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Co-culture with neurotrophic factor secreting cells induced from adipose-derived stem cells: Promotes neurogenic differentiation

Shahnaz Razavi [a,*], Mohamad Reza Razavi [b], Mahsa Kheirollahi-Kouhestani [c], Mohammad Mardani [a], Fatemeh Sadat Mostafavi [a]

[a] Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan 81744-176, Iran
[b] Molecular Parasitology Laboratory, Pasteur Institute of Iran, Tehran 1316943551, Iran
[c] Institute of Genetic Medicine, International Centre for Life, Central Parkway, Newcastle upon Tyne, NE1 3BZ, United Kingdom

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ABSTRACT

Adipose-derived stem cells (ADSCs) and bone marrow stem cells (BMSCs) can be equally proper in the treatment of neurodegenerative diseases. However, ADSCs have practical benefits. In this study, we attempted to induce the secretion of neurotrophic factors (NTF) in human ADSCs. We then evaluated the effects of co-culture with NTF secreting cells in neural differentiation of human ADSCs. Isolated human ADSCs were induced to neurotrophic factors secreting cells. To evaluate the *in vitro* effects of NTF-secreting ADSCs on neurogenic differentiation of ADSCs, we used neurogenic induction medium (control group), the combination of neurogenic medium and conditioned medium, or co-cultured NTF-secreting ADSCs which were encapsulated in alginate beads (co-culture) for 7 days. ELISA showed increased (by about 5 times) release of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in NTF-secreting ADSCs compared to human ADSCs. Real time RT-PCR analysis revealed that NTF-secreting ADSCs highly expressed NGF and BDNF. In addition, co-culture with NTF-secreting ADSCs could also promote neuronal differentiation relative to gliogenesis. Overall, NTF-secreting ADSCs secrete a range of growth factors whose levels in culture could promote neuronal differentiation and could support the survival and regeneration in a variety of neurodegenerative diseases.

1. Introduction

Mesenchymal stem cell (MSC)-based therapies may be efficient in the treatment of neurological diseases. The efficiency of MSCs as a clinical therapeutic implement for neurodegenerative diseases has been recently investigated in two points, transdifferentiation into neural cells and replacing damaged cells [1] or production of cytokines as trophic factors for support and regeneration of damaged nervous tissue [2–4].

Bone marrow stromal cells (BMSCs) were the first type of adult stem cells to successfully differentiate into neuronal cells and promote neuronal survival in cerebral ischemia [2,5]. They have also been used in the functional recovery following spinal cord injury [6]. So; transplantation of MSCs can be enhancing regeneration of damaged neural tissues through secretion of cytokines and trophic factors [7].

Recent evidence suggests that the neurotrophic and neuroprotective effects of induced BMSCs can enhance survival and regeneration in a variety of neurodegenerative diseases [8–12].

However, BMSC harvest is an invasive and painful procedure that might yield few MSCs [13]. Therefore, an alternative cell source is preferred. Unlike bone marrow, adipose tissue is abundant and easily accessible. Moreover, its harvesting does not require any invasive and painful procedures [14].

Adipose-derived stem cells (ADSCs) with similar characteristics can successfully differentiate into chondrocytes, adipocytes, osteoblasts, myocytes, and neuronal linage [15–17]. In addition, compared to BMSCs, a higher proportion of ADSCs express nestin which is a marker of progenitor neural cells [18]. Several bioactive protein factors secreted by ADSCs have been identified [19]. ADSCs secrete a range of growth factors whose levels achieved in culture provided significant protection to neurons [20–23]. Zhao et al. reported that ADSCs possessed potent neuroprotective activity against neuronal excitotoxicity [24]. The protective effect of ADSCs was found to be highly dependent on the presence of brain-derived neurotrophic factor (BDNF), an important neurotrophic factor which prevents neuronal degeneration and is involved in neuronal development [25].

Moreover, conditioned media of MSCs culture have induced neuritogenesis in PC12 cells as well as neuroprotective effects against neurotoxicity agents via neurotrophin contains [24–26].

* Corresponding author. Fax: +98 311 792 2517.
E-mail address: razavi@med.mui.ac.ir (S. Razavi).

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However, only selected molecules have been dosed in ADSCs as possible neurotrophic candidates [27,28].

