1 **A new method to effectively and rapidly generate neurons from SH-SY5Y cells**

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Abstract

It is well known that neurons differentiated from SH-SY5Y cells can serve as cell models for neuroscience research; i.e., neurotoxicity and tolerance to morphine *in vitro*. To differentiate SH-SY5Y cells into neurons, RA (retinoic acid) is commonly used to produce the inductive effect. However, the percentage of neuronal cells produced from SH-SY5Y cells is low, either from the use of RA treatment alone or from the combined application of RA and other chemicals. In the current study, we used CM-hNSCs (conditioned medium of human neural stem cells) as the combinational inducer with RA to prompt neuronal differentiation of SH-SY5Y cells. We found that neuronal differentiation was improved and that neurons were greatly increased in the differentiated SH-SY5Y cells using a combined treatment of CM-hNSCs and RA compared to RA treatment alone. The neuronal percentage was higher than 80% (about 88%) on the 3rd day and about 91% on the 7th day examined after a combined treatment with CM-hNSCs and RA. Cell maturation and neurite growth of these neuronal cells were also improved. In addition, the use of CM-hNSCs inhibited the apoptosis of RA-treated SH-SY5Y cells in culture. We are the first to report the use of CM-hNSCs in combination with RA to induce neuronal differentiation of RA-treated SH-SY5Y cells. Our method can rapidly and effectively promote the neuronal production of SH-SY5Y cells in culture conditions.

Key words: SH-SY5Y cells, RA, CM-hNSCs, neurons, β -tubulin III

Introduction

Neurons differentiated from SH-SY5Y neuroblastoma cells by RA (retinoic acid) treatment are widely used as *in vitro* cell models in neuroscience research topics including neurotoxicity and tolerance to morphine [4, 5, 9, 12, 17, 19, 20]. SH-SY5Y is a human-derived cell line, with the capacity to expand in culture prior to differentiation. In the literature, RA is the most commonly implemented and best-characterized agent to differentiate SH-SY5Y cells into neurons *in vitro*. However, only about 20% of SH-SY5Y cells can be differentiated into neurons with RA treatment alone [15]. In addition, it takes time (an average of 7 days) to produce a neuronal differentiation of SH-SY5Y cells with RA treatment alone [5, 16]. Recently, some investigators proposed that other chemicals (i.e., herbimycin A (herb-A), 12-O-tetradecanoyl-phorbol-13 acetate (TPA), and dibutyryl cyclic AMP [dbcAMP]) or neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) with or without extra cellular matrix (ECM) gel, can further improve neuronal differentiation and maintain these conditions in cultures of RA-treated SH-SY5Y cells [3, 6, 10, 15]. Cultivating SH-SY5Y cells in primary neuronal cultures or NSC (neural stem cell) culture medium with a B27 supplement has been shown to enhance neuronal differentiation with RA treatment [17]. However, the percentage of neuronal cells in the differentiated SH-SY5Y cells and the time needed to induce neuronal differentiation remain unsatisfactory under these combined conditions. Furthermore, these methods place an increased financial burden on experimental expenses. Thus, it is necessary to find an effective method to increase neuronal differentiation and production and at the same time to reduce the neuronal differentiation time of SH-SY5Y cells, thus making differentiated SH-SY5Y cells an ideal cell model.

NSCs (neural stem cells) have been successfully isolated from human brain tissue and can be expanded *in vitro* for an extended time in neural basal culture medium supplemented with B27 and mitotic factors bFGF (basic fibroblast growth factor) and

EGF (epidermal growth factor) [18]. Human NSC (hNSC) lines are currently commercially available for research use. It has been reported that NSCs can secrete many neurotrophic factors, such as BDNF, NGF, GDNF (glial cell line-derived neurotrophic factor), and NT-3 (neurotrophin-3), as well as other soluble factors in culture conditions [2, 8, 13]. Thus, CM-NSCs (conditioned medium collected from hNSC cultures) containing these already known neurotrophic factors may also contain unknown factors released by hNSCs. In previous studies, our group successfully generated self-renewable functional NSC-like cells from hBM-MSCs (human bone marrow-derived mesenchymal stem cells) cultivated in CM-NSCs [7, 14], demonstrating that CM-NSCs plays a crucial role in promoting the neuronal differentiation of these connective tissue cells. We hypothesized that CM-NSCs used to culture SH-SY5Y cells with an RA combination may increase neuronal differentiation and production of these cells.

