The Proliferation Study of Hips Cell-Derived Neuronal Progenitors on Poly-Caprolactone Scaffold

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A B S T R A C T

Introduction: The native inability of nervous system to regenerate, encourage researchers to consider neural tissue engineering as a potential treatment for spinal cord injuries. Considering the suitable characteristics of induced pluripotent stem cells (iPSCs) for tissue regeneration applications, in this study we investigated the adhesion, viability and proliferation of neural progenitors (derived from human iPSCs) on aligned poly-caprolactone (PCL) nanofibers.

Methods: Aligned poly-caprolactone nanofibrous scaffold was fabricated by electrospinning and characterized by scanning electron microscopy (SEM). Through neural induction, neural progenitor cells were derived from induced pluripotent stem cells. After cell seeding on the scaffolds, their proliferation was investigated on different days of culture.

Results: According to the SEM micrographs, the electrospun PCL scaffolds were aligned along with uniformed morphology. Evaluation of adhesion and viability of neural progenitor cells on plate (control) and PCL scaffold illustrated increasing trends in proliferation but this rate was higher in scaffold group. The statistical analyses confirmed significant differences between groups on 36h and 48h.

Discussion: Evaluation of cell proliferation along with morphological assessments, staining and SEM finding suggested biocompatibility of the PCL scaffolds and suitability of the combination of the mentioned scaffold and human iPSC cells for neural regeneration.

Key Words:
Poly-Caprolactone Scaffold, HipS Cells, Neuronal Progenitors, Proliferation Assay.

1. Introduction

The people who suffer from spinal cord injury (SCI) often have permanent functional deficits because of no repair in the mentioned tissue. Hence, promoting axonal regeneration is one of the critical aims for successful repair from SCI (Okano & Sawamoto, 2008). Recently, many studies focused on this field; cell transplantation is one of the most interesting strategies (Ziraksaz, Nomani, Soleimani, Bakhshandeh, Arefian et al., 2013). In this regard, selection of the cell type is a challenging subject. Human embryonic stem cells (hESCs), derived from the inner cell mass of embryos, can competently differentiate into functional neurons and glia by a mechanism similar to in vivo development (Thomson, Itskovitz-Eldor, Shapiro, Waknitz, Swiergiel et al., 1998). Functional engraftment of human ES...
cell-derived dopaminergic neurons enriched by co-culture with telomerase-immortalized midbrain astrocytes (Watanabe, Ueno, Kamiya, Nishiyama, Matsumura et al., 2007, Yang, SooPaa, Adler, Roepke, Kattman et al., 2008, Hu, Du, Li, Ayala, & Zhang, 2009). Unfortunately, ethical concerns as well as potential immune rejection after transplantation of allograft transplantation limited the application of hESCs (Bakhshandeh, Soleimani, Hafizi, Paylakhi, & Ghaemi, 2012). Human induced pluripotent stem cells (hiPSCs) are known as a replacement for hESCs and a main candidate cell source for regenerative medicine aims (Narsinh, Plews, & Wu, 2011). HiPSCs are induced from a non-pluripotent cell by “reprogramming” of somatic cells into a pluripotent state through the over expression of a key set of transcription factors such as Oct4, Sox2, Klf4 and c-MYC (Takahashi & Yamanaka, 2006). HiPSCs are similar to hESCs, in terms of their morphology, feeder dependence surface marker expression and the ability to differentiation into derivations of the three embryonic germ layers (Yu, Vodyanik, Smuga-Otto, Antosiewicz-Bourget, Frané et al., 2007, Takahashi, Tanabe, Ohnuki, Narita, Ichisaka et al., 2007, Moretti, Bellin, Welling, Jung, Lam et al., 2010).

