



Differentiation of adult human mesenchymal stem cells into dopaminergic neurons

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Abstract

The striatal dopamine (DA) deficiency is known as the main cause of the clinical picture of Parkinson's disease (PD). The disease is a progressive degeneration of dopaminergic neurons in the striatum. The treatment of PD is based on compensation for the brain's supply of DA lost by drug therapy, deep brain stimulation, surgery, gene and cell therapies. Clinical studies have focused on the utility of stem cell-based therapies in PD. Embryonic and mesenchymal stem cells (MSCs) are widely used. Recently, human adipose derived stem cells (hADSCs) have been considered as a suitable source of tissue for this purpose. In this project, hADSCs differentiated into dopaminergic neurons and the specificity of the cell preparations was examined. Human adipose tissues were collected from healthy volunteers undergoing liposuction and hADSCs were isolated by collagenase-based enzymatic method. Flow cytometry was performed using the surface cluster of differentiation (CD) markers to confirm the cell typical properties. Then hADSCs were differentiated to dopaminergic neurons in neurobasal medium in the presence of differentiation factors and confirmed by immunocytochemistry via neuronal and dopaminergic markers. The isolated hADSCs were cultured and identified by the expression of MSCs surface markers including CD90, and CD44. These cells did not express hematopoietic surface markers such as CD45 and CD14. Differentiated cells express neuronal marker NeuN and dopaminergic marker tyrosine hydroxylase (TH). It is concluded that hADSCs can be easily taken from the patient's own body and differentiated into dopaminergic cells having a lower risk of transplant rejection.

Keywords: Adipose derived stem cells; Differentiation; Dopaminergic neurons.

INTRODUCTION

Dopamine (DA) is one of the most eminent catecholamine neurotransmitters in the brain because of its role in motor control as well as cognition, learning, and memory. The biosynthesis of DA takes place in the cytosol of dopaminergic neurons and starts with the hydroxylation of L-tyrosine catalyzed by tyrosine hydroxylase (TH) to yield dihydroxyphenylalanine (levodopa). TH is the rate-limiting enzyme in DA biosynthesis and plays an important role in the regulation of DA levels in the striatum (1). Misregulation of DA signaling is associated with several

neurological disorders such as schizophrenia and Parkinson's disease (PD). Innervation of DA neuron in the striatum adjusts voluntary movements and preferentially degenerates in PD. The disease is a chronic progressive neurodegenerative disorder of central nervous system with clinical motor symptoms such as akinesia, muscle rigidity, tremor, and postural instability, resulting from the extensive degeneration of dopaminergic neurons in substantia nigra pars compacta to the striatum (2-5).

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The treatment of PD is based on compensation for the brain's supply of dopamine lost in the substantia nigra by drug therapy, deep brain stimulation, surgery, and gene and cell therapies. Some of these therapies not only did not have positive effects on the progress of the disease, but also caused new problems and complicated side effects for the patients. Due to the extensive degeneration of dopaminergic neurons, DA depletion, and non-effective prevention of the clinical progression of PD, cell replacement therapy has been proposed as an alternative treatment for the disease (6,7). However, it is important to delineate the specificity of the differentiated cell types before such cells can be safely used for clinical transplantation.

Embryonic stem cells (ESCs), neural stem cells (NSCs), and induced pluripotent stem cells (iPSCs), have shown to represent a promising unlimited cell source to replenish functional neurons for the treatment of PD (2,8,9). In contrast to iPSCs, adult or mesenchymal stem cells (MSCs) are demonstrated to be multi-potent immunosuppressive cells having non-tumorigenic potentials upon transplantation and are free of ethical restrictions (10). MSCs can be isolated from various tissues such as bone marrow, dental pulp, amnion, placenta, umbilical cord blood, and adipose tissue transdifferentiated into dopaminergic neurons in PD (11-17). Therefore, our goal was to generate dopaminergic neurons from human adipose derived stem cells (hADSCs) that can express TH, a specific dopaminergic neuronal marker in DA synthesis and regulation (5). For this purpose, we induced low passage (P4) of adult ADMSCs to dopaminergic neurons via direct differentiation method to investigate the specificity of the differentiated hADSCs to dopaminergic neurons.