Materials and Methods

1. Collection of CM-hNSCs

hNSCs were purchased from Lonza (Walkersville, MD, USA). The detailed method for the growth and maintenance of undifferentiated hNSC spheroids and for collecting CM-hNSCs was performed according to the protocol we established previously [14]. In brief, hNSCs were cultured in a T-75 flask containing 15ml complete culture medium including DMEM/F12 (Invitrogen, CA, USA), human recombinant epidermal growth factor (EGF; 20 ng/ml) and basic fibroblast growth factor (bFGF; 20 ng/ml) (R&D Systems, Minneapolis, MN, USA), B27 (serum-free medium supplements formulated to provide optimal growth condition for NSC expansion, 1 : 50; Invitrogen), heparin (5 µg/ml; Sigma, St Louis, MO, USA), 2 mM L-glutamine, and an antibiotic–antimycotic mixture (1 : 100; Invitrogen) at 37°C in a 5% CO₂ humidified incubation chamber (Fisher, Pittsburgh, PA, USA). Fifty percent of the culture medium was exchanged with fresh culture medium every 3 days. Larger neuro-spheroids were cut into small spheroids observed under a microscope (Olympus,

Japan) every 2 weeks. At each medium change, CM-hNSCs were collected and filtered through a membrane with a 0.22 μm in diameter pore size (Millipore, Billerica, MA, USA). The filtered conditioned mediums were centrifuged at 1000 RPM x 10 minutes at RT (room temperature) and observed under a microscope to make sure there was no cell contamination.

2. Neuronal differentiation of SH-SY5Y cells

SH-SY5Y cells were purchased from Sigma (St Louis, MO, USA). The cells were cultured in DMEM/F12 (Invitrogen), including 15% FBS, 2 mM L-glutamine, and an antibiotic–antimycotic mixture (1:100; Invitrogen) at 37°C in a 5% CO₂ humidified incubation chamber. SH-SY5Y cells used for the experiment were controlled between passages 25 and 30. The cells (1×10^5) plated onto poly-lysine coated coverslips (10mm in diameter) in 1ml culture medium were used for immunocytochemical studies while the cells (2×10^6) seeded into 10 cm tissue culture dishes in 10ml culture medium were used for Western Blot analysis, cell counting, and PI/annexin V staining. In total, four groups were divided. A & B: Cells were cultured in DMEM/F12 (Invitrogen) containing 3% FBS and 10 μM RA (Sigma) for 3 days (A) and 7 days (B) as control groups. C & D: The cells were cultured in CM-hNSCs containing 10 μM RA (Sigma) for 3 days (C) and 7 days (D) as experimental groups. Every day, the fresh medium was changed and each experiment was performed in duplicate.

3. PI (propidium iodide)/annexin V staining

After 7 days of differentiation, the cells were harvested from the tissue culture dishes by trypsin and prepared as a single cell suspension. We counted the number of differentiated cells by Countess™ Automated Cell Counter (Invitrogen) before performing PI/annexin V staining. The protocols for conducting PI/annexin V staining were according to the manufacturer's instructions (BD, 556547). In brief, 1×10^6 cells were re-suspended into 1ml 1 x binding buffer containing 5 μl annexin V and 5 μl PI

and incubated for 15min in the dark. Prior to flow cytometry (Dako, Denmark), a supplement of 400µl 1 x binding buffer was added to each sample.

4. Immunocytochemistry

At the 3rd and 7th day of the differentiation in culture, cells on the coverslips were washed twice using PBS and fixed in 4% PFA (paraformaldehyde, Sigma) for 30 min at RT. After washing twice in PBS, cells were blocked in PBS containing 5% goat serum for 1h at RT, followed by incubation with rabbit anti β-tubulin III (1:1000, Abcam) overnight at 4°C. The second day, cells were incubated with TRITC-conjugated goat anti-rabbit IgG (1:200 Jackson ImmunoResearch Lab) for 2 h in the dark at RT after washing in PBS twice. Finally, cells were coverslipped with the medium for fluorescence with DAPI (Vector Labs) and viewed by fluorescence microscopy (Olympus, Japan). The number of positive cells stained by specific antibodies was counted at x200 magnification. Data were averaged, expressed as the mean ± SEM, and compared between groups.