Another concern for cell transplantation is the matrix for cell transferring and seeding. The artificial scaffolds can mimic the tissue specific extracellular matrix (ECM). The ECM plays a vital role in regulating cellular functions through affecting cells by biochemical signals and topographical cues (Berthiaume, Moghe, Toner, & Yarmush, 1996). Regarding recent reports, these scaffolds provide a permissive substrate for axons to penetrate into the injured area in central nervous system (CNS) tissue engineering (Jain, Kim, McKeon, & Bellamkonda, 2006). Electrospinning is a simple method for fabrication of nanofibrous scaffolds using a wide range of materials including natural and synthetic polymers, composites and ceramics (Chew, Wen, Dzenis, & Leong, 2006, Venugopal, Low, Choon, & Ramakrishna, 2008, Barnes, Sell, Boland, Simpson, & Bowlin, 2007, Bakhshandeh, Soleimani, Ghaemi, & Shabani, 2011). The application of nanofibrous scaffolds as cell culture platforms is a promising strategy in CNS repair, supplying neurons while at the same time, inhibiting glial scar formation for its proper topographical and biochemical signaling (Cao, Liu, & Chew, 2009). In a study, the neural differentiation of mouse embryonic stem (ES) cells on the electrospun nanofibers into neurons, oligodendrocytes and astrocytes was investigated (Xie, Willerth, Li, Macewian, Rader et al., 2009). The potential of scaffolds in cell transferring and their biocompatibility are two main characteristics.

In this study, we investigated the combination of human iPS cells and poly-caprolactone (PCL) aligned nanofibrous scaffold for tissue engineering applications.

2. Methods

2.1. HiPSC Cell Culture and Embryoid Body (EB) Formation

HiPSCs (a gift from Stem Cells Technology Research Center, Tehran, Iran, supplementary Doc.1) were cultured on inactivated SNL feeder cell line (by mytomycin) in the medium containing DMEM/F12 supplemented with 20% knockout serum replacement (Invitro gen), 1% nonessential aminoacid (Inviro gen), 1 mM L-glutamine (Gibco) and 20ng/ml FGF2 (Peprotech) for 7 days. The medium was exchanged daily.

For generation of EBs, the human iPSCs were detached by 1 mg/ml collagenase IV (Gibco) followed by culturing in bacterial petridish (low attachment plates) in presence of above mentioned medium except FGF2 for 4 days.

2.2. Neural Progenitor Derivation and Propagation

For derivation and propagation of neural progenitor (NP) cells, EBs were cultured on Laminin (1mg/cm²) coated 35 mm tissue culture plates; 48h later, the medium was exchanged with new medium containing insulin (5µg/ml), Apo-Transferrin (50µg/ml), Sodium-selenite (30nM) and Human Fibronectin (2.5µg/ml) in DMEM/F12. After 7 days, outgrowing cells were triturated to a single cell suspension by trypsin/EDTA treatment.

2.3. Scaffold Fabrication

The nanofibrous scaffolds were prepared via electrospinning as previously reported by our laboratory (Bakhshandeh, Soleimani, Ghaemi, & Shabani, 2011). PCL (Sigma Aldrich, St. Louis, MO) was dissolved in chloroform (4% (w/v), Merck, Germany). The solution was fed into a 20-gauge needle through an extension tube by a syringe pump. The needle was located at a distance of 15 cm from a grounded collector, and a 15-kV voltage applied to this setup using a high-voltage direct-current power supply (Stem Cell Technology Research Center, Tehran, Iran). Having reached a thickness of about 200 µm, the mat was detached from the collector and placed in a vacuum for evaporation of the residual solvent. Then, oxygen plasma treatment was performed by a low-frequency plasma generator of 44 GHz frequency with a cylindrical quartz reactor (Diener Electronics, Ebhausen, Germany). Pure oxygen was introduced into the reaction
chamber at 0.4 mbar pressure, and the glow discharge ignited for 5 min.

2.4. Scanning Electron Microscopy

The scaffolds were mounted on aluminum stumps, and then coated with gold in a sputtering device for 1.5 min at 10 mA and examined under a scanning electron microscope (SEM, JSM-6360LA, JEOL, Japan).

2.5. Cell Seeding on the Scaffolds

Scaffolds were sterilized in 70% ethanol for 2 hours. Subsequently, the scaffolds were soaked in a culture medium overnight prior to cell seeding in order to facilitate protein adsorption and cell attachment on the nanofiber surface. Then, the medium was removed and the cells were seeded on the scaffold (5 × 10^4 cells/cm^2). Staining of the nucleus by DAPI was performed for cell detection.

2.6. Cell Proliferation Assay

Proliferation of the cells on PCL nanofibrous scaffolds or tissue culture polystyrene (TCP, control) was measured by 2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) assay according to the manufacturer’s instructions. The cells were cultured with a density of 2 × 10^4 cell/ml. After 1 and 3 days of culture, 100 µl MTT solution were added to each well. The cells were allowed to incubate in the dark at 37°C (5% CO2) for 4 h. Then, the medium was removed and the formazan reaction products dissolved in dimethyl sulfoxide and the plates were shaken for 20 min. The optical density of the formazan solution was read on an ELISA plate reader (Dynex MRX) at 570 nm. This assay was replicated 5 times.

