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Original Article

Differentiation of Mouse Stem Cells into Neural Cells on PLGA Microspheres Scaffold

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Abstract

The cellular therapy and nerve tissue engineering will probably become a major therapeutic strategy for promoting axonal growth through injured area in central nervous system and peripheral nervous system in the coming years. The stem cell carrier scaffolds in nerve tissue engineering resulted in strong survival of cells and suitable differentiation into neural cells, so this pathway should be created a favorable environment for axon regeneration. Poly lactic-co-glycolic acid (PLGA) has been widely used for manufacturing three dimentional scaffolds for tissue engineering. The pluripotent nature and proliferative capacity of embryonic carcinoma cells such as P19 also makes them an attractive cell source for tissue engineering. This study was initiated to evaluate potential of biodegradable PLGA microspheres for P19-derived neurons for neural tissue engineering and axon regeneration. The PLGA microspheres were prepared by using solvent evaporation, water in oil in water, technique. The water phase was polyvinyl alcohol (PVA) solution and the oil phase was PLGA solution. Retinoic acid (RA) was added to bacterial dishes as a differentiation factor inducer. P19 cells were attached to the PLGA microspheres and differentiated into neural cells on them. PLGA microspheres were characterised for size and surface morphology by scanning electron microscopy. The *in vitro* experimental studies were performed via immunoflouresent staining, scanning electron microscopy (SEM), RT-PCR, and histology. The photomicrograph and histology staining show the surrounded microspheres by P19 cells. The SEM results demonstrated the attachment and axon formation. Immunoflouresent staining and RT-PCR analysis for MapII, β-Tubulin, Nestin and Pax6 indicated the differentiation of P19 cells into neural cells. This report shows that high surface area also allows rapid cell expansion and increases cell attachment on PLGA microspheres, so each microsphere contains high cell density that resulted in survival of transplantation into the straitum of host animals, therefore, PLGA microspheres can help the differentiation of P19 cells into neural cells.

Keyword: Nerve tissue engineering; Neural differentiation; PLGA microspheres scaffold; Pluripotent stem cell.
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1.Introduction

The term "tissue engineering" was coined at a committee meeting at the National Science Foundation during the fall of 1987 [1]. Tissue engineering is an interdisciplinary technology that applies the principles and methods of bioengineering and life science to assemble and synthesize biological substitutes that will restore, maintain and improve tissue functions following damage by either disease or thrauma. Basically it involves the combination of living cells with either natural or synthetic materials to establish a 3-dimonsional construct that is structurally functional and mechanically equal to, or better than the damaged organ or tissue to be replaced [2]. Research in this field rapidly expanded and resulted in development of different designs and materials used in the preparing of 3-dimensional scaffolds suitable for tissue engineering.

A vast majority of biodegradable polymers belonging to the polyester family including polyglycolides and polylactides have been studied. Poly L-lactic-co-glycolic acid (PLGA), a biocompatible and biodegradable copolymer, has been used mostly for tissue engineering scaffold and drug delivery carriers because of good mechanical properties, non-toxicity, processibility and biodegradability [5, 6].

Scaffolds can be prepared in various shapes such as film, fiber, foam and particle. Special structure properties of microspheres such as high surface area and capability to be injected resulted in expanding of cells, rapidly [3]. The key elements in nerve tissue engineering are supportive scaffolds, cells, and trophic factors. It was noted that a permanent material is more likely to provoke a chronic immunological rejection and has a higher risk of infection as well as the potential to compress the axons over time; thus, their scaffolds should be biodegradable but not bioactive, to ensure safety. In addition, they should be cell adhesive to facilitate subsequent proliferation and differentiation [1].

Nerve tissue repair is a precious treatment concept in human health care as it directly impacts the quality of life. It is well known that the adult central nerve system can not be regenerated on its own after trauma or disease. Therefore, restoring its function is a challenge for neurobiologists and neurologists [4].

Stem cells have the capacity of self-renewal and differentiation to all types of cells. Like other embryonal carcinoma and embryonic stem cells, P19 cells are developmentally pluripotent and appear to differentiate using the same mechanisms as normal embryonic cells [7]. P19 embryonic carcinoma cells have the potential to differentiate into cell types of all three germ layer and form all tissues in the body. Teratocarcinomas can develop in some mouse

