Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research

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ABSTRACT

Human neuroblastoma SH-SY5Y is a dopaminergic neuronal cell line which has been used as an in vitro model for neurotoxicity experiments. Although the neuroblastoma is usually differentiated by all-trans-retinoic acid (RA), both RA-differentiated and undifferentiated SH-SY5Y cells have been used in neuroscience research. However, the changes in neuronal properties triggered by RA as well as the subsequent responsiveness to neurotoxins have not been comprehensively studied. Therefore, we aim to re-evaluate the differentiation property of RA on this cell line. We hypothesize that modulation of signaling pathways and neuronal properties during RA-mediated differentiation in SH-SY5Y cells can affect their susceptibility to neurotoxins. The differentiation property of RA was confirmed by showing an extensive outgrowth of neurites, increased expressions of neuronal nuclei, neuron specific enolase, synaptophysin and synaptic associated protein-97, and decreased expression of inhibitor of differentiation-1. While undifferentiated SH-SY5Y cells were susceptible to 6-OHDA and MPP+, RA-differentiation conferred SH-SY5Y cells higher tolerance, potentially by up-regulating survival signaling, including Akt pathway as inhibition of Akt removed RA-induced neuroprotection against 6-OHDA. As a result, the real toxicity cannot be revealed in RA-differentiated cells. Therefore, undifferentiated SH-SY5Y is more appropriate for studying neurotoxicity or neuroprotection in experimental Parkinson’s disease research.

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1. Introduction

Parkinson’s disease (PD) is an aging-related disease with no effective treatments (Kedar, 2003). In studies of the properties of neurotoxins and development of new therapeutic compounds for disease management, in vitro cell culture models are often used (Segura-Aguilar and Kostrzewa, 2006), among which neuroblastoma SH-SY5Y cell line has commonly been chosen to study the pathogenesis of neurodegeneration (Chang et al., 2002; Xue et al., 2006; Zheng et al., 2006) and for drug screening (Levites et al., 2002a). 6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-pyridinium ion (MPP+) are commonly used neurotoxins in experimental PD.

As derived from neuroblastoma, SH-SY5Y cells are often induced to differentiate by All-trans-retinoic acid (RA) to obtain more neuron-like properties, including neurite outgrowth and morphological changes (Pahlman et al., 1984), so as to mimic responses of neurons in studies. RA has also been shown to down-regulate the mRNA and protein levels of the differentiation-inhibiting basic helix-loop-helix (Id) transcription factors (Lopez-Carballo et al., 2002). RA also activates survival signaling in neuroblastoma (Pailaud et al., 2002; Lee et al., 2006), promoting cell survival and reducing cell susceptibility to neurotoxins (Cavanaugh et al., 2006; Fernandez-Gomez et al., 2006). Therefore, we hypothesize that RA-mediated differentiation affects cell susceptibility to neurotoxicity study in PD research.

While RA-differentiated SH-SY5Y cell model has long been used for studies in neuroscience, undifferentiated SH-SY5Y has also been chosen as model cell line (Levites et al., 2002b; Levites et al., 2003; Xue et al., 2006; Lee et al., 2006). However, a report has shown that undifferentiated SK-N-SH cells (the sister cell line of SH-SY5Y cells) do not exhibit significant difference in their neuronal properties compared to that of the RA-differentiated SK-N-SH cells (Lombet et al., 2001). This raises a question on choosing between differentiated and undifferentiated cells in neuroscience research. It is still controversial about the necessity of differentiating SH-SY5Y cells with RA. Therefore, we aim to re-evaluate the changes of neuronal properties of human neuroblastoma SH-SY5Y cells upon...
RA-induced differentiation and cellular responses to different neurotoxins with or without differentiation.

To address this question, we compared immunoreactivity of different neuronal markers in undifferentiated and RA-differentiated SH-SYSY cells by Western-blot and immunocytochemical analysis. Next, we determined intracellular signaling altered by RA by Western-blot analysis. Finally, we examined the differential responses to 6-OHDA and MPP+. The results have implication in using RA-differentiated SH-SYSY cells for in vitro study of neurotoxicity.

