Inhibitor of PI3K/Akt Signaling Pathway Small Molecule Promotes Motor Neuron Differentiation of Human Endometrial Stem Cells Cultured on Electrospin Biocomposite Polycaprolactone/Collagen Scaffolds

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Received: 8 February 2016 / Accepted: 3 March 2016 © Springer Science+Business Media New York 2016

Abstract Small molecules as useful chemical tools can affect cell differentiation and even change cell fate. It is demonstrated that LY294002, a small molecule inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway, can inhibit proliferation and promote neuronal differentiation of mesenchymal stem cells (MSCs). The purpose of this study was to investigate the differentiation effect of Ly294002 small molecule on the human endometrial stem cells (hEnSCs) into motor neuron-like cells on polycaprolactone (PCL)/collagen scaffolds. hEnSCs were cultured in a neurogenic inductive medium containing 1 μM LY294002 on the surface of PCL/collagen electrospin fibrous scaffolds. Cell attachment and viability of cells on scaffolds were characterized by scanning electron microscope (SEM) and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The expression of neuron-specific markers was assayed by real-time PCR and immunocytochemistry analysis after 15 days post induction. Results showed that attachment and differentiation of hEnSCs into motor neuron-like cells on the scaffolds with Ly294002 small molecule were higher than that of the cells on tissue culture plates as control group. In conclusion, PCL/collagen electrospin scaffolds with Ly294002 have potential for being used in neural tissue engineering because of its bioactive and three-dimensional structure which enhances viability and differentiation of hEnSCs into neurons through inhibition of the PI3K/Akt pathway. Thus, manipulation of this pathway by small molecules can enhance neural differentiation.

Keywords PI3K/Akt signaling · Small molecule · Differentiation · Motor neuron cells · PCL/collagen scaffold

Introduction

Motor neurons (MNs) are cells located in specific areas of the central nervous system, such as brain cortex (upper motor neurons), brain stem, and spinal cord (lower motor neurons), which maintain control over voluntary actions. Stem cell-derived MNs represent a promising research tool in disease modeling, drug screening, and development of therapeutic approaches for motor neuron diseases and spinal cord injuries [1, 2]. They could provide a replacement for dying cells and a trophic support within the central nervous system (CNS) [1]. The uterine endometrium is one of the rich sources of mesenchymal stem cells (MSCs), an alternative source of multipotent stem cells [3]. These cells are easily available and culture, and
they have high affinity of being differentiated to different types of cell lineages such as neuron-like cells [4–7], oligodendrocyte cells [8–11], adipocyte cells [12], osteoblast cells [13], and pancreatic islet beta-cell [14]. These cells can be characterized by the expression of specific gene markers such as CD44, CD146, CD90, and CD105 [8]. However, one of the most prominent hurdle in stem cell therapy is low survival rate of cells upon transplantation. Scientists tried to jump over this obstacle by using natural and synthetic biomaterial scaffolds [15, 16]. A scaffold not only bridges the gap of the lesion for contact guidance but also acts as mimics of cellular environment cues in the extracellular matrix (ECM) [17] to provide appropriate signals for improving the cellular viability and differentiation toward specific cell lineages. A biomaterial scaffold synthesized from either a natural or synthetic polymer can promote prevention of scar formation in tissue [18]. It can also concentrate growth factors [19], while promoting axonal regeneration [20]. It has been shown that surface properties of scaffolds have significant effect on cellular behaviors such as adhesion, proliferation, and differentiation. Nowadays, electrospinning is a method for providing fibers in nanoscale diameters to mimic native ECM that is a great advantage for neural tissue engineering applications. Electrospun nanofibers have large surface area with high porosity that are suitable for efficient nutrient delivery and cellular communication [21, 22]. Many strategies have been investigated to optimize the scaffolds’ efficiency to promote axonal regeneration. The phosphatidylinositol 3-kinase (PI3K) is an enzyme implicated in signal transduction by associating with receptor and nonreceptor tyrosine kinases that evolutionarily conserved from yeast to mammals [23]. Akt kinase, one of downstream transmitters of PI3K pathway, regulates diverse cellular processes including metabolism, proliferation, differentiation, apoptosis, and autophagy [23, 24]. Small molecules are low molecular weight molecules and have lower cost with greater ease of producing that include lipids, monosaccharides, second messengers, other natural products, and metabolites, as well as drugs and other xenobiotics. They are distinct from macromolecules such as proteins and can be therapeutic candidate in the future [25]. LY294002 as a small molecule shows a highly selective inhibitor of PI3K/Akt signaling pathway that has been shown to regulate differentiation in some cell lineages, for example, cardiomyocyte and neurons, but its effectiveness in cells is more different. In previous studies, the relationship between PI3K signaling pathway and neuronal differentiation of MSCs has been investigated [25, 26]. In spite of difference protocols for motor neuron differentiation, the use of low-cost protocol is preferred. Here, we substituted sonic hedgehog (Shh) with LY294002 to reduce cost for the final maturation in polycaprolactone (PCL)/collagen scaffolds and have found that combination of molecule PI3K/Akt inhibitor LY294002 and scaffolds can make a highly efficient method for deriving motor neuron-like cells from MSCs.

