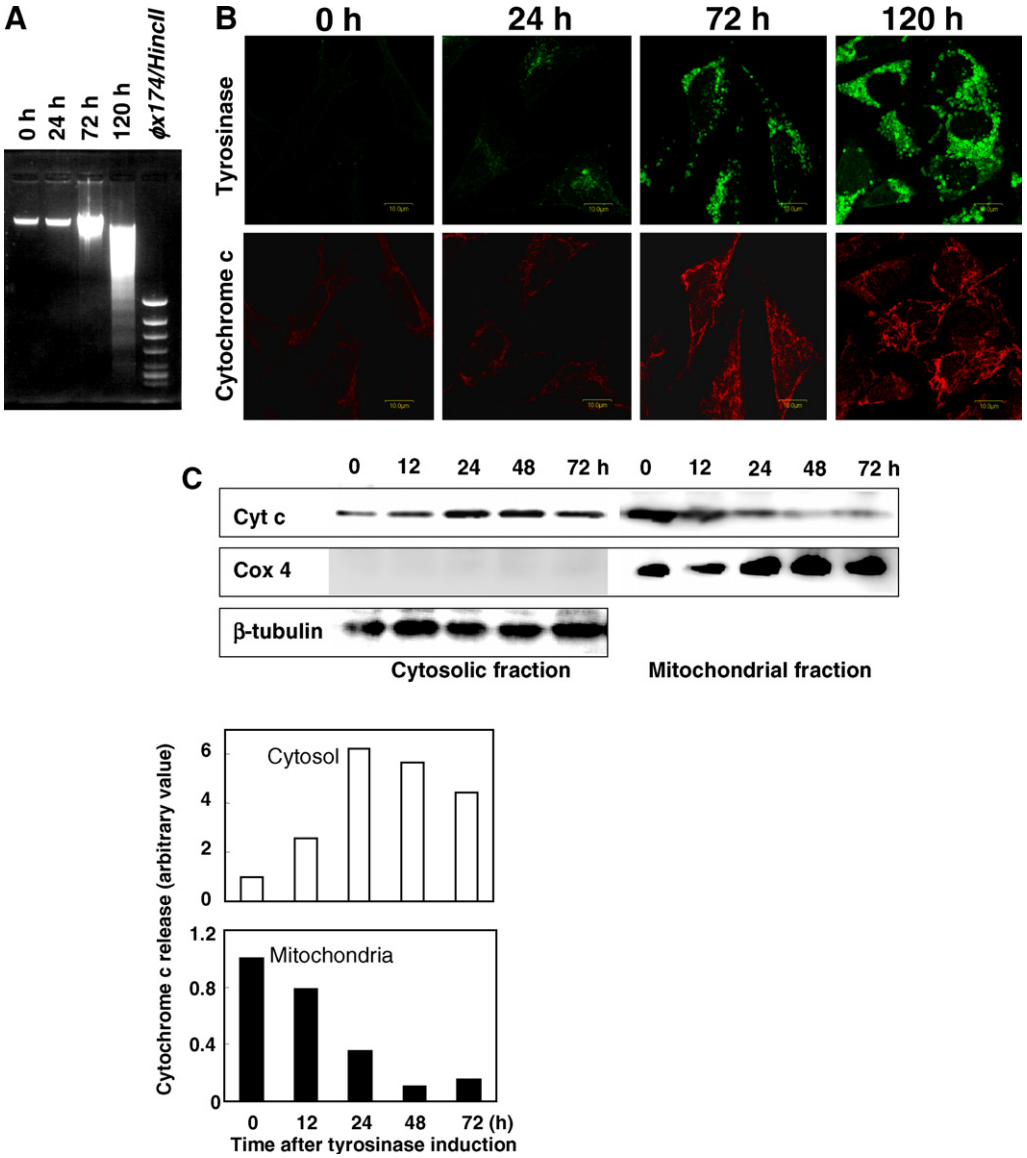

Fig. 2. Overexpression of tyrosinase induces mitochondria-mediated apoptosis in SH-SY5Y neuroblastoma cells (A). Typical internucleosomal DNA fragmentation is observed after 120 h of tyrosinase induction. Time-dependent cytosolic accumulation of cytochrome *c* confirmed by immunocytochemical (B: tyrosinase, green; cytochrome c, red, scale bar = 10 μm) and Western blot analyses (C). Expression of β-tubulin and cox-4 are measured to confirm successful fractionation. Densitometric analysis shows the intensity of the cytochrome *c* bands of cytosolic and mitochondrial fractions.