2. Experimental procedures

2.1. Materials

6-OHDA was purchased from Sigma (Saint Louis, USA) whereas MPP+ was purchased from RBI (Wayland, MA, USA). Materials used for neuronal cell culture were purchased from Gibco-BRL (Invitrogen, NY, USA). Other chemicals used in this study were purchased from companies listed as follow: RA, dimethyl sulphoxide (DMSO), Triton X-100, 1,4-dithiobis (DTT), paraformaldehyde, protease inhibitor cocktail, phosphatase inhibitor cocktail, Tween-20, Temed, 30% acrylamide, mouse anti-β-actin monoclonal antibody and rabbit anti-SAP97 from Sigma; cyto toxicity detection kit (LDH) and cell proliferation kit (MTT) from Roche Diagnostics (Mannheim, Germany); colorimetric caspase-3 substrate (Ac-DEVD-pNA) from Calbiochem, Inc. (La Jolla, CA, USA); caspase-3 activity kit from Biosource (Camarillo, CA, USA); Alexa Fluor-488-conjugated goat anti-mouse IgG antibody, Alexa Fluor-488-conjugated goat anti-rabbit IgG antibody and prolong gold antifade reagent from Invitrogen (Molecular Probes, Oregon, USA); mouse anti-neuronal nuclei (NeuN), mouse anti-neurofilament monoclonal antibody and mouse anti-human gamma neuron specific enolase (NSE), mouse anti-synaptophysin, rabbit anti-tyro sine hydroxylase from Chemicon (Temecula, CA, USA); rabbit polyclonal antibodies for JNK, phospho-JNK, Akt, phospho-Akt, Erk, phospho-Erk, PKC, mTOR, phospho-mTOR, MAP2, Akt inhibitor LY600124 and JNK, phospho-JNK, Akt, phospho-Akt, Erk, phospho-Erk, phospho-PKC, mTOR, phospho-mTOR, MAP2, Akt inhibitor LY600124 and Erk1/2 inhibitor U0126 from Cell Signaling Technology (Beverly, MA, USA); rat anti-dopamine transporter (DAT) from Abcam (Cambridge, MA, USA); rabbit anti-inhibitor of DNA binding 1 (Id1) from Santa Cruz (CA, USA); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies from DAKO (Glostrup, Denmark); PVDf membrane and protein assay kit were from Bio-Rad (Richmond, CA, USA); Biomax X-ray film from Kodak (Tokyo, Japan); enhanced chemiluminescence (ECL) detection kit from Amersham (Buckinghamshire, UK).

2.2. Cell culture

The procedures for growing SH-SYSY cells has been described elsewhere (Chang et al., 2002; Suen et al., 2003). Briefly, SH-SYSY cells were cultured with 10% complete medium (minimum essential medium, MEM, 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) in a humidified, 5% CO2, 37°C incubator. Forty-eight hour after seeding, serum levels of the medium were reduced to 3% for the assay. The reaction underwent in dark environment for 30 min prior to measurement. Changes in absorbance were measured at 492 nm by a multicycle reader (Labsystem). Results were expressed as percentage of control.

2.3. Treatments

All treatments were performed under dark condition unless otherwise stated. A final concentration of 6-OHDA (25 μM) or MPP+ (1 mM) was used to treat SH-SYSY cells with or without differentiation. To investigate the role of Akt in attenuating 6-OHDA neurotoxicity after differentiation, undifferentiated or RA-differentiated SH-SYSY cells were pre-treated with LY600124 (5 μM) for 1 h, before subsequent treatment of 6-OHDA (25 μM).

2.4. Measurement of the release of LDH assay

General toxicity was measured by LDH assay. The procedures of the assay were followed according to the methods published elsewhere (Suen et al., 2003; Yu et al., 2005; Yu et al., 2006a; Ho et al., 2007). Briefly, culture medium was collected after treatment for the assay. The reaction underwent in dark environment for 30 min prior to measurement. Changes in absorbance were measured at 492 nm by a multicycle reader (Labsystem). Results were expressed as percentage of control.