Material and Methods

Preparation of Electrospun Fibrous Scaffolds

PCL (10 % w/v; Mw 80,000 g/mol, Sigma-Aldrich, USA) polymer was dissolved in mixture of dichloromethane (DCM)/N,N-dimethylformamide (DMF) at the ratio of (1:3) and stirred for 24 h at room temperature. Rat tail collagen type I (1 mg/mL, Sigma, USA) was dissolved in acid acetic at a ratio of 1:1 (v/v) and stirred overnight to form a 12.5 % solution. The polymer solutions were mixed together with ratio of 50:50 into a 1-mL standard syringe attached to a 25G blunted stainless steel needle using a syringe pump at a flow rate of 1.3 mL/h. A high voltage of 15 kV was applied. The needle was located at the distance of 12 cm from the grounded collector and fibers collected on an aluminum foil-wrapped collector.

Characterization of the Scaffolds

Scanning electron microscope (SEM; Philips XL30, Netherlands) operated at 15 kV was used for morphological analysis and surface topography of the PCL/collagen nanofiber scaffolds. The average fiber diameters of nanofibers were measured in 20 different point locations for each sample using ImageJ analysis software (National Institutes of Health, USA). The hydrophilicity of the electrospun scaffolds was determined by water contact angle measurement using sessile drop method with an optical bench-type contact angle goniometer (OCA DataPhysics model, Germany). Of water droplets, 0.5 μL were dispensed onto the scaffold, and the contact angle was determined automatically. The contact angles were done at least three times per each scaffold.

Human Endometrial Stem Cell Isolation and Cell Seeding on PCL/Collagen Scaffolds

Human EnSCs were obtained by our previous study protocol [8]. Briefly, tissues were maintained in Hanks’ balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) with 10 % fetal bovine serum (FBS) and 1 % (v/v) penicillin/streptomycin. Tissue was enzymatically dissolved with collagenase type I (1 mg/mL, Sigma-Aldrich) at 37 °C for 60 min. The suspension was dissolved with Dulbecco’s modified Eagle’s medium F12 (DMEM/F12; Invitrogen) containing 10 % FBS (Sigma-Aldrich), passed through 70- and 40-μm cell strainers. Finally, the pellet of stromal cells was suspended in medium consisting of DMEM/F12, 10 % FBS, and 1 % penicillin/streptomycin (P/S; Sigma-Aldrich) and incubated in 37 °C in incubator.
Cell Attachment and Morphology Analysis on the Scaffolds After Cell Seeding

Following the sterilization by exposure with UV radiation for 1 h, scaffolds were incubated in DMEM/F12 containing 10% FBS for 24 h before cell seeding. Then, 100 μL of cell suspension containing 5 × 10^4 cells was added to each well and incubated at 37 °C in 5% CO₂ for 2 h. Then, 500 μL DMEM/F12 containing 10% FBS was added to each well and incubated for 48 h to allow the cells to attach to the surfaces of the scaffolds. The cell attachment and cell morphology of seeded cells on scaffolds were visualized by SEM at the third day of culture period. Cell-seeded scaffolds were fixed in 2.5% glutaraldehyde for 1 h, rinsed three times in phosphate-buffered saline, and then dehydrated in increasing concentrations of ethanol from 30, 50, 70, 80, 90, and 100% for 10 min per each. Finally, samples were sputter-coated with gold and observed using a scanning electron microscope (Philips XL-30, Netherlands), operated at 15 kV.