We hypothesized that human ADSCs act as source of variety of neurotrophic factors and can release more neurotrophic factors after induction. Therefore, we decided to induce human ADSCs to secret neurotrophic factors. In this study, we attempted to induce NTF secretion in human ADSCs. We then evaluated the effects of co-culture with NTF secreting cells on neural differentiation of human ADSCs.

2. Materials and methods

2.1. Preparation and culture of human MSCs

All chemicals, except where specified otherwise, were purchased from Sigma–Aldrich (St. Louis, MO). Human adipose tissue was obtained from lipoaspirate samples of abdominal fat from female donors (age range: 23–41 years old) who had provided informed consent. The tissue was cultured as described previously [29]. The cells used in the present study were from passages 3–6.

Flow cytometry analysis of the isolated cells was carried out as previously described [30].

2.2. Induction of human ADSCs into NTF secreting cells

The induction of human ADSCs into NTF secreting cells was carried out according to a previously described method [9]. We used glial fibrillary acidic protein (GFAP) and S100β to confirm the effectiveness of human ADSCs differentiation into NTF secreting cells. In order to detect the ability of the induced cells to secrete neurotrophin, CTNF, GDNF, NGF, and BDNF were assessed by real-time RT-PCR and ELISA.

We evaluated the *in vitro* effects of NTF-secreting ADSCs on neurogenic differentiation of ADSCs using neurogenic induction medium (control group), the combination of neurogenic medium and conditioned medium (CM-NTF) or co-cultured NTF-secreting ADSCs which were encapsulated in alginate bead (co-culture) for 7 days. Nestin, MAP2, and GFAP were used as markers to confirm neurogenic differentiation of human ADSCs using both immunostaining and real time reverse transcriptase PCR analysis.

2.3. Preparation of NTF-secreting ADSCs conditioned medium

Confluent NTF-secreting ADSCs were cultured for 3 days with no serum. The media was then removed and stored at −70 °C prior to use in culture experiments.

2.4. Neurogenic differentiation of human ADSCs

Human ADSCs were dissociated with 0.25% trypsin/0.02% EDTA. They were plated on low-attachment plastic tissue culture plates in DMEM, 2% B27, 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml hEGF with the formation of floating bodies. The cells were treated with aforementioned factors every 2 days up to 7 days. The spheres were then dissociated and became single by pipetting in trypsin/EDTA. After centrifugation, the cell pellet was cultured in neurobasal medium containing 5% FBS, 1% L-glutamine, 1% non-essential amino acids, 2% N_2 supplement, and 2% B27 for 1 week.

2.5. Preparation of encapsulated alginate beads containing NTF-secreting ADSCs

Alginate contains a natural linear polymer of 1,4-linked β-D-mannuronic acid (M) and α-glucuronic acid (G) with various compositional and sequential structures [31]. Such composition makes it a suitable hydrogel for cell microencapsulation.

Cell encapsulation was carried out by mixing a pellet of NTF-secreting ADSCs with alginate (50:50) and keeping it in a 0.1 M calcium chloride solution for 10 min. The encapsulation yielded beads with a final concentration of 1,000,000 cells/mL in 1% w:v alginate (approximately 20,000 cells per bead). Beads were cultured in 24-well plates containing 20,000 human ADSCs. They were allowed to differentiate in the presence of neurobasal medium supplemented with 1% pen/strep, 1% non-essential amino acids, 2 mM L-glutamine, 2% B27, and 2% N2 for 7 days.

In order to compare the effects of conditioned media of NTF-secreting ADSCs and NTF-secreting ADSCs on enhancing neurogenic differentiation, we considered 3 groups. In group 1, neurogenic differentiation of human ADSCs was performed in neurobasal medium containing 1% pen/strep, 1% non-essential amino acids, 2 mM L-glutamine, 2% B27, and 2% N2. In group 2, neurogenic differentiation of human ADSCs was conducted in the neurogenic medium and conditioned media of NTF-secreting ADSCs (1:1 v/v). In group 3, neurogenic differentiation of human ADSCs was carried out in neurogenic medium which co-cultured with 3 NTF-secreting ADSCs encapsulated alginate beads for 7 days (2×10^4 of NTF-secreting ADSCs encapsulated in each alginate bead). After 7 days, the beads that contained NTF-secreting ADSCs were removed and differentiated ADSCs were assessed for neural markers using immunocytochemical and real-time PCR.