MATERIALS AND METHODS

Mesenchymal stem cells isolation and culture

The lipoaspirate samples were collected from four adult healthy volunteer donors by liposuction technique with informed consent signed by each donor. The procedure of MSCs isolation was carried out using an enzymatic

procedure as described by Zuk *et al.* (18). The freshly lipoaspirate was washed three times with sterile phosphate buffered saline (PBS) and incubated in a solution containing 0.1% collagenase type IA (Sigma Aldrich, St. Louis, Missouri, USA) for 60 min at 37 °C in a shaking water bath to digest the extracellular matrix. This was centrifuged at $400 \times g$ (1200 rpm) for 10 min to separate stromal vascular fraction from primary adipocytes and lysed red blood cells. The stromal vascular fraction was then suspended in growth medium including Dulbecco's modified eagle/F12 medium (DMEM/F12), 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco, Thermo Fisher Scientific, Waltham in eastern Massachusetts, USA) in the tissue culture flasks and incubated at 37 °C humidified with 5% CO₂. After attachment phase of 48 h, non-adherent cells were removed by rinsing with PBS and attached MSCs were cultured in fresh medium. The culture medium was changed every 4 days and cells were sub-cultured on reaching 80%-90% confluence using 0.05% trypsin EDTA solution (Gibco, Thermo Fisher Scientific, Waltham in eastern Massachusetts, USA) (19,20). For all the following *in vitro* experiments, hADSCs in passage four were used. The isolated MSCs were characterized morphologically using a Leica, Wetzlar, Germany inverted microscope examination (Fig. 1).

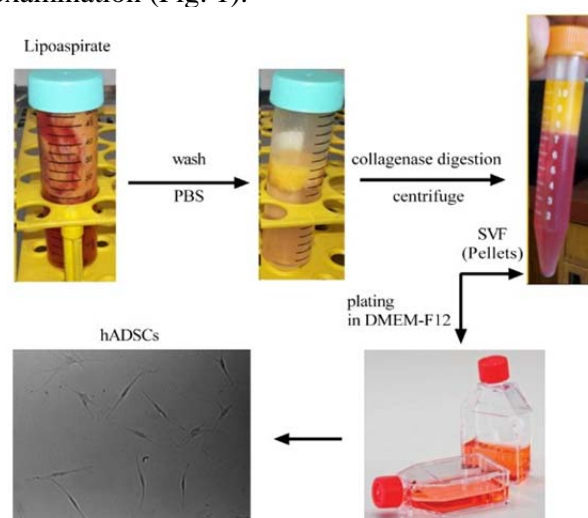


Fig. 1. Processing of lipoaspirate, isolation, and culturing of human adipose derived mesenchymal stem cells (hADMSCs).

Flow cytometric analysis for mesenchymal stem cells confirmation

The isolated cells were confirmed as being MSCs through flow cytometry to identify the surface specific cluster of differentiation (CD) markers. Fourth-passaged harvested adherent cells were counted and fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, Missouri, USA) for 10 min. Then, cells were washed twice with 1% cold bovine serum albumin (BSA)/PBS and incubated in darkness with fluorochrome-conjugated antibodies for positive and negative detection of CD90-phycoerythrin (Sigma Aldrich, St. Louis, Missouri, USA), CD44- fluorescein isothiocyanate (FITC) and CD45-FITC, CD14-FITC (Abcam, California, USA) respectively. After 30 min the cells were washed and re-suspended in 1% BSA/PBS. The same amounts of unstained MSCs were considered as control. All steps were carried out at 4 °C. The specific fluorescence intensity of each sample was observed by fluorescence-activated cell sorting Calibur flow cytometry (BD Biosciences, Bergen County, New Jersey, USA). Corresponding isotypes were used as an isotype control. The percentage values of each surface CD markers were analyzed by cell quest software (BD Biosciences, Bergen County, New Jersey, USA) (12,21).