Determination of Cell Viability by MTT Assay

Cell viability of the hEnSCs cultured on the electrospun PCL/collagen scaffolds were studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on days 1, 3, 5, and 7. For MTT assay, cells were seeded at a density of 1 × 10^4 cells/scaffolds in 96-well plates and maintained at 37 °C under 5% CO₂ for 1, 3, 5, and 7 days. The culture medium of each cultured specimen was removed, and 100 μL of MTT solution (0.5 mg/mL) was added to each well. After incubation for 4 h, the MTT solution was removed and formazan crystals dissolved in 100 μL DMSO and the plates left at room temperature in dark place for 10 min on a rotary shaker. The absorbance of plates was measured at 570 nm using an ELIZA reader (Expert 96, Asys Hitch, Ec Austria).

Differentiation of hEnSCs into Motor Neuron-Like Cells with LY294002 as a Small Molecule

For differentiation of hEnSCs into motor neuron-like cells, the cells in the third passages were seeded at 5000 cells/cm² on PCL/collagen scaffolds and 24-well tissue culture polystyrene (TCP). The cells were incubated with DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 1 mg/mL streptomycin for 24 h. Differentiation of cells was started by exposing the cells to the first medium containing DMEM/F12 (1:1), 20% FBS, 2% B27, 10 ng/mL fibroblast growth factor 2 (FGF2), 250 μM isobutylmethylxanthin, 100 μM 2-metcaptoethanol for 24 h at 37 °C, and 5% CO₂ incubation. The treated cells were then cultured in induction media containing DMEM/F12 (1:1), 0.2% B27, 1 μM of LY294002, and 0.01 ng/mL retinoid acid (RA) for 1 week. Then, the induced media was replaced with a medium composed of DMEM/F12 (1:1), 0.2% B27, and 200 ng/mL brain-derived neurotrophic factor (BDNF) for another 1 week. As a control, a group of hEnSCs was cultured on the scaffolds or TCP in the absence of differentiation factors for 15 days. The medium was changed every 3 days.

Molecular Analysis Using Real-Time PCR

Total RNA was extracted by using RNeasy Plus Mini Kit (Qiagen, USA, 74134), and complementary DNA (cDNA) synthesis from 1 μg of RNA was performed by Revert Aid First Strand cDNA Synthesis Kit (Takara, USA, K1632). Real-time PCR reactions were carried out in the 48-well optical reaction plates on StepOneTM Real-Time PCR machine. In each PCR reaction, 30 ng synthesized cDNA was used to PCR by mixing with 10 μL of Power SYBER Green Master Mix (2x, Applied Biosystems) and 0.5 μM of each primer (Table 1) in a total volume of 20 μL at the annealing temperature. The comparative Ct method, 2^-DDCt, was used for relative gene expression analysis.

Immunofluorescence Analysis

After differentiation period time, cells were fixed 20 min with 4% paraformaldehyde (PFA; Sigma-Aldrich) at room temperature. The fixed cells were permeabilized with 0.1% TX-100 in TBS and blocked for 30 min at room temperature with 5% BSA and incubated with primary antibodies including NF-H(SMI-32) (mouse monoclonal anti-human; Abcam, USA, 1:200), Tuj-1 (mouse monoclonal anti-human; Abcam, 1:200), choline acetyltransferase (Chat) (mouse monoclonal anti-human; Abcam, 1:200), and Islet-1 (mouse monoclonal anti-human; Abcam, 1:200) overnight at 4 °C. After three...
rinses in phosphate-buffered saline (PBS), cells were incubated in the dark with secondary antibodies that included Alexa Fluor 488 donkey anti-mouse (1:500; Gibco, A-11058) or Alexa Fluor 594 donkey anti-rabbit (1:700; Gibco, A-21207) for 1 h at room temperature. After three rinses in PBS, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). To quantify the number of positive cells for each antibody, at least ten microscopic fields per well were counted randomly and reported in relative to whole DAPI-stained nuclei as percentage.