2.6. MTT assay

To examine the viability of NTF-secreting cells after induction, 5 mg of MTT was dissolved in 1 ml of PBS. The stock solution was added to the culture medium at a dilution of 1:10. The plates were incubated at 37 °C for 4 h. The medium was then aspirated and 200 μl of dimethyl sulfoxide (DMSO) was added to extract the MTT formazan. The absorbance of each well was detected by a microplate reader (Hiperion MPR 4+, Germany) at the wavelength of 540 nm.

2.7. Immunocytochemistry

After fixation with 4% paraformaldehyde (PFA)/PBS, cells were treated with blocking solution (PBS containing 4% goat serum and 0.1% Triton X-100) for 45 min at RT. Then, cells were incubated in primary antibodies in PBS/0.1% Triton X-100 and 1% goat serum overnight at 4 °C. Anti-GFAP (1:300; Abcam, UK) and anti-S100β (1:500; Abcam, UK), anti-Nestin (1:300, Abcam, UK), mouse anti-microtubule-associated protein (MAP2, MAP2 and 1:300, Abcam, UK), mouse anti-glial fibrillary acidic protein (GFAP, 1:300, Abcam, UK) were used. After washing with PBS, the slides were exposed to secondary antibodies, i.e. rabbit anti-mouse fluorescein isothiocyanate (FITC) (1:500; Abcam, UK) and rabbit anti-mouse phycoerythrin (PE) (1:200; Abcam, UK)-conjugated. They were incubated at room temperature for 1 h. Diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. For negative controls, primary antibody was omitted from the reaction series in each experiment. Cells were observed using a fluorescence microscope (Olympus BX51, Japan). To perform quantitative analysis, the numbers of positive cells were counted on each acquired image by ImageJ1.42 (NIH), and the ratio to the number of nuclei was analyzed for each antigen. The number of immunopositive cells was counted in a minimum total of 200 cells per slide. All immunocytochemical experiments were repeated twice.