2.5. Measurement of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined by a mitochondria enzyme dependent reaction of MTT as described elsewhere (Fang et al., 2005). Briefly, MTT was added to SH-SYSY cells after treatment in 96-well plates. Metabolic active cells cleaved the yellow tetrazolium salt MTT to purple formazan crystals. The formazan formed was solubilized and the absorbance was measured by a multiplate reader at 570 nm. Results were expressed as percentage of control.

2.6. Measurement of caspase-3-like activity assay

Apoptosis was determined by caspase-3-like activity assay. The procedures of caspase-3-like activity assay has been described elsewhere (Suen et al., 2003; Lin et al., 2004; Lai et al., 2006; Yu et al., 2006b; Yu et al., 2007b). Briefly, cellular proteins were harvested in lysis buffer after treatment. Proteins were separated by centrifugation at 14,000 g for 30 min at 4°C. Supernatant was collected and protein concentration was determined by protein assay kit (Bio-Rad). Equal amount of protein from each sample was incubated with caspase-3 substrate for 2 h at 37°C. The caspase-3-like activity was determined by the absorbance at 405 nm of the yellowish product (pNA) cleaved from the substrate. Specific activity (s.a., unit = pmol/min/μg) were calculated and reported in text. Results were expressed as percentage of control.

2.7. Western-blot analysis

Procedures of Western-blot were described elsewhere (Yu et al., 2004; Yu et al., 2007a; Lai et al., 2008). After treatment, SH-SYSY cells were harvested and lysed in ice-cold lysis buffer containing Tris (10 mM, pH 7.4), NaCl (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), Na2P2O7 (20 mM), Na2VO4 (2 mM), Triton X-100 (1%), glycerol (10%), SDS (0.1%), deoxycholate (0.5%). Phenylmethylsulfonyl fluoride (1 mM), protease inhibitor cocktail, and phosphatase inhibitor cocktail were added. Protein extracts (50 μg) were separated in 6 or 12.5% SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was blocked by 5% non-fat dry milk with BSA in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. It was then incubated with rabbit anti-Akt (1:1000 dilution), rabbit anti-phosphorylated Akt at serine 473 (1:1000 dilution), rabbit anti-phosphorylated PKCpan (1:1000 dilution), rabbit anti-JNK (1:1000 dilution), rabbit anti-phosphorylated JNK (1:1000 dilution), rabbit anti-Erk1/2 (1:1000 dilution), rabbit anti-phosphorylated caspase-3 activity kit from Biosource (Camarillo, CA, USA); Alexa Fluor-488-conjugated goat anti-mouse IgG antibody, Alexa Fluor-488-conjugated goat anti-rabbit IgG antibody and prolong gold antifade reagent from Invitrogen (Molecular Probes, Oregon, USA); mouse anti-neuronal nuclei (NeuN), mouse anti-neurofilament monoclonal antibody and mouse anti-human gamma neuron specific enolase (NSE), mouse anti-synaptophysin, rabbit anti-tyro sine hydroxylase from Chemicon (Temecula, CA, USA); rabbit polyclonal antibodies for JNK, phospho-JNK, Akt, phospho-Akt, Erk, phospho-Erk, PKC, mTOR, phospho-mTOR, MAP2, Akt inhibitor LY600124 and Erk1/2 inhibitor U0126 from Cell Signaling Technology (Beverly, MA, USA); rat anti-dopamine transporter (DAT) from Abcam (Cambridge, MA, USA); rabbit anti-inhibitor of DNA binding 1 (Id1) from Santa Cruz (CA, USA); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies from DAKO (Glostrup, Denmark); PVDf membrane and protein assay kit were from Bio-Rad (Richmond, CA, USA); Biomax X-ray film from Kodak (Tokyo, Japan); enhanced chemiluminescence (ECL) detection kit from Amersham (Buckinghamshire, UK).
anti-phosphorylated Erk1/2 (1:1000 dilution), rabbit anti-mTOR (1:1000 dilution), rabbit anti-phosphorylated mTOR (1:1000 dilution), rabbit anti-Id1 (1:1000 dilution), mouse anti-human NSE (1:500 dilution), mouse anti-NF (1:1000 dilution), rabbit anti-MAP2 (1:1000 dilution), mouse anti-NeuN (1:500 dilution), rabbit anti-Th (1:500 dilution), rat anti-DAT (1:500 dilution), mouse anti-synaptophysin (1:5000 dilution), rabbit anti-SAP97 (1:1000 dilution), or mouse anti-β-actin (1:5000 dilution) for 4 h at room temperature. Subsequently the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000, 1:2000 or 1:10000 dilution) for 45 min at room temperature. Bands were visualized on a Biomax X-ray film (Kodak) using an enhanced chemiluminescence (ECL) kit.