mulated suggesting that disialoganglioside GD3 is indispensable for the activation of the mitochondrial pathway of cell death [25,26,32–36], the role of ganglioside GD3 in neuronal apoptosis is still obscure. Likewise, the potential role of ganglioside-modifying enzymes in the process of neurodegeneration remains to be elucidated. In the present study, we have addressed this issue in the tyrosinase-overexpressing neuroblastoma cell lines [28]. Human tyrosinase catalyzes both the hydroxylation of tyrosine to L-DOPA and the subsequent oxidation of L-DOPA and dopamine (DA) to their *o*-quinone-metabolites (Fig. 1) [28,37]. These catechol-oxidized metabolites are highly cytotoxic and it has been shown that the artificial expression of tyrosinase in cultured cells triggers the cascade of melanin synthesis and produces toxic intermediates followed by growth retardation or cell death [38,39]. Indeed, we previously demonstrated that following the induction of tyrosinase in SH-SY5Y cells, small pigmented granules similar to neuromelanin appeared in the cytoplasm. After 72 h of tyrosinase induction, cell growth gradually declined and began to detouch from the culture plate [27]. Consistent with these previous observations, 120 h after induction of tyrosinase, internucleosomal DNA fragmentation, a hallmark of apoptosis, was observed in this cellular model (Fig. 2A). Control cells expressing empty vector alone did not show any change in cell growth (data not shown).

The toxicity of DA and L-DOPA is well documented using cellular and animal models [40]. Previous report has provided evidence that addition of DA in culture media could induce SH-SY5Y cells to release cytochrome *c* from mitochondria in a time-dependent manner [41]. In addition, cytosolic cytochrome *c* binds to apoptotic protease activating factor-1 (Apaf-1) and subsequently, triggers the sequential activation of caspase-9 and caspase-3 [42]. Using subcellular fractionation and Western blotting analysis, we confirmed that the overexpression of tyrosinase resulted in cytosolic accumulation of cytochrome *c* (Fig. 2B and C), which in turn could effect the activation of caspase-9. Of note, caspase-3 inhibition potently blocks DA-induced apoptosis but does not affect cytochrome *c* release [41], indicating that caspase-3 activation is not required for cytochrome *c* release and therefore DA-induced cytochrome *c* release precedes the onset of apoptosis. These data suggest that cytochrome *c* release is not a consequence but rather an initiator of catechol-oxidized metabolites induced activation of caspases and subsequent apoptotic cell death. Taken together, our data clearly showed the overexpression of human tyrosinase in SH-SY5Y cells increased intracellular catechol-oxidized metabolites and ultimately led to apoptotic cell death via the activation of mitochondrial pathway.

3.2. Catechol-oxidized metabolites induce the trafficking of ganglioside GD3 to mitochondria in SH-SY5Y cells

It has been known that mitochondrion is a key destination for the apoptogenic ganglioside GD3, as shown by the reports that apoptosis induced by ceramide and tumor necrosis factor-α caused targeting of GD3 to mitochondria [36,43]. To determine whether the mitochondrial targeting of GD3 occurs in response to catechol-oxidized metabolites, we monitored the colocalization of GD3 in our cellular model following the tyrosinase overexpression by immunocytochemical methods (Fig. 3A). As predicted by previous reports [36], in basal condition, most of ganglioside GD3 in SH-SY5Y cells was predominantly located at the plasma membrane. However, after the induction of tyrosinase, GD3 underwent a striking redistribution to intracellular compartment accompanied by its disappearance from the plasma membrane. Furthermore, 120 h after the tyrosinase induction, a magnified image showed that GD3 fluorescence appeared to be largely colocalized with mitochondrial marker, MitoTracker (Fig. 3B). The redistribution of GD3 to mitochondria during apoptotic process has been detected in other cell lines such as rat hepatocyte [36],