Direct dopaminergic induction of human adipose derived stem cells

Dopaminergic differentiation was performed according to the method described by Trzaska *et al.* (22). hADSCs were sub-cultured in T-25 flasks, which pre-coated with poly-L-lysine (Sigma Aldrich, USA). At 80% confluence of passage 4, hADSCs medium was replaced with neurobasal medium (Invitrogen) and 0.5% B27 supplement (Gibco, Waltham in eastern Massachusetts, USA) including induction cocktail (Sigma Aldrich, USA); 250 ng/mL sonic hedgehog (SHH), 100 ng/mL fibroblast growth factors (FGF)-8 and 50 ng/mL bFGF for a period of 12 days. As a control, hADSCs were cultured with neurobasal medium and B27 supplement alone, without the induction cocktail (22).

Immunocytochemistry/immunofluorescence for neuronal and dopaminergic markers

To determine whether treated hADSCs express the proteins related to dopaminergic

neurons, immunocytochemical analyses were performed for neuronal marker (a neuronal biomarker: NeuN) and dopaminergic marker (TH) as described by Trzaska *et al.* (22). The differentiated cells were examined for the presence of hexaribonucleotide binding protein-3 (a neuronal biomarker, NeuN) and TH. The passage 4 of hADSCs seeded into 12-well plates in the present of 80% confluence. Control MSCs and differentiated MSCs were washed with 1% BSA/PBS and fixed with paraformaldehyde 4% for 10 min. Subsequently permeabilized and blocked with 0.1% tween 20 (Sigma Aldrich, USA) and 10% goat serum (Sigma Aldrich, USA) in 1% BSA/PBS for 2 h in room temperature. Primary antibodies, anti-NeuN (Abcam, ab177487) and anti-TH (Abcam, ab6211) antibodies, diluted with a solution of 1% BSA/PBS containing 0.1% tween 20, 1:300 and 1:1000 respectively. All cells were incubated with diluted antibodies overnight at 4 °C. At the following day the wells were washed with 1% BSA/PBS and treated with secondary antibodies goat anti-rabbit IgG-phycoerythrin (1:400, Sigma Aldrich, St. Louis, Missouri, USA), goat anti-rabbit IgG-FITC (1:400; Sigma Aldrich, St. Louis, Missouri, USA). 4,6-diamidino-2-phenylindole (DAPI, Sigma, D9542) was used for nuclear staining of the cells. Finally the images were visualized under a confocal laser scanning microscope (Zeiss, Germany).

RESULTS

Confirmation of mesenchymal stem cells by flow cytometry

The flow cytometric examination indicated that isolated MSCs express the surface markers, CD90 and CD44, and entirely lack of CD45 and CD14 which are hematopoietic lineage markers (Fig. 2). These observations proved that the isolated MSCs contain surface structure which can differentiate into mature cell phenotypes. Isolated MSCs were approximately 90% positive for two MSCs surface CD markers, CD90 and CD44. But isolated MSCs were 100% negative for two hematopoietic lineage CD markers, CD45 and CD14.