2.8. Immunocytochemical analysis of neuronal specific markers

Effects on neuronal characteristics by differentiation were investigated by immunocytochemical analysis of neuronal markers. The staining procedures were based on previous publication with modification (Chang et al., 2000; Chang et al., 2001). Briefly, cells were washed with Tris-buffered saline followed by fixation in 4% paraformaldehyde on ice or methanol in −20°C for 20 min. Non-specific binding of antibody was blocked by 1% BSA in Tris-buffered saline. The cells were subsequently incubated with mouse anti-NF (1:200 dilution), mouse anti-human NSE (1:200 dilution), rabbit anti-Th (1:200 dilution), mouse anti-synaptophysin (1:400 dilution) or rabbit anti-SAP97 (1:200 dilution) at 4°C overnight. Cells were then incubated with Alexa Fluor-488-conjugated goat anti-mouse or anti-rabbit IgG antibody. Fluorescent intensity was examined under a confocal microscope (Bio-Rad, Radiance2000). Laser scanning was performed under a 600× magnification to measure the average fluorescent intensity. Photos were taken from three randomly selected fields for each experiment and at least three independent experiments were performed.

2.9. Measurement of nitro blue tetrazolium (NBT) assay

Generation of intracellular reactive oxygen species was determined by a NBT as described elsewhere (Vrablic et al., 2001). Briefly, 200 μl NBT (1.0 mg/ml) was added to the medium of SH-SYSY cells at the end of the treatment period, followed by additional incubation of 2 h at 37°C. Intracellular superoxide anion catalyzed the conversion of NBT to purple formazan. Metabolic active cells cleaved the yellow tetrazolium salt MTT to purple formazan crystals. Cells were washed with PBS, and then harvested with 100 μl DMSO. The lysates were then dissolved in 100 μl 2 M KOH, and the absorbance at 570 nm was determined by using spectrophotometric method.

2.10. Statistical analysis

The results are expressed by mean ± S.E.M. from at least three independent experiments. For statistical comparisons, quantitative data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-test according to the statistical program SigmaStat® (Jandel Scientific, Chicago, IL, USA). A p-value less than 0.05 was regarded as significant.

3. Results

3.1. Differentiation properties of retinoic acid

To demonstrate that SH-SYSY cells can be differentiated by RA as in other reports, we first showed that extension of neurites, a typical neuronal phenotype, was observed 24 h after application of RA (data not shown) and such phenomenon was retained till day 7 (Fig. 1B), while undifferentiated cells maintained relatively short neurites (Fig. 1A). Protein expression of Id1 was significantly reduced after differentiation by RA, as revealed by Western-blot (Fig. 1C) and immunocytochemical analysis (Fig. 1D). Moreover, without differentiation, Id1 was shown to distribute throughout the cell but re-localized in cytoplasm after RA-induced differentiation (Fig. 1D).

3.2. Western-blot analysis on the changes in neuronal marker levels by RA

After showing successful differentiation by RA, we next sought to examine the effects of RA on neuronal properties. We chose to investigate neuronal markers including NSE, synaptophysin, postsynaptic associated protein-97 (SAP97), NeuN, NF, MAP2 and dopaminergic neuronal markers such as DAT and Th. Protein levels of neuronal markers in undifferentiated and RA-differentiated SH-SYSY cells were compared by Western-blot analysis (Fig. 2). We observed a significant increase in the expression of NSE, synaptophysin, SAP97 and NeuN in RA-differentiated cells. There was no significant change in the expression of NF, MAP2 and the dopaminergic neuronal markers DAT and Th between undifferentiated and RA-differentiated cells.