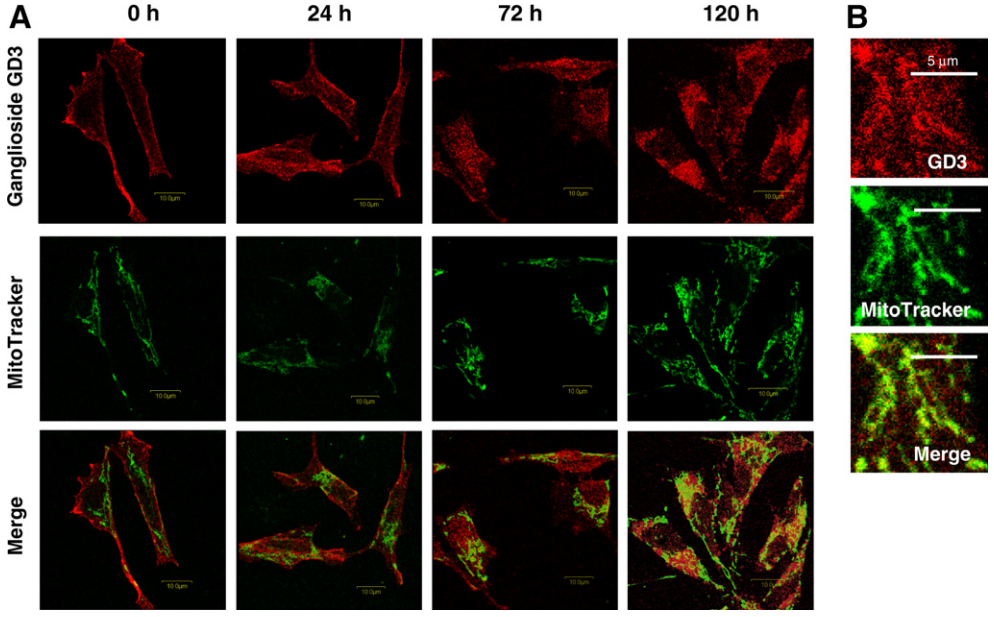

Fig. 3. Induction of tyrosinase causes a striking redistribution of GD3 to intracellular compartment accompanied by its disappearance from the plasma membrane (A). After 120 h, the magnified image shows that GD3 fluorescence (red) appeared to be largely colocalized with mitochondrial marker, MitoTracker (green) (B). Scale bar = 10 μm.

suggesting that this phenomenon is not a cell type-dependent event.

3.3. Early decrease of Neu4L expression during the apoptotic neuronal cell death induced by tyrosinase overexpression

The primary targets that are critical to the toxicity induced by catechol-oxidized metabolites is not understood, but likely candidates include many of the proteins important for the maintenance of normal mitochondrial functions [44,45]. Several reports documented that mitochondrion is a key organelle for the apoptotic process triggered by ganglioside GD3 [32,33,42]. Actually, GD3 is known as a cell death effector causing the loss of mitochondrial membrane potential accompanied by the release of several molecules such as Apaf-1, apoptosis inducing factor (AIF), caspase-9, and cytochrome *c* [25,26,35]. With regard to its substrate specificity and subcellular localization, it is plausible that Neu4L may regulate the level of mitochondrial GD3. To our surprise, among mammalian sialidase genes expressed in the nervous system [14,20,46,47], the expression of Neu4L began to decrease remarkably during the first 24 h of tyrosinase induction, and the partial decrement of Neu4 (L plus S) expression seemed to reflect the striking decrease of Neu4L expression (Fig. 4A). In control cells expressing empty vector, the expression level of Neu4L was unchanged (data not shown). In agreement with these findings, the ganglioside patterns by thin-layer chromatography revealed the time-dependent increase of endogenous GD3, GD1a, GM1 and GM2 after tyrosinase induction (Fig. 4B). In SH-SY5Y cell lines, there is a controversy about the endogenous expression of GD3 [48] presumably due to a low content of this molecule. It is also interesting to note that sialidase-resistant GM1 was increased