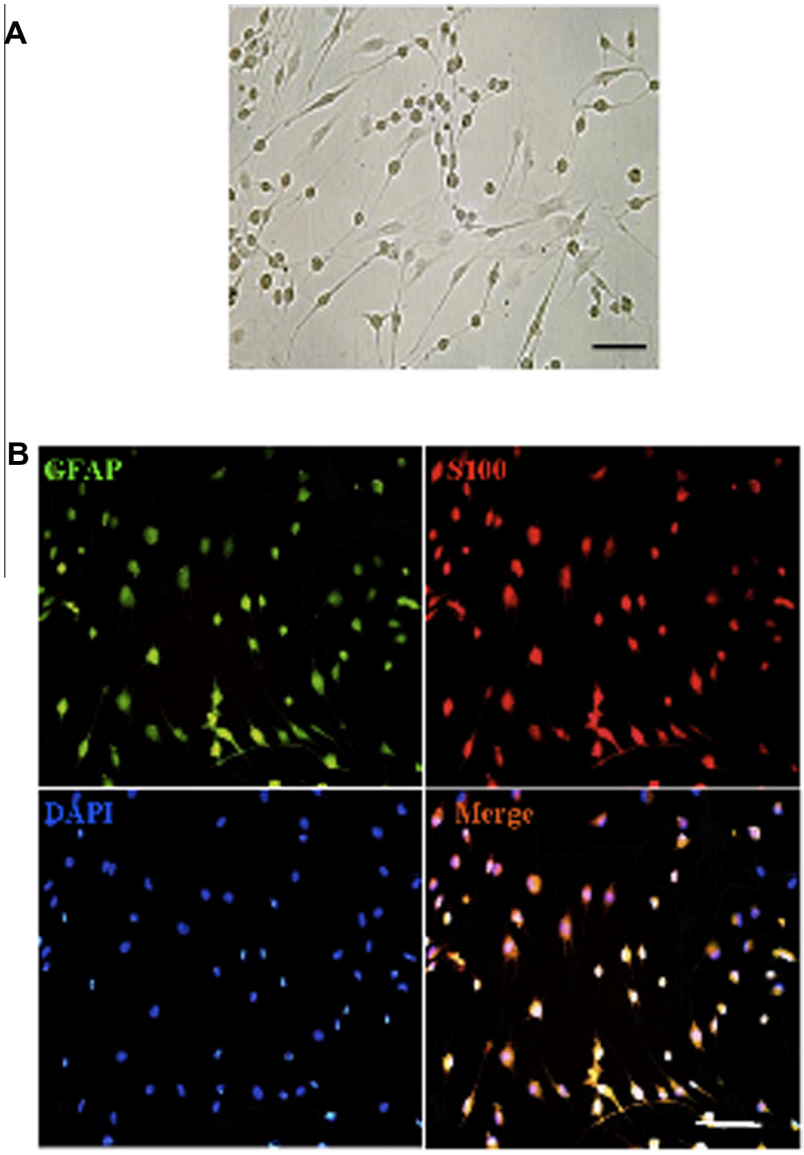

Fig. 1. Differentiated human adipose-derived stem cells (ADSC) demonstrated astrocyte characteristics. (A) Phase contrast image of neurotrophic factor (NTF)-secreting ADSCs showed the characteristic typical satellite-like morphology of following differentiation; (B) Co-expression of GFAP and S100 in NTF-secreting ADSCs. Scale bar: a = 50 μm, b = 100 μm.

2.8. Real time reverse transcriptase PCR

Total ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Equal amounts of total RNA were reverse transcribed into complementary deoxyribonucleic acid (cDNA) using oligo-dT and Revert-Aid First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using a thermal cycler (Rotor-Gene 6000, QIAGEN), with 12.5 μl SYBR Green PCR Master Mix (QIAGEN), 5 pM of each of forward and reverse primers, and 1.5–2 μl cDNA for each reaction in final volume of 20 μl. Cycle conditions were carried out according to the manufacturer's instructions (QIAGEN). Relative gene expression was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). All samples were normalized to levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) which was used as the internal control. All measurements were done in triplicates. The following primers were used:

- GDNF, 5′-TCAAATATGCCAGAGGATTATCCTG-3′, 5′-GCCATT TGTTTATC TGGTGACCTT-3′;
- BDNF,5′-AGCTCCGGGTTGGTATACTGG-3′,5′-CCTGGTGGAACTTC TTTGCG-3′;
- NGF, 5′-CATGCTGGACCCAAGCTCA-3′, 5′-GACATTACGC TATGCACCTCAGTG-3′;
- CNTF, 5′-CCTGACTGCTCTTACGGAATCCTAT-3′, 5′-CCATCCG CAGAGTCCAG-3′;
- MAP2, 5′-TCAGAGGCAATGACCTTACC-3′,5′-GTGGTAGGCTCTTG GTCTTT-3′;
- GFAP, 5′-CCTCTCCCTGGCTCGAATG-3′,5′GGAAGCGAACCTTCTCG ATGTA-3′;
- Nestin, 5′- AGCCCTGACCACTCCAGTTTAG -3′, 5′-CCCTCT ATGGCTGTTTCTTTCTCT-3′;
- GAPDH, 5′-GAAATCCCATCACCATCTTCCAGG-3′, 5′- GAGCCC CAGCCTTCTCCATG-3′.

2.9. ELISA

Human ADSCs at passages 3–6 and induced cells were cultured. Aliquots (100 μl) of the culture supernatant were analyzed. The protein level of CTNF, GDNF, NGF, and BDNF was measured using