2.7. Reverse Transcriptase PCR

Total RNA was extracted from hiPSCs- derived neural cells, using the RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen). CDNA was synthesized with M-MuLV reverse transcriptase and random hexamers as primers, according to the manufacturer’s instructions (Fermentas). PCR amplification was performed using a standard procedure with denaturation at 94 °C for 15 s, annealing at 58–61 °C (depending on the primers) for 30 s, and extension at 72 °C for 45 s. Amplified DNA fragments were electrophoresed on a 2% agarose gels, stained with ethidium bromide (10 µg/mL) and photographed on a UV trans-illuminator (uvidoc, UK). The data were normalized to an endogenous control gene (HPRT). The specific primers illustrated in table 1.

| Gene | Primer Sequences Sense, Top; Antisense, Bottom | Len |
|------|-----------------------------------------------|-----|
| HPRT | CCT GGC GTG GTG ATT AGT G TCA GTC CGT TCC ATT AGT CC | 125 |
| Nestin | GAA GGT GAA GGG CAA ATC TG CCT CTT CTT CCC ATA TTT CCT G | 96 |
| Map2 | AGT TCC AGC AGC GTG ATG CAT TCT TCT TAC CTT CTC | 97 |
| Tubulin | GAT CGG AGCCAA GTG CAT CGT CCC AGG TTC | 177 |

2.8. Statistical Analysis

Statistical difference was assessed with Student’s t – test, with significance set at P < 0.05 and the data were illustrated as mean± standard deviation (SD).

3. Results

3.1. IPS Cells Culture, EB Formation and Neural Progenitor Derivation

Morphological evaluation of iPS cells showed large nuclei with narrow cytoplasm. IPSCs colonies on SNL were tightly packed and flat (figure 1A). After 7 days, suspended culture of iPSCs colonies formed ball-shaped
spherical aggregates known as embryoid bodies (figure 1B); this ability assumed as an indicator of differentiation capacity of iPSCs. Culture of EBs on Laminin under neurogenic induction resulted in the neural progenitor cells (figure 1C, red arrow). RT-PCR analysis revealed that the neural progenitor cells in this stage expressed nestin, a common neural progenitor marker. With further culturing in the neural induction medium, differentiated cells were positive for β-tubulin and Map2 (figure 2).

3.2. Characterization of the Scaffolds

According to the SEM micrographs, the electrospun PCL scaffolds were aligned with uniformed morphology; these characteristics were similar to the dimension of major ECM component collagen. SEM micrograph of electrospun PCL aligned nanofibers is shown in Figure 3. Average fiber diameter was estimated to be 369.42 nm with diameter ranging from 100-1,000 nm. SEM examination showed that the seeded cells were adhered to scaffolds. This issue indicated that PCL scaffolds possessed biocompatibility for attachment of neural progenitors in vitro. The SEM image also indicated arrangement of the cell body along with the nanofibers and revealed cell attachment on the nanofiber scaffold.

3.3. Cell Proliferation Analysis

The adhesion and viability of neural progenitor cells on TCP (control) and PCL scaffold were studied using MTT chromometry assays after 24, 48, and 72 h of cultivation. The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of the viable cells. Both groups illustrated increasing trends in proliferation but this rate was higher in scaffold group. The statistical analyses confirmed significant differences between groups on 36h and 48h (figure 4). For 72h groups, the differences were not significant.

4. Discussion

Nerve regeneration is a complex process and a challenging field for researchers. During the past decades, impressive progress was made in the neural healing, however, much remains unexplored. Recently, many studies focused on the stimulated of the regenerative potential of axons through growth factor receptors or
manipulation of internal signaling pathways for spinal cord healing. On the other hand, stem cells could provide motor neurons for some other neural disorders.