**Statistical Analysis**

The data presented as means ± standard deviation of the means \((n = 3)\). Statistical analysis was carried out using one-way ANOVA. The value of the variance \(P\) was <0.05, meaning statistical significance was accepted at the 95 % confidence level.

**Result**

**Synthesis and Characterization of Nanofibrus Scaffolds**

PCL/collagen (50:50) nanofiber scaffolds were prepared using the electrospun method and characterized using SEM. Figure 1 shows uniform and smooth nanofibers without beads with average fiber diameter of 239 ± 0.6375 nm. Surface wettability is an important property of biomaterials and can affect the attachment, proliferation, migration, and viability of cells. Wettability of nanofibrus scaffolds can be determined by contact angle of water. The PCL polymer is hydrophobic, whereas collagen is hydrophilic. When collagen add to PCL, the hydrophilicity of scaffold becomes better than PCL alone. In this study, it was observed that the water drop was absorbed into the fibers and resulted in a zero contact angle after 15 s. So, these scaffolds were hydrophilic and can be used for cell seeding and differentiation. The morphology and attachment of cells on PCL/collagen electrospun scaffold were scanned at 3 days after cell seeding. Images demonstrated that cells attached, grew, and spread on the PCL/collagen nanofibrous scaffolds (Fig. 2a).

**Isolation and Identification of Human EnSCs**

Flow cytometry analysis which was published in our previous report [8] showed that CD90+ (80 %), CD105+ (79 %), and CD146+ (97 %) were highly expressed, and CD31, CD34, CD133, and CD45 were extremely low.

**Assessment of Cell Viability and Proliferation**

The viability of cells cultured on PCL/collagen scaffolds was assayed by MTT at 1, 3, 5, and 7 days. As shown in Fig. 2b, until day 3, the cells cultured on TCP showed higher viability than cells cultured on the PCL/collagen scaffold but were not statistically significant. However, in days 5 and 7, the viability of cells cultured on PCL/collagen scaffold significantly enhanced relative to cells cultured on two-dimensional group (Fig. 2b). The results obtained from MTT assay showed that PCL/collagen nanofibrous scaffolds were suitable substrates in relation to cell attachment and proliferation. Collagen is a natural polymer that enhances hydrophilicity of scaffolds and prepares suitable condition for cell attachment and proliferation.

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**Fig. 1** Scaffold characterization analysis. a Scanning electron micrographs showing PCL/collagen scaffolds with three different magnifications. Histogram shows diameters of the PCL/collagen nanofibers.
Evaluation of hEnSC Differentiation into Motor Neuron-Like Cell by Exposing with LY294002 as a Small Molecule

After hEnSCs cultured on TCP and PCL/collagen scaffolds treated with induction media containing of LY294002 for 15 days, cells showed neural morphological characteristics with neurite-like processes. In this study, these morphological changing results were confirmed with the induction of neuronal marker expression by real-time PCR and immunocytochemistry for specific neural markers such as nestin, Pax6, NF-H, TUJ-1, Chat, Islet-1, and HB9. Real-time RT-PCR data showed that messenger RNA (mRNA) levels of NF-H, TUJ-1, Chat, Islet-1, and HB9 significantly increased in hEnSCs induced by LY294002 (Fig. 3). According to our results, while the expression of Islet-1, Chat, NF-H, and HB9 increased significantly during differentiation, the expression of nestin and Pax6 were downregulated following induction. The comparison results between TCP and PCL/collagen groups showed that in the PCL/collagen group, the expression of Chat, HB9 \( P > 0.01 \), Islet-1 \( P > 0.05 \), and NF-H were higher than in the TCP group and were statistically significant as shown (Fig. 3). Cells were fixed and stained for the motor neuron markers, and differentiation rate was determined by counting TUJ-1, NF-H, Chat, and Islet-1-positive cells as percentage of total number of DAPI-stained cells. Immunofluorescence analysis showed that Chat and Islet-1-specific motor neuron markers were found widely distributed in the cell cytoplasm. The ratio of Chat and Islet-1-positive cells was also increased by the treatment with LY294002 and cultured on PCL/collagen scaffolds compared to TCP group. Also, the proportion of NF-H and Tuj-1-positive cells was also increased in PCL/collagen scaffolds (Fig. 4). These results confirmed that treatment with LY294002 could induce differentiation of hEnSCs into motor neuron-like cells and PCL/collagen nanofibrous provide a suitable condition to cell attachment and differentiation.