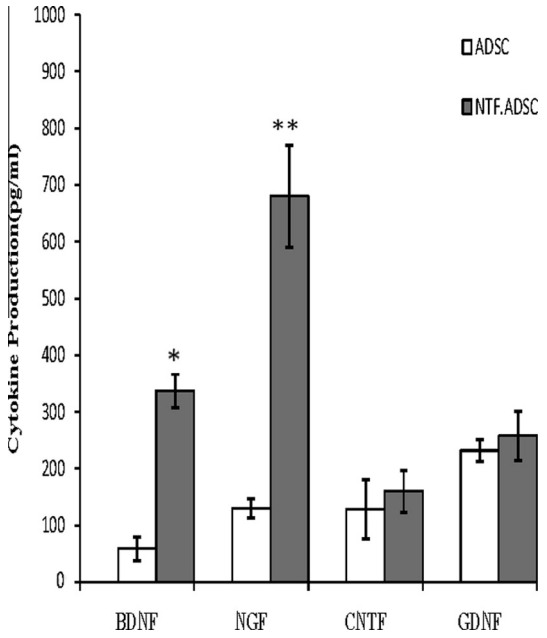

Fig. 2. Comparative analysis of BDNF, NGF, CNTF, and GDNF secretion levels in conditioned media of human ADSCs and NTF-secreting ADSCs cultures examined by ELISA. The levels of all of these factors elevated in NTF- secreting ADSCs compared to human ADSCs, the levels of BDNF and NGF secretion, significantly elevated in NTF- secreting ADSCs cultures compared to human ADSCs cultures. (mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.)

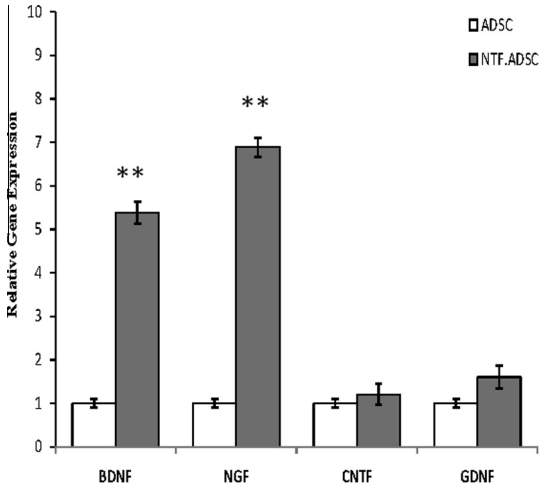

Fig. 3. Comparative analysis of BDNF, NGF, CNTF, and GDNF markers in human ADSCs and NTF-secreting ADSCs cultures examined by real time RT-PCR. The expression level of BDNF and NGF genes, significantly evaluated, while the increased levels of CNTF and GDNF in NTF-secreting ADSCs cultures in comparison to human ADSCs cultures were insignificantly. (mean ± SEM, $^{**}p < 0.01$.)

a sandwich ELISA procedure (DuoSet, R&D System for human CTNF, GDNF, NGF, and BDNF). The absorbance at 450 nm was recorded using a microplate reader (Hiperion MPR 4+, Germany). The results were calculated for *P*g/ml. All samples were analyzed in triplicate.

2.10. Statistical analysis

Data are presented as mean ± standard error (SE) from 4 to 5 independent cell cultures. Kruskal–Wallis one way analysis of variance (ANOVA) and Dunn's multiple comparison test were used to determine the statistical significance between data.

3. Results

3.1. Isolation of human ADSCs

The isolated human ADSCs were expanded after plating and grown to confluence. Cells became more uniform and grew in a more spindle-shaped, typical fibroblast-like morphology.

3.2. Characterization of NTF-secreting ADSCs

Human ADSCs grown in expansion medium exhibited the typical flat fibroblast-like morphology. They were then further grown in the induction medium and demonstrated astrocyte-like morphology (Fig. 1a).

3.3. Cell viability

We examined the survival ability of NTF-secreting ADSCs by MTT assay. The cell viability of NTF-secreting ADSCs was more than human ADSCs (0.76 ± 0.25 vs. 0.51 ± 0.12). It was found that induction of NTF-secreting cells significantly promoted the proliferation of the induced cells in comparison with human ADSCs ($p < 0.05$).

3.4. Immunocytochemical staining

Using immunocytochemical staining, we assessed the protein expression of astrocyte and neurotrophic factor markers to examine the efficiency of the induction protocol. We found that the induced ADSCs were positive for GFAP and S100 β (64.21 ± 17.4 and 70.39 ± 19.5, respectively) (Fig. 1b).

3.5. ELISA measurements

We performed a quantitative cytokine analysis, the most important test for the functional result of the differentiation. The secretion of the neurotrophic and growth factors was proved by this protocol. Therefore, the supernatants were collected from cultured human ADSCs and the induced cells were analyzed by ELISA. The secretion level of BDNF, NGF, CNTF, and GDNF in these media was analyzed. The levels of BDNF and NGF secretion, significantly elevated in NTF-secreting ADSCs cultures compared to human ADSCs cultures ($p < 0.05$) (Fig. 2).