5. Western Blot

The Western blot procedure was performed according to the protocol described in publication [4]. At the 3rd and 7th day of the differentiation, cells on the tissue culture dishes were washed twice in PBS, treated in 0.25% trypsin and EDTA for 1 min at 37°C in a 5% CO₂ humidified incubation chamber and the suspending cells were collected. After that, cells were incubated in ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, 2.5 mM EDTA) including protease inhibitors (1mM, Beyotime, China) for 30 min at 4 °C. The cells were then centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was collected and protein concentration was measured using a BCA protein assay kit (Beyotime, China). Proteins were separated on 10% SDS polyacrylamide gels according to the molecular weight of the detection proteins and transferred onto PVDF membranes by electrophoresis. The membranes were blocked with 5% non-fat milk in TBST (TBS containing 0.1% Tween-20) for 1 h at RT and incubated with

rabbit anti- β -tubulin III (1:600, Abcam) and mouse anti- β -actin (1:1000, Santa Cruz) primary antibodies overnight at 4°C, followed by staining with goat anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish-peroxidase (HRP, Beyotime, China) for 1 h at RT. Finally, proteins were detected by chemiluminescence assay. Blots were repeated three times for every set of experiments. Bands were normalized to β -actin levels and the density of the bands were measured using Image J analysis software (NIH, USA).

6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad). Data were expressed as means with $\pm$ SEM. A two-sample t-test was used to analyze the apoptotic percentage between groups. A one-way ANOVA was performed to analyze other data unless specifically stated otherwise. $P < 0.05$ was used as the significance level.

Results

1. CM-hNSCs significantly improved the survival of RA-treated SH-SY5Y cells

To confirm whether CM-hNSCs have a protective effect on the survival of RA-treated SH-SY5Y cells, we counted the cell number by a Countess™-automated cell counter after different treatments. We found that RA resulted in a decrease in the number of treated SH-SY5Y cells, however, the cell number was significantly higher in the presence of CM-hNSCs compared to those in the absence of CM-hNSCs (Fig. 1 A, $**p < 0.01$), suggesting that CM-hNSCs have a beneficial effect on the survival of RA-treated SH-SY5Y cells. To investigate whether the decreased number of

RA-treated SH-SY5Y was attributable to cell apoptosis, we applied propidium iodide (PI) and annexin V staining for flow cytometry analysis. On the 7th day of RA treatment, the apoptotic percentage of differentiated SH-SY5Y cells in the presence of CM-hNSCs was about 19%±4.5% while the apoptotic percentage of differentiated SH-SY5Y cells in the absence of CM-hNSCs was about 76% ± 3.8% (Fig1 B, C & D). Taken together, these data suggest that RA can lead to the apoptosis of treated SH-SY5Y cells whereas CM-hNSCs can greatly counteract the cell apoptosis resulting from RA treatment.

2. CM-hNSCs significantly increased the neuronal production of RA-differentiated SH-SY5Y cells.

To assess the potential of RA-treated SH-SY5Y cells to differentiate into neurons in CM-hNSCs, we performed fluorescent immunocytochemical staining in cultured cells for the neuron-specific marker β -tubulin III. We observed that CM-hNSCs significantly increased the percentage of neuronal cells in RA-treated SH-SY5Y cells compared to that of neuronal cells in SH-SY5Y cells treated by RA alone (Fig. 2 A & C, **P<0.01). In the presence of CM-hNSCs, neuronal percentage in differentiated SH-SY5Y cells was increased from 10% to 88% after 3 days of differentiation and from 20% to 91% after 7 days of differentiation compared to RA treatment alone. No significant difference was detected between the percentage of neuronal cells differentiated for 3 and 7 days in the presence of CM-hNSCs and RA, suggesting that CM-hNSCs have substantially lessened the time for neuronal differentiation of

RA-treated SH-SY5Y cells. In addition, we observed that β -tubulin III-positive neurites were extensively long in the group using CM-hNSCs and RA treatment. However, very few β -tubulin III -positive neurites were detected in the group with RA treatment alone (Fig. 2 A). To confirm the increased neuronal differentiation of RA-treated SH-SY5Y cells with CM-hNSCs, we further examined the protein levels of neuron-specific marker β -tubulin III by Western blot. Our results showed that the protein levels of β -tubulin III with CM-hNSCs and RA treatment were significantly higher than those with RA treatment alone (Fig. 2 B & D, *P<0.05). Collectively, these data indicate that the combined application of CM-hNSCs and RA rapidly and effectively increased neuronal production of SH-SY5Y cells in culture compared to RA treatment alone.