Discussion

Our main goal in this study was to investigate the differentiation capability of hEnSCs on PCL/collagen nanofibrous scaffold into motor neuron-like cells in the presence of LY294002, as small molecule inhibitor of PI3K/Akt signal pathway. In this study, we found that inhibition of PI3K/Akt signal pathway by means of LY294002 results in promotion of hEnSC differentiation to motor neuron-like cells. Real-time PCR and immunocytochemistry were used to show the expression of specific markers such as Tuj-1, Chat, Islet-1, NF-H, HB9, Pax6, and nestin in mRNA levels and protein. The results of immunocytochemistry and real-time PCR represented the higher level of hEnSC differentiation to motor neuron-like cells in cultured cells on PCL/collagen nanofibrous scaffold relative to those on a TCP surface. It is well-documented that biomaterials aim to provide a suitable substrate and appropriate microenvironment to enhance cellular viability [27–30]. In accordance with previous studies, our MTT assay results...
demonstrated that the viability of hEnSCs cultured on PCL/collagen nanofibrous scaffold dramatically enhanced relative to cells cultured on TCP surface. Importantly, for those kinds of neurological disorders [31–33] in which motor neurons are selectively targeted for degeneration [34], a large population of generated new motor neurons may need to promote therapeutic efficiency [35]. Thus, inhibition of those signaling pathways that prevent differentiation of motor neurons, such as PI3/AKT, can potentially increase the number of motor neurons and also effectiveness of cell transplants, as overall, fewer cells need to be injected because of a higher number of motor neurons [36, 37].

It has shown that electrospun nanofibrous scaffolds provide a matrix for cell integration with surrounding tissue [38]. Collagen as a natural ECM protein has an excellent cell adhesion properties, and its combination with PCL by means of electrospinning can produce a scaffold with higher biocompatibility [39]. In other words, collagen acts like an ECM-mimicking nanoscaffold because it has specific ligands for cell adhesion [40, 41]. In addition, the mixture will provide a small fiber diameter and hydrophilic mesh with high porosity which are desirable for neural tissue engineering [39].

LY294002 is one of the main synthetic components [42] and has been used for studying the involvement of PI3K pathway in different biological systems [43, 44]. For instance, it was shown that LY294002 inhibits neural precursor cell proliferation in rat [45], which suggested that PI3K/Akt signaling pathway may play a critical role in cell cycle regulation and cellular proliferation. Moreover, LY294002 negatively regulate the proliferation of cultured MSCs, and it is sufficient to commit MSCs to neural fate. It also significantly increases a5b1 integrin expression and FAK phosphorylation on neuron-like cell via upregulation of glycogen synthase kinase 3β (GSK-3β) phosphorylation [26]. The GSK-3β, a serine/threonine protein kinase, is required in different cellular
processes, such as glucose metabolism, proliferation, and differentiation [46]. In line with previous achievements, our results also showed that LY294002 significantly accelerates differentiation of hEnSCs into motor neuron-like cells with PCL/collagen nanofibrous scaffolds and it may be caused by crucial impact of PI3K/AKT/GSK-3 axis on expression of adhesion molecules during differentiation of hEnSCs.

**Conclusion**

All in all, we have demonstrated that hEnSCs were cultured on PCL/collagen nanofibrous scaffolds and induced to differentiate into motor neuron-like cells with LY294002. Expression of motor neuron markers such as Chat, Islet-1, and NF-H in RNA and protein levels by real-time PCR and immunocytochemistry indicated that LY294002 as a small molecule can promote differentiation of hEnSCs into motor neuron-like cells on PCL/collagen scaffold and can replace expensive recombinant proteins that were initially used in the pioneering protocols. Thus, this work provides a new method for motor neuron derivation from hEnSCs in vitro and hence would lead to an efficient strategy for stem cell-based therapy of neurodegenerative diseases in future.

**Acknowledgments** The authors thank Tehran University of Medical Sciences to support this research with grant number “93-04-159-28027”.

**References**

are required for adult neurogenesis in the brain subventricular zone. Cell Death Dis 5:e1403