3.3. Immunocytochemical analysis on the changes in neuronal marker levels by RA

To confirm the changes in the expression of neuronal markers revealed by Western-blot analysis, immunocytochemical analysis was then performed. Fluorescent intensity of NSE, synaptophysin, SAP97, NF, and Th was compared between undifferentiated and RA-differentiated SH-SYSY (Fig. 3). In undifferentiated SH-SYSY, NSE immunoreactivity was mainly detected in the cytoplasm. After differentiation, there was a significant increase in fluorescent intensity of NSE, both in soma and neurites. Significant increases in fluorescent intensity in soma and neurites after differentiation were also observed in synaptophysin and SAP97. In agreement with the results from Western-blot, no significant change was observed in fluorescent intensity of NF and Th (Fig. 3).

3.4. Activation of survival signaling after differentiation by retinoic acid

To elucidate whether RA regulates survival signaling pathways, the phosphorylation of Akt, mTOR, Erk1/2 and PKC in SH-SYSY cells was examined by Western-blot analysis (Fig. 4A). We observed a marked increase in the phosphorylation of Akt at serine 473 after differentiation. However, there was no significant change in its downstream target mammalian target of rapamycin (mTOR). Furthermore, RA stimulated an increase in Erk 1/2 phosphorylation and reduced the phospho-PKC level in SH-SYSY cells.

3.5. Activation of c-Jun N-terminal kinase (JNK) pathway after differentiation by retinoic acid

Recent reports have shown that JNK pathway is involved in RA-mediated differentiation (Yu et al., 2003). Therefore, we investigated the effects of RA on JNK pathway by Western-blot analysis (Fig. 4B). In undifferentiated SH-SYSY, phosphorylated form of both JNK1 and JNK2 was barely detectable. However, phospho-JNK2 level was markedly increased 7 days after differentiation, while the increase in phospho-JNK1 was marginal. The phosphorylation state of the downstream c-Jun was also increased.
3.6. Differentiation effects of retinoic acid on the susceptibility of SH-SY5Y cells to 6-OHDA-induced neurotoxicity

We have demonstrated that RA-induced differentiation regulates both survival and stress signaling. Therefore, we sought to examine whether this also modulates the susceptibility of SH-SY5Y cells to neurotoxins. Cells with or without RA-induced differentiation were exposed to 6-OHDA (25 μM) for 24 h and then assayed for cell viability by LDH release, MTT assay and caspase-3-like activity. Release of LDH triggered by 6-OHDA was reduced by two folds in differentiated group (Fig. 5A). We also observed a corresponding increase in cell viability. After differentiation, MTT assay showed a near 0.3-fold increase in the number of viable cells compared to undifferentiated cells 24 h after exposure to 6-OHDA (Fig. 5B). In addition, the caspase-3-like activity was reduced by 0.5-fold in differentiated group (Fig. 5C).

3.7. Differentiation effects of retinoic acid on the susceptibility of SH-SY5Y cells to MPP+-induced neurotoxicity

The differentiation effects on another Parkinsonism mimetic toxin MPP+ were also investigated. Similarly, SH-SY5Y cells with or without differentiation were exposed to MPP+ (1 mM) for 24 h and then assayed for cell viability by LDH release, MTT assay and caspase-3-like activity. Release of LDH triggered by 6-OHDA was reduced by two folds in differentiated group (Fig. 6A). Accordingly, MTT assay showed a near 0.3-fold increase in the number of viable cells compared to undifferentiated cells 24 h after exposure to 6-OHDA (Fig. 6B). In addition, the caspase-3-like activity was reduced by 0.5-fold in differentiated group (Fig. 6C).