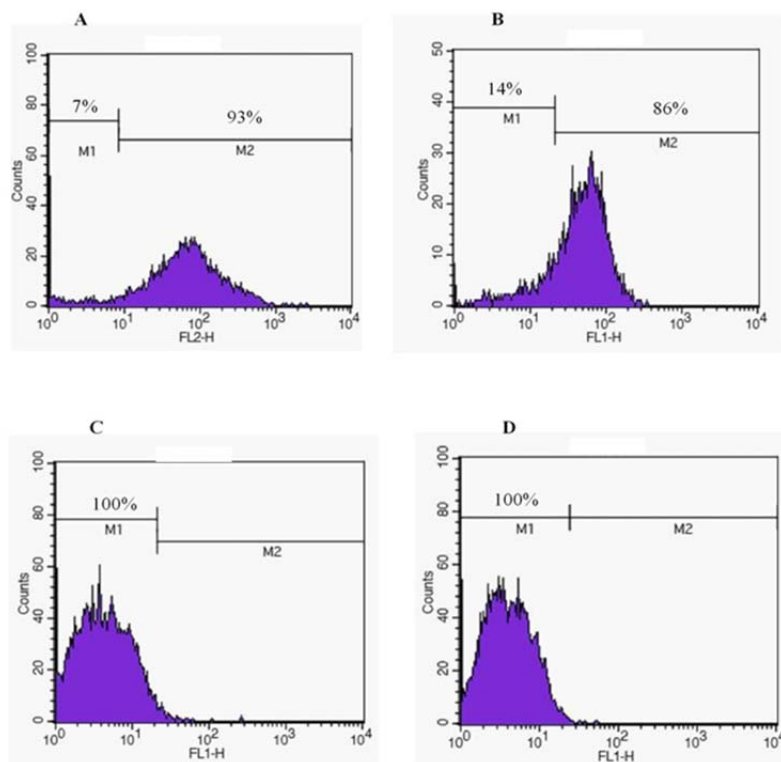


Fig. 2. Flow cytometry analysis. Human MSCs were isolated from adipose tissue, lipoaspirate and analyzed after four cell passages. (A and B) Isolated MSCs were positive for the MSCs markers CD90 and CD44, (C and D) negative for hematopoietic markers CD45 and CD14. MSC, Mesenchymal stem cells; CD, cluster of differentiation.

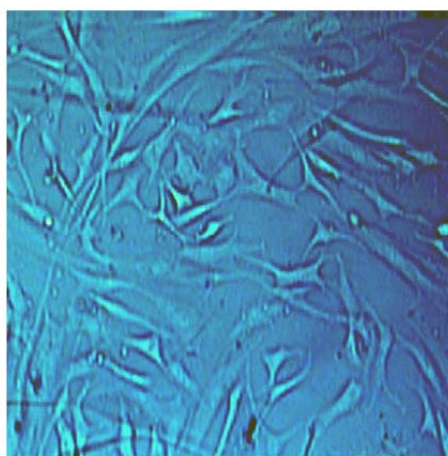


Fig. 3. Bright-field images of induced-hADSCs in culture. By day 12, morphological changes were observed in passage four of hADSCs after induction with FGF-2, FGF-8 and SHH. HADSCs cultured with neurobasal medium and B27 only and considered as a control. hADSC, Human adipose derived stem cells; FGF, fibroblast growth factors; SHH, sonic hedgehog.

Direct induction of human adipose derived stem cells to dopaminergic neurons

The dopaminergic neurons were generated directly from hADSCs using a cocktail of differentiating factors in neurobasal medium in a short period of time. As shown in Fig. 3, during 12 days, neurogenic induction resulted

in a change in hADSCs morphology assuming a neuron-like phenotype. Induced hADSCs are spindle shaped and exhibited the cell bodies with multiple long thin extensions (Fig. 3). In the absence of differentiating factors (FGF-2, FGF-8, and SHH), the morphology of hADSCs cultured in neurobasal medium and B27 alone was not changed.

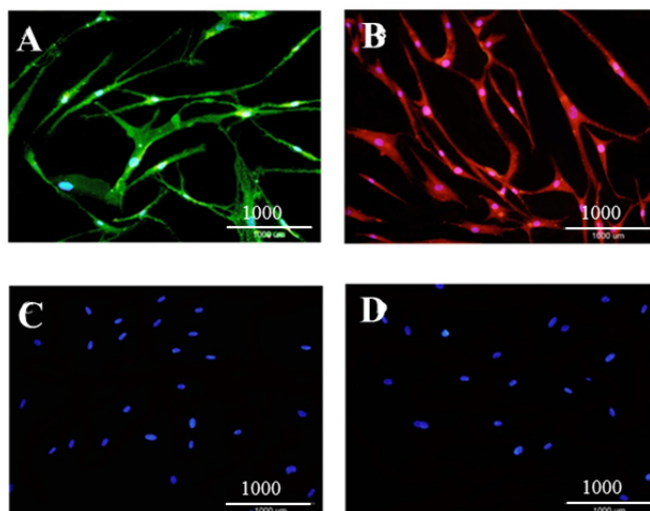


Fig. 4. Immunocytochemistry analysis. (A) Induced-hADSCs displays expression of NeuN (FITC) on day 12, indicated by the green, (B) induced-hADSCs displays expression of tyrosine hydroxylase (tyrosinase) on day 12, indicated by the red, and (C and D) non-induced-hADSCs display the nuclear label DAPI (blue). hADSC, Human adipose derived stem cells; FITC, fluorescein isothiocyanate.