Discussion

To date, the methodology to rapidly and effectively generate a good percentage of neuronal cells from SH-SY5Y cells for research purposes has been lacking although much effort has been expended, including the combined application of RA and other chemicals or neurotrophic factors, such as BDNF and NGF [3, 6, 10]. In the current studies, we demonstrated that the combined application of CM-hNSCs and RA in culture not only results in rapid and effective neuronal differentiation but also significantly increased the percentage of neuronal cells in the differentiated SH-SY5Y cells. In addition, CM-hNSCs demonstrated a beneficial effect on the survival of

differentiated SH-SY5Y cells and significantly decreased the apoptotic portion of the RA-treated SH-SY5Y cells.

It is well known that SH-SY5Y cells, morphologically characterized by neuroblast-like and non-polarized cell bodies with few and truncated processes, can continuously proliferate in culture but lack neuronal markers prior to differentiation [6]. β -tubulin III is a neuron-specific marker in which levels increase with the rate of neuronal differentiation and maturation. Previous studies reported that SH-SY5Y cells can exhibit this property for neurons in molecular and protein levels at 7 or more days when cultured in low serum culture conditions with 10 μ M RA supplements [16]. Our studies found that the percentage of β -tubulin III-positive cells in differentiated SH-SY5Y cells was only 10% on the 3rd day and about 20% on the 7th day when treated by RA alone (Fig. 2 A & C). These data suggest that there is an increased neuronal differentiation of SH-SY5Y cells with the increased duration of RA treatment. However, the percentage of apoptotic cells was also found to be increased with an increased duration of RA treatment, as demonstrated in our studies (Fig. 1). In addition, we found few neurites or fibers of differentiated neurons at 3 or 7 days after RA treatment (Fig. 2 A). These observations suggest that most neuronal cells resulting from RA-treated SH-SY5Y cells are immature. In contrast, the combined application of CM-hNSCs and RA in culture produced neurons with a round cell body and long processes; i.e., the morphological characteristics of typically mature neurons (Fig. 2A). Furthermore, the neuronal percentage detected from differentiated SH-SY5Y cells

was as high as 80% at 3 days only and about 91% at 7 days after combined RA and CM-hNSC treatment (Fig. 2. A & C). There was no significant difference in neuronal percentage between 3 and 7 days of RA and CM-hNSC treatment (Fig. 2 A & C). These data suggest that 3 days is sufficient for neuronal production of SH-SY5Y cells cultured in CM-hNSCs and RA and our protocol significantly shortened the time that SH-SY5Y cells needed to produce neuronal differentiation. In addition, the increased number of neuronal cells in the combined treatment of CM-hNSCs and RA is confirmed by the augmented expression of β -tubulin III in those differentiated SH-SY5Y cells with Western blot assay (Fig. 2A & B).

It was reported that RA can activate the survival signal pathways of differentiated SH-SY5Y cells by phosphorylation of Akt at serine 473 [4, 15]. In contrast to previous studies, our results showed that the total number of differentiated SH-SY5Y cells was significantly decreased following RA treatment. However, we also found that the number of RA-treated SH-SY5Y cells was significantly high in the presence of CM-hNSCs compared to that in the absence of CM-hNSCs (Fig. 1 A $^{**}P<0.01$), suggesting that CM-hNSC has a beneficial effect on the survival of differentiated SH-SY5Y cells. A previous *in vivo* study by other investigators showed that CM-hNSCs can reduce the number of caspase 3-positive apoptotic profiles of spinal cord injuries [11]. Consistently, our results further confirmed that the decreased number of differentiated SH-SY5Y cells is due to apoptosis resulting from RA treatment, whereas CM-hNSCs can protect these RA-differentiated SH-SY5Y cells

from apoptosis, as demonstrated by the PI/annexin V staining results (Fig. 1 B, **P<0.01).