3.8. Effect of retinoic acid on the generation of 6-OHDA-induced reactive oxygen species in SH-SY5Y cells

6-OHDA can induce oxidative stress by generating reactive oxygen species (Soto-Otero et al., 2008). We sought to investigate...
Fig. 3. Immunocytochemical analysis of neuronal markers in SH-SY5Y cells with (RA) or without (Control) RA-differentiation. Changes in expression of NeuN, SAP97, NSE, synaptophysin, NF and Th were determined by their fluorescent intensity using confocal microscope. Scale bar = 50 μm.
the effect of retinoic acid differentiation on 6-OHDA-induced oxidative stress. By NBT assay, we observed a significant increase in the production of intracellular reactive oxygen species in SH-SY5Y cells after treatment of 6-OHDA. In RA-differentiated SH-SY5Y cells, there was a slight decrease in oxidative stress in both 6-OHDA and MPP+ treated group. However, such effect was not statistically significant (Fig. 7).

3.9. Attenuation of the RA-mediated neuroprotection in response to 6-OHDA toxicity by inhibiting Akt but not Erk1/2

We have shown that RA up-regulated Akt signaling, so we sought to examine whether Akt inhibitor would attenuate RA-mediated protection to neurotoxins. Undifferentiated or RA-differentiated SH-SY5Y cells were exposed to Akt inhibitor LY600124 (5 μM) for 1 h prior to 6-OHDA (25 μM) treatment for 24 h. Neurotoxicity in term of apoptosis was measured by caspase-3-like activity assay (Fig. 8). Inhibitor alone did not cause significant change in caspase-3-like activity. 6-OHDA caused marked increase in caspase-3-like activity in both undifferentiated (1.53 folds) and RA-differentiated cells (1.25 folds). Pre-treatment of Akt inhibitor exaggerated the toxicity of 6-OHDA by showing a 0.5-fold increase in undifferentiated group and 1-fold increase in differentiated group. More importantly, RA-mediated neuroprotection against 6-OHDA was removed, as pre-treatment of inhibitor led to a comparable toxicity in both differentiated and undifferentiated cells.

4. Discussion

The study demonstrates that RA-induced differentiation of SH-SY5Y cells modulates their responsiveness to neurotoxins by altering survival signaling pathways. Since both differentiated and undifferentiated SH-SY5Y cells have been widely used in neuroscience research, our study has high implication in using this cell line for neurotoxicity and neurotoxin studies.

The differentiation property of RA is well established (Goodall et al., 1997; Tosetti et al., 1998; Guarneri et al., 2000; Hashemi et al., 2003). It has been stated that neuroblastoma cells have to be differentiated in vitro for at least 7 days for experiment (Sarkanen et al., 2007). We re-confirm the differentiation property of RA in
SH-SY5Y cells by investigating the changes in morphology, differentiation markers and neuronal markers. Morphologically, we observed extensive neurites outgrowth as a typical neuronal phenotype in SH-SY5Y cells by RA (Fig. 1B), which is similar to other reports (Vesanen et al., 1994; Clagett-Dame et al., 2006). RA-differentiation showed a down-regulation and re-localization in Id1, which has been demonstrated both by our result and others (Lopez-Carballo et al., 2002).

Previously, Lombet and co-workers have demonstrated no significant change in expression of gamma subunit of NSE after RA-mediated differentiation on SK-N-SH cells, the sister cell line of SH-SY5Y cells (Lombet et al., 2001). However, in our system, we observed a significant increase in NSE protein expression after RA-mediated differentiation by both immunocytochemical and Western-blot analyses. The difference may be due to the low concentration of RA (3 μM) that they used for differentiation compared to our system.

To address whether RA-differentiated SH-SY5Y cells are functionally mature, protein expression of pre-synaptic protein synaptophysin has been studied. Synaptophysin is an abundant integral membrane protein on synaptic vesicles involved in the release of neurotransmitters (Rehm et al., 1986). It has been reported to be re-localized after differentiation by RA and cholesterol (Sarkanen et al., 2007). Similarly, we observed an increase in synaptophysin expression along neurites after differentiation by RA.