Application of stem cells in tissue regeneration is under focus of many recent studies (Havasi, Nabioni, Soleimani, Bakhshandeh, & Parivar, 2013). Despite stem cells, the iPSCs make it possible for patient-specific cell therapies, which avoid immune rejection issue and ethical concerns of using embryonic stem cells (ESCs) (Moretti et al., 2010). The differentiation tendency of hiPSCs to neural (Hu, Weick, Yu, Ma, Zhang et al., 2010), cardio-vascular (Narsinh, Sun, Sanchez-Freire, Lee, Almeida et al., 2011) and hemangioblastic lineages (Feng, Lu, Klimanskaya, Gomes, Kim et al., 2010) emerged their potential for disease modeling and cell replacement therapies. Regarding such characteristics, we investigated human iPS cells for neural regeneration. The morphological evaluation of iPS cells and EB formation test presented in figure 1. Induction of cultured EBs into neural lineage resulted in the neural progenitor cells, which acquired for following investigations (figure 1C).

The fibrous structure closely resembles the natural environment that cells grow in and provides appropriate physical cues for manipulating cellular functions. Nano-fiber matrices provide a necessary 3D structure similar to natural ECM and result in better cell performance than 2D substrates. Current understanding of the effects of fibrous architecture on cellular function is still limited to the evaluation of cell morphological and viability changes. Electrospinning is a versatile and economical way of mass-producing fibrous constructs. It also enables incorporation of biochemicals to provide a synergistic effect in promoting nerve regeneration. Biofunctional nanofibrous scaffolds hold great potential as direct implantable devices and as basic neural cell biological study platforms that may be necessary for neural tissue engineering (Cao, Liu, & Chew, 2009). It is confirmed that nanotopographical features significantly alter cell behavior and influence cellular adhesion (Glass-Brudzinski, Perizzolo, & Brunette, 2002), morphology (Karuri, Lienensiek, Teixeira, Abrams, Campbell et al., 2004, Min, Kim, Kim, Paik, Jung et al., 2012) and proliferation (Dalby, Riehle, Johnstone, Affrossman, & Curtis, 2002) of various cell types. PCL scaffold as a temporary ECM enables the cells to adhere, spread and proliferate. Many studies investigated nanofibers of PCL to support growth, proliferation and migration of various cells (Bakhshandeh, Soleimani, Ghaemi, & Shabani, 2011, Venugopal & Ramakrishna, 2005, Achille, Sundaresh, Chu, & Hadjiargyrou, 2012, Ahvaz, Soleimani, Mobasher, Bakhshandeh, Shakhsalim et al., 2012). Many investigations of PCL scaffolds in neural tissue engineering (Min et al., 2012, Capkin, Cakmak, Kurt, Gumusderelioğlu, Sen et al., 2012, Prabhakaran, Venugopal, Chyan, Hai, Chan et al., 2008, Wang, Forsythe, Nisbet, & Parish, 2012) en-

![Figure 4. Evaluation of the cell proliferation that were cultured on TCP (control) or scaffold by MTT assay in 3 days; Asterisks show significant difference with p < 0.05](image-url)
encouraged us to electrospin PCL aligned nanofibers for this study. Evaluation of the nanofibers by SEM illustrated the homogeneity and aligned orientation (figure 3A). DAPI staining of the seeded-cell nuclei on TCP and scaffolds confirmed the adhesion and biocompatibility of the scaffold (figure 3B, C).

Evaluation of proliferation and viability of neural progenitor cells on PCL scaffold and TCP (as control) showed that the rate of proliferation in scaffold group is significantly higher than control till 48h after incubation. Insignificant difference between the groups on 72h could be explained by more penetration of the cells into the scaffold and inaccessibility to MTT reagents; therefore, this finding may be due to low sensibility and preciseness of the MTT method. Summarily, we confirmed the biocompatibility of the PCL scaffolds and suggested the suitability of the combination of the mentioned scaffold and human iPS cells for neural regeneration.

Supplementary: IPS Cells Generation Procedure:

For reprogramming induction, polycistronic lentiviral based vector carrying Sox2, Klf4, Oct4 and cMyc genes was used. For generation of virus, polycistronic vector co-transfected with PAX2 and PMD2 vectors in HEK 293T cell line by calcium phosphate method was used. Isolated foreskin fibroblasts trypsinized, centrifuged and the viral supernatant was added on cell pellet, and incubated in 37°C. After one hour, transduced cells were transferred into T75 cell culture flask and incubated in 5% CO2 and 37°C. The medium was changed after 24h with fresh medium and viral supernatant re-added at days 3 and 5. Reprogrammed clones were appeared after about two weeks and the medium replaced by embryonic medium containing DMEM F12, ES qualified serum replacement, NEAA, 2ME 4nM, and bFGF 40ng/ml. Media should be changed every day and regular passage for pluripotency maintenance and prevention of differentiation is necessary.

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