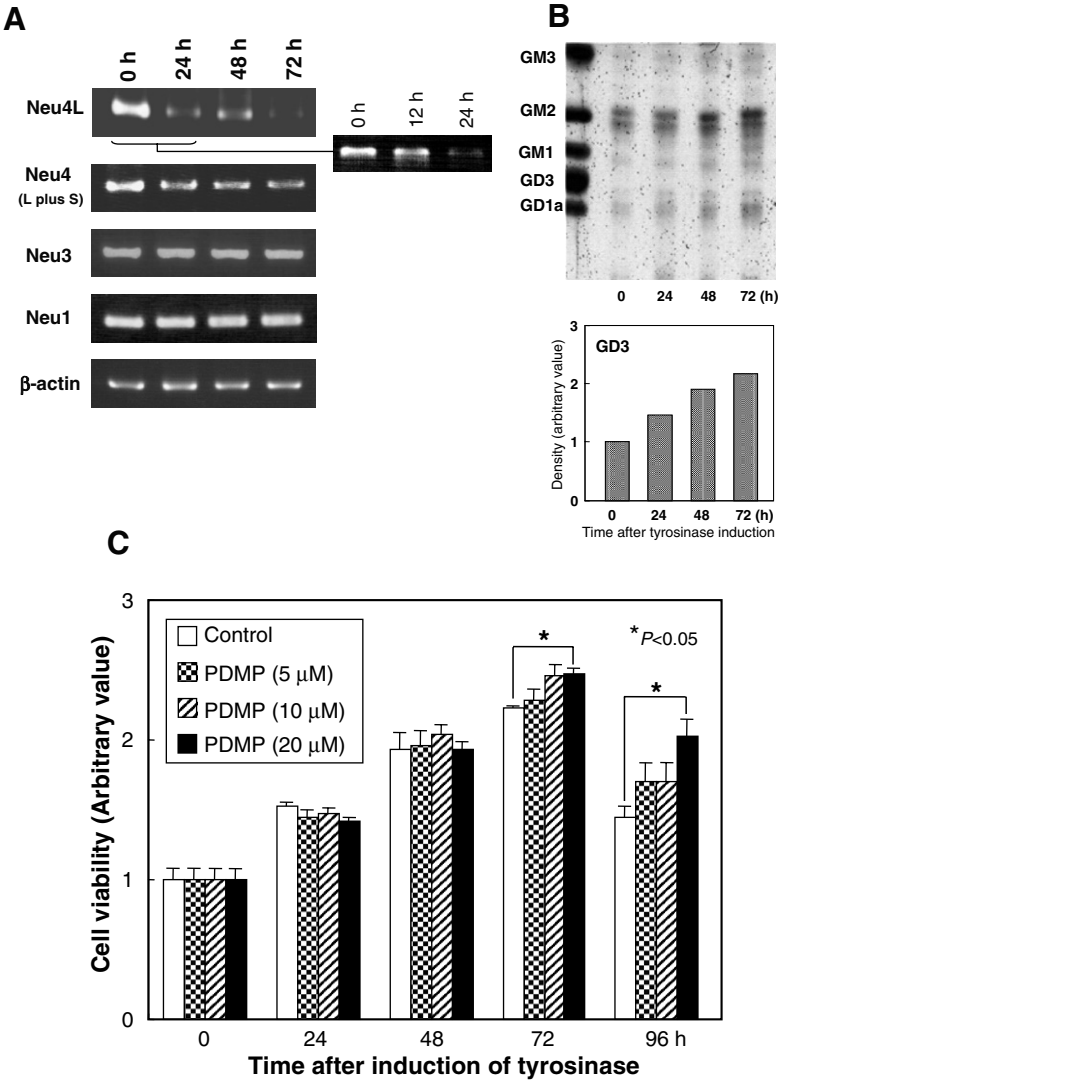

Fig. 4. RT-PCR analyses of three mammalian sialidases during the apoptotic process reproduced in this cellular model (A). Note the early decrement of Neu4L expression while the expression levels of other sialidases were not significantly altered. Expression of β-actin is measured as an internal control. Thin layer chromatographic analysis of ganglioside pattern in SH-SY5Y cells after the induction of tyrosinase (B, upper panel). Densitometric scanning analysis shows the intensity of the GD3 bands (lower panel). Pretreatment of D-PDMP for 12 h before the tyrosinase induction partially recovered cell viability in a dose-dependent manner (C). Cell survival rates were evaluated by MTT assay. Values shown represent means ± S.E. ($n = 8$, $P < 0.05$).

in spite of the downregulation of Neu4L, suggesting that GM1 biosynthesis might also be modulated in the apoptotic process.

Moreover, in accordance with previous observations [25,36], pretreatment of D-PDMP, a potent inhibitor of glucosylceramide synthase, partially recovered cell viability in a dose-dependent manner (Fig. 4C). Thus, these data imply that down regulation of Neu4L sialidase may induce the accumulation of mitochondrial GD3 followed by the induction of apoptotic cell death. Although further evidence is needed to establish critical roles of GD3 in neuronal degeneration, previous studies also suggested its involvement in apoptotic signaling. For example, it was reported that β-amyloid peptide induces the upregulation of intracellular GD3 in rat cortical neurons, which triggers the subsequent development of apoptosis [49]. In primary cultures of rat cerebellar granule neurons, early and transient increase of the ganglioside GD3 was shown to contribute to the development of apoptosis [35].

In conclusion, our data provide evidence that Neu4L and its substrate GD3 are involved in the neuronal apoptosis induced by catechol metabolites. Further experimental work is needed to check if downregulation of Neu4L is, in fact, the principal executioner of the apoptotic cascade in neuronal cells.

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