We showed that RA-mediated differentiation affects neuronal properties by further regulating other well established neuronal markers. Among the studied protein markers such as NeuN, NF,
MAP2 and dopaminergic neuronal markers DAT and Th, NeuN is expressed in neurons upon maturation neuron and therefore served as a marker for mature neurons (Mullen et al., 1992; Weyer and Schilling, 2003). In agreement with what have been reported, we found that RA-differentiation increased the expression of NeuN. Protein levels of NF and MAP2 have been reported to increase in short period (3–6 days) of RA-mediated differentiation (Sharma et al., 1999; Li et al., 2000; Pan et al., 2005). However, Encinas and co-workers obtained contrasting observations by showing a comparable expression pattern of NF and MAP2 between RA-differentiated and undifferentiated SH-SY5Y cells (Encinas et al., 2000). In agreement to their results, we did not observe significant change in the expression of NF and MAP2 with or without differentiation.

SH-SY5Y cells express dopaminergic markers so that they are widely used in PD research (Kitao et al., 2007; Kyratzis et al., 2007; Sung et al., 2007). We showed that the expression of DAT and Th is independent to the differentiation by RA. Together, our results suggest that there are limited changes in neuronal marker expressions after RA-mediated differentiation of SH-SY5Y cells.

The signaling involved in cell differentiation has been widely studied (Lopez-Carballo et al., 2002; Takeda and Ichijo, 2002; Lee and Kim, 2004; Moran et al., 2005; Song et al., 2005; Fontan-Gabas et al., 2007). Regarding the signaling involved in RA-mediated differentiation, PI3k/Akt, JNK and p35/cdk5 have been reported to play important roles in neurite outgrowth, cell cycle arrest and differentiation (Yu et al., 2003; Miloso et al., 2004). We also showed that RA-differentiation regulates some of these proteins. Interestingly, although mTOR is one important downstream protein of PI3k/Akt pathway, our results demonstrated that mTOR does not involve in RA-mediated differentiation process. Instead, we observed a significant decrease of active PKC after differentiation by RA, which has been shown to negatively regulate Akt signaling pathway in mouse keratinocytes (Li et al., 2006). Furthermore, inhibition of PKC has been reported to promote neuronal cells survival through Akt-dependent pathway (Zhu et al., 2004).

Apart from cell differentiation, RA can trigger survival signaling in different cell types (Paillaud et al., 2002; Lee et al., 2006). This leads to a suggestion that RA-mediated differentiation may also affect cellular response to neurotoxins. Our results show that RA-differentiated cells are less susceptible to Parkinsonism mimetic than undifferentiated cells. The resistance to Parkinsonism mimetic after differentiation by RA in our system was not due to dopaminergic neuronal markers DAT as we did not observe any significant change in DAT expression by RA. We also found that differentiation did not protect the cells from 6-OHDA-induced oxidative stress but it increased the mitochondrial activity, as revealed by NBT assay and MTT assay, respectively. However, there are reports showing that up-regulation of survival signaling (Akt and Erk1/2) can protect neurons from Parkinsonism mimetic (Jiang and Yu, 2005; Cavanaugh et al., 2006; Fernandez-Gomez et al., 2006; Fujita et al., 2006; Huang et al., 2007). We focus on the role of Akt in RA-mediated neuroprotection in response to 6-OHDA. After inhibition of Akt, caspase-3-like activity triggered by 6-OHDA in RA-differentiated cells is comparable to that of the undifferentiated cells, suggesting that Akt plays a role in RA-mediated neuroprotection in response to 6-OHDA toxicity during differentiation.

Taken together, RA differentiates SH-SY5Y neuroblastoma cells by altering specific neuronal markers. However, with the consideration that there is no significant change in dopaminergic properties and RA modulates the Akt pathway resulting in higher tolerance to 6-OHDA toxicity, undifferentiated SH-SY5Y may be more appropriate for studying neurotoxicity or neuroprotection in experimental PD research. Cautions should be taken if one attempts to use this cell type for investigating neurotoxic responses of drugs.

Conflict of interest statement

There is no potential conflict of interest or competing interest.

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