3.6. Real time reverse transcriptase PCR results

Real time RT-PCR analysis of NTF-secreting ADSCs showed that messenger RNA (mRNA) of BDNF and NGF was higher by 5 and 7 folds, respectively, in the NTF-secreting ADSCs than in the ADSCs ($p < 0.01$). However, up regulation of CNTF and GDNF in the NTF-secreting ADSCs were not significantly in compared to human ADSCs (Fig. 3).

3.7. Co-culture of NTF-secreting ADSCs with human ADSCs

As shown by immunostaining, the mean percentage of MAP2 and nestin positive cells was increased in the conditioned medium and co-cultured groups in comparison to the control group. However, the mean percentage of GFAP positive cells in co-cultured group was significantly decreased compared to the other groups (Fig. 4). Real time RT-PCR provided further evidence that co-culture with NTF-secreting cells provided significant neuronal differentiation (nestin and MAP2 markers) relative not only to the control cultures but also to the cultures supplemented with conditioned media from NTF-secreting ADSCs ($p < 0.05$). It should be noted that

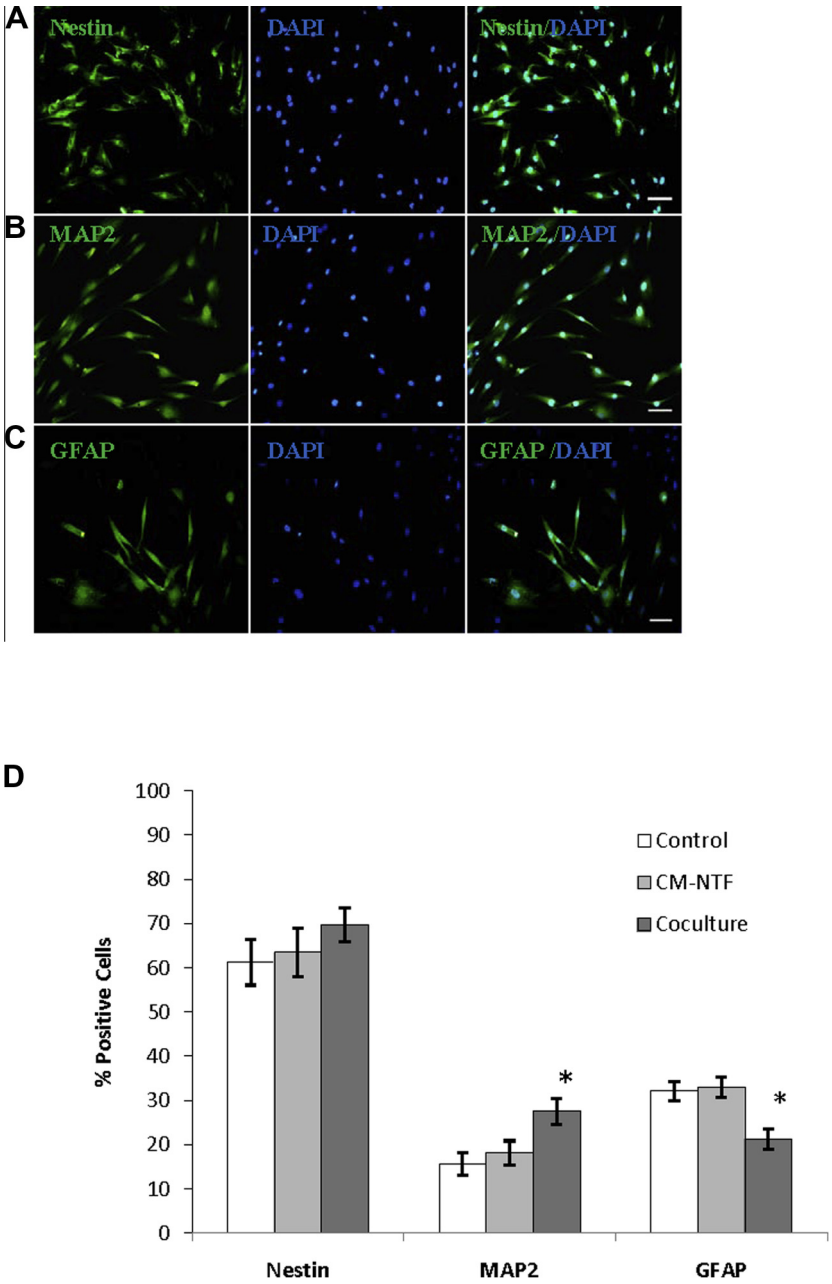

Fig. 4. Comparative immunocytochemistry analysis of neural markers, Nestin (A), MAP2 (B) and GFAP (C) in neurogenic differentiated ADSCs. The mean percentage of positive cells for all markers insignificantly increased in the combination of neurogenic medium with conditioned medium (CM-NTF) relative to control cultures, while the mean percentage of MAP2 positive cells was significantly increased in co-cultured of NTF-secreting ADSCs (co-culture) compared to control cultures. In addition, the mean percentage of GFAP positive cells was significantly decreased in co-cultured of NTF-secreting ADSCs (co-culture) compared to control cultures (D). (mean ± SEM, $^{*}p < 0.05$).

expression of MAP2 was increased more than two folds relative to GFAP in the co-cultured group.

4. Discussion

There are an increasing number of reports describing the trophic effects of ADSCs on the protection, survival, and differentiation of neural cells and tissues. In addition, recent studies have indicated that ADSCs-induced repair may act through cell differentiation as well as secreting trophic factors [32].

Several bioactive protein factors secreted by ADSCs have been identified. ADSCs have been shown to secrete bioactive growth factors that contribute to the angiogenic activity of the cells after transplantation [19]. It has been reported that ADSCs secret BDNF, a neurotrophin rescuing type of neurons. Furthermore, conditioned medium from ADSC cultures have been found to possess significant levels of BDNF to prevent glutamate-induced neuronal death [33].