Induced human adipose derived stem cells express neuronal and dopaminergic markers

Expression of the neuron nuclei (NeuN) and the neuron-specific enzyme for DA synthesis (TH) in the striatum was confirmed in the induced-hADSCs using immunocytochemical method. Figure 4A and 4B display expression of NeuN and TH designated by green and red spindle shape at the passages 4 on day 12, respectively. In contrast, non-induced hADSCs, probed with NeuN and TH antibodies were negative and only showed the nuclear stain DAPI (Fig. 4C and 4D).

DISCUSSION

In this study, hADSCs were differentiated to dopaminergic neurons directly. MSCs were enzymatically (collagenase) isolated and cultured from human adipose tissues. Then, hADSCs were confirmed by flow cytometry. Isolated MSCs were positive for two MSCs surface CD markers, CD90 and CD44, but not for CD45 and CD14. The results were consistent with the other reports (21-24). The transplanted cells differentiated into the dopaminergic neurons using a cocktail of differentiating factors in neurobasal medium and B27 that included

FGF-2, FGF-8, and SHH. Results are consistent with that observed by Trzaska *et al.* (22), which uses the induction of MSCs derived from the adult human bone marrow during 12 days. The inducing hADSCs retain their original phenotype having a capacity to differentiate into the stem cell lineages neural cells. Immunohistochemical evaluations reliably demonstrated the existence of cells positive for neural marker of NeuN and the enzyme marker TH, which catalyze the first reaction of DA biosynthesis in the striatum (24-26). Our results suggested that hADSCs have great capacity to differentiate into the dopaminergic neuron. This is in agreement with previous studies in which human adipose tissue-derived stem cells differentiate into endothelial cells (23). However, this is inconsistent with observation recently reported by Marei *et al.* (21), who examined adipose-derived MSCs differentiation in a different induction medium containing FGF2, EGF, BMP-9, retinoic acid, and heparin, resulting a mixture of cholinergic and dopaminergic neurons. Considering the neuronal specificity, the presence of the cholinergic neurons in the clinical use could be challenging (27).

Results suggested that hADSCs have great capacity to differentiate into the dopaminergic

neuron. This valuable feature paves the way for the therapeutic application of hADSCs for neurodegenerative maladies such as Parkinson's disease (PD). As an effective treatment for PD, stem cell-based therapies have been rapidly expanding. However, the clinical use of ESCs and NSCs has presented several physiological problems including ethical constraints, teratoma formation, histocompatibility, inadequate tissue supply, low efficiency of differentiation, lineage polarization, and inefficacy of migration toward damaged brain regions (28-30). On the other hand, limited survival and being vulnerable to neurodegeneration of post-transplantation of stem cells has been a main problem attributed to neurotoxic factors and deficiencies in trophic factors in PD brain (28). Unlike ESCs, iPSC, NSCs, and MSCs are easily accessible and can be transdifferentiated into dopaminergic neurons in PD and exert their therapeutic effects via the immunomodulatory, anti-inflammatory properties, cytokines, and neurotrophic factor secretion, which are impaired in PD (11). Furthermore, MSCs are able to migrate toward the location of damaged DA neurons in PD, which enables them to work as a targeted delivery of cytokines and neurotrophic agents (31,32). hADSCs present many advantages over than other MSCs, including simple extraction procedures using non-invasive surgical methods, convenient cultivation, high abundance, high rate of proliferation, high level of neurotrophic factors releasing, long term neurogenic, neuroprotective, and immunomodulatory effects (33-35).

CONCLUSION

The results presented in this study suggest that hADSCs as a source of pluripotent MSCs able to differentiated to dopaminergic neurons simply and directly. It is concluded that although hADSCs may be considered as a potential source for differentiation of dopaminergic neurons, further characterizations of the cell are needed before its clinical application.

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