Neuronally differentiated SH-SY5Y cells are effective *in vitro* cell models for various neuroscience experiments that need to express specific markers for neurons such as β -tubulin III. However, there are other specific markers for neuronally differentiated SH-SY5Y cells used to confirm that these cell models are suitable for specific disease research. For example, RA-treated SH-SY5Y cells can express adult splicing forms of tau with neuronal localization as well as tyrosine hydroxylase (TH) [1, 10], making these cells suitable for *in vitro* cellular models of Alzheimer's disease (AD) and Parkinson's disease (PD) research. In addition, they can also serve as a cell model for research in morphine tolerance, since the differentiated SH-SY5Y cells are able to express the opioid receptor μ [19]. It would be interesting to investigate the expression of these specific markers in neurons differentiated from SH-SY5Y cells under these culture conditions with a combination of RA and CM-hNSC applications.

Our studies have clearly shown that SH-SY5Y cells cultured in CM-hNSCs containing RA can effectively and rapidly differentiate into a number of neuronal cells. The CM-hNSCs used in culturing SH-SY5Y cells in the current studies are cell free, thus the possibility of neural cell contamination can be excluded. These results suggest that soluble neurotrophic factors released from hNSCs in the CM are important for the resulting neuronal production of SH-SY5Y cells. These soluble neurotrophic factors may include those already known; i.e., BDNF, NGF, GDNF, and

NT-3 [2, 8, 13], as well as many other unknown soluble factors. Although the combined application in cultures of RA and primary neuronal cells or the use of NSC culture medium supplemented with B27 or BDNF or NGF can help improve the neuronal production of SH-SY5Y cells [3, 6, 10], the degree and extent of the increased neuronal percentage in these studies are still low compared to that produced using our protocol, suggesting that the combination of the known and unknown soluble factors and/or some unknown soluble factors in the CM-hNSCs may have significantly contributed to the increased neuronal differentiation and production of SH-SY5Y cells in culture. In the current preliminary studies, however, we did not perform experiments to focus on those unknown soluble factors that exist in CM-hNSCs. Further studies are warranted to identify those unknown soluble factors by global proteomic analysis of CM-hNSC contents in the future, which may help elucidate the mechanism of CM-hNSCs in the neuronal differentiation of SH-SY-5Y cells.

In conclusion, we are the first to demonstrate that a combined application of RA and CM-hNSCs can not only rapidly and effectively promote the neuronal differentiation of SH-SY5Y cells but can also greatly increase the percentage of neurons in the treated SH-SY5Y cells. CM-hNSCs has also a beneficial effect on the survival of the differentiated SH-SY5Y cells and can protect these cells from the apoptotic processes resulting from RA treatment.

Acknowledgements

This research is supported by grants of 1) DoD (PR100499P1), 2) The Boothroyd Foundation, USA, 3) 5150 Program of Jinan City, and 4) Taishan Overseas Scholars Program of Shandong Province, China to T. QU.

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Legend

1. CM-hNSCs significantly improved the survival of RA-treated SH-SY5Y cells.

A. Bar graph showing that the cell number at the 3rd day was higher than that at the 7th day following RA treatment in the presence or absence of CM-hNSCs. However, note that the presence of CM-hNSCs significantly improved the cell survival rate, with a higher cell number than that in the absence of CM-hNSCs, at both of the 3rd and 7th day following RA treatment. One-way ANOVA, **P<0.01

B. Bar graph showing that the apoptotic percentage of RA-treated SH-SY5Y cells was

significantly decreased in the presence of CM-hNSCs. Two-sample *t*-test, ***P*<0.01

C & D. The dot plots showing the representative flow cytometric results of PI/annexin V staining in the absence (C) and presence (D) of CM-hNSCs at the 7th day following differentiation of the RA-treated SH-SY5Y cells.

2. CM-hNSCs significantly increased the neuronal production of RA-treated SH-SY5Y cells.

A. Representative fluorescent immunocytochemical results showing β -tubulin III-positive cells (red) in the population of differentiated SH-SY5Y cells examined at the 3rd and 7th day of RA treatment in the presence and absence of CM-hNSCs. Bar: 200 μ m.

B. Representative Western blot results showing the expression levels of β -tubulin III in the differentiated SH-SY5Y cells examined at the 3rd and 7th day of RA treatment in the presence and absence of CM-hNSCs.

C & D. Bar graph showing that when examined at the 3rd and 7th day following differentiation, a combined application of CM-hNSCs and RA significantly increased the number of β -tubulin III-positive cells (C) and the expression levels of β -tubulin III (D) in SH-SY5Y cells compared to RA treatment alone. One-way ANOVA, ***P*<0.01, **P*<0.05.

Figure1

Figure2