Several studies have demonstrated that neurotrophic factors including BDNF, insulin-like growth factor 1 (IGF-1), and GDNF are useful in preventing neuronal death in many neurodegenerative disorders [34–36]. Previous studies have suggested that ADSCs and BMSCs may be equally beneficial in the context of treatment of neurodegenerative diseases [37,38]. However, ADSCs have practical advantages.

We believed that human ADSCs may act as source of variety of neurotrophic factors and can release more neurotrophic factors after induction. Therefore, we decided to induce human ADSCs to

secret neurotrophic factors. Then, it was assessed the effects of co-culture with NTF secreting cells on neural differentiation of human ADSCs.

The release of NTFs after induction was assessed by ELISA analysis. Compared to human SDSCs, we found the induced cells to secrete significantly more BDNF and NGF (about 5 times), almost the same amount of CNTF and GDNF. In addition, real time reverse transcriptase PCR revealed that NTF-secreting ADSCs highly expressed NGF and BDNF. In both analyses, the expression of NGF was higher than other NTFs even in the pre-induction phase.

The potential of NTF-secreting ADSCs for promoting neurogenic differentiation of ADSCs was studied. For this purpose, neurosphere cells from induced ADSCs were either cultured in NTF-secreting ADSCs conditioned media or co-cultured with NTF-secreting ADSCs.

It was show that the level of neurotrophic factors in conditioned media was not sufficient for promoting neurogenic differentiation. On the other hand, in the co-cultured group, NTF-secreting ADSCs released more soluble products than human ADSCs and could promote neuronal differentiation. This phenomenon could be due to lost function of NTFs in conditioned media gradually. However, in the co-cultured group, constant NTF secretion leads to more neurotrophic factor access for neurogenic differentiating ADSCs. Moreover, co-culture with NTF-secreting ADSCs could promote neuronal differentiation more than gliogenesis.

Our previous study demonstrate that NTF-secreting factor cells derived BMSCs and ADSCs could secret a range of different growth factors [39]. We show that the levels of secretion BDNF and NGF in NTF-secreting ADSCs was more than other neurotrophic factors. The NTF-secreting ADSCs released sufficient amount of neurotrophic factor for promoting neuronal differentiation. Furthermore, co-culture with NTF-secreting ADSCs could promote neuronal differentiation more than gliogenesis. Notably, NTF-secreting ADSCs do not solely release BDNF, NGF, CNTF, and GDNF. They may release other trophic factors or active biomolecules which are useful in the treatment of neurodegenerative disease. Other trophic factors secreted by NTF-secreting ADSCs need to be further evaluated in the future.

Acknowledgments

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