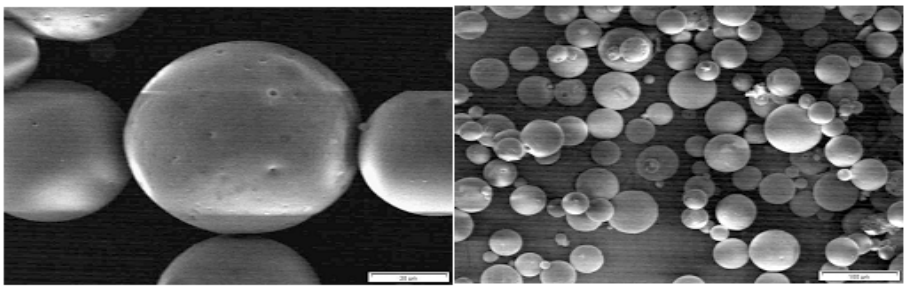

Figure 1. A scanning electron micrograph showing the surface morphology of the PLGA microspheres.

strains from early embryos transplanted from the uterus into ectopic sites. The differentiation of these cells can be controlled by nontoxic drugs. P19 cells treated with retinoic acid differentiate into a different spectrum of cell types that include neurons, astroglia and microglia cell types normally derived from the neuroectoderm [7, 8].

Injuries to the nervous system are the result of mechanical, thermal, chemical or congenital pathologies and if function is not restored, they lead to loss of function, pain and impair sensation. Nerve injuries complicate successful rehabilitation because mature neurons like many other cells in the body do not replicate; that is, they don't undergo cell division [9]. Spinal cord injury interrupts the nerve connections between the brain and the rest of the body and results in paralysis and loss of sensation below the level of injury. Because in most cases of human spinal cord injury there is significant loss of spinal cord tissue, cavity formation is an important obstacle, impeding axonal regeneration [1]. Therefore, repair of the injured human spinal cord in many cases will require not only neural survival, axonal growth and remyelination, but also reconnection across the trauma cavity by means of bridging grafts [9]. There have been encouraging reports of deficit reduction and axonal regrowth by blocking inhibitory molecules and antagonizing secondary injury mechanism; myelin replacement by stem cell, schwann cell and olfactory ensheating cells; delivery of growth factor and small molecules; and implantation of fetal tissue and various types of scaffolds, such as fibers, films, microparticle and nanoparticle scaffolds, as yet there is no practical treatment for spinal cord injury [10].

The goal of this project was to investigate the use of biodegradable PLGA microspheres as microcarriers for pluripotent cells. In this study retinoic acid (RA) was used which is known as the vitamin an acid as inducer. It affects the differentiation of P19 cells into neuron, astrocytes and oligodendrocytes and cell types normally derived from the neuroectoderm.

2. Materials and methods

2.1. Preparation of PLGA microspheres

PLGA polymer with uncapped end groups (type RG504H) was purchased from Boehringer-Ingelheim company and polyvinyl alchohol (PVA) (87-89% hydrolyzed, Mol. wt. 31000-50000 g/mol) was purchased from Aldrich.

The most commonly used method for preparing PLGA microspheres is the solvent evaporation technique based on the formation of a double emulsion (water-in-oil-in-water). Therefore, in this study, PLGA microspheres

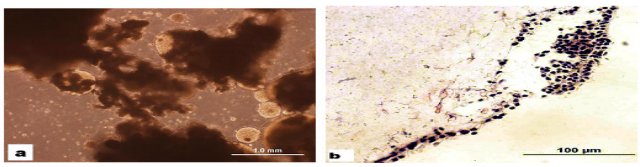

Figure 2. Phase contrast of P19 cells cultured on the surface of PLGA microspheres (a) and histological examination with H & E stain (b) showing cell adhesion and attachment on the PLGA microspheres.

were prepared by solvent evaporation, water-in-oil-in-water technique [11, 12]. Briefly, 100 mg of PLGA were dissolved in 500 µl chloroform. This polymer solution was combined with 2 ml of a 2% w/v solution of PVA in distilled deionized water (ddH_2O) and homogenized for 2 min. This emulsion was then added to a 30 ml of stirring 2% w/v PVA/ddH_2O and continued to be stirred for 4 h. The microspheres were then collected via centrifugation using a centrifuge set at 11000 rpm, 20 °C, for 10 min. The PLGA microsphieres were then washed twice with ddH_2O and centrifuged under the previously stated conditions. The resulting microspheres were then freeze-dried for 2-3 days and stored at -20 °C before use.

2.2. Characterization of microspheres

The diameters and surface morphology were determined using scanning electron microscopy (SEM) (Vega@ Tescan). Briefly, 5vmg of freeze-dried microspheres were resuspended in 500 µl of ddH_2O and air-dried onto aluminum plates. These samples were coated with gold-palladium at a thickness of 20 nm and were then viewed using a SEM.

2.3. Cell seeding

P19 cell line was obtained from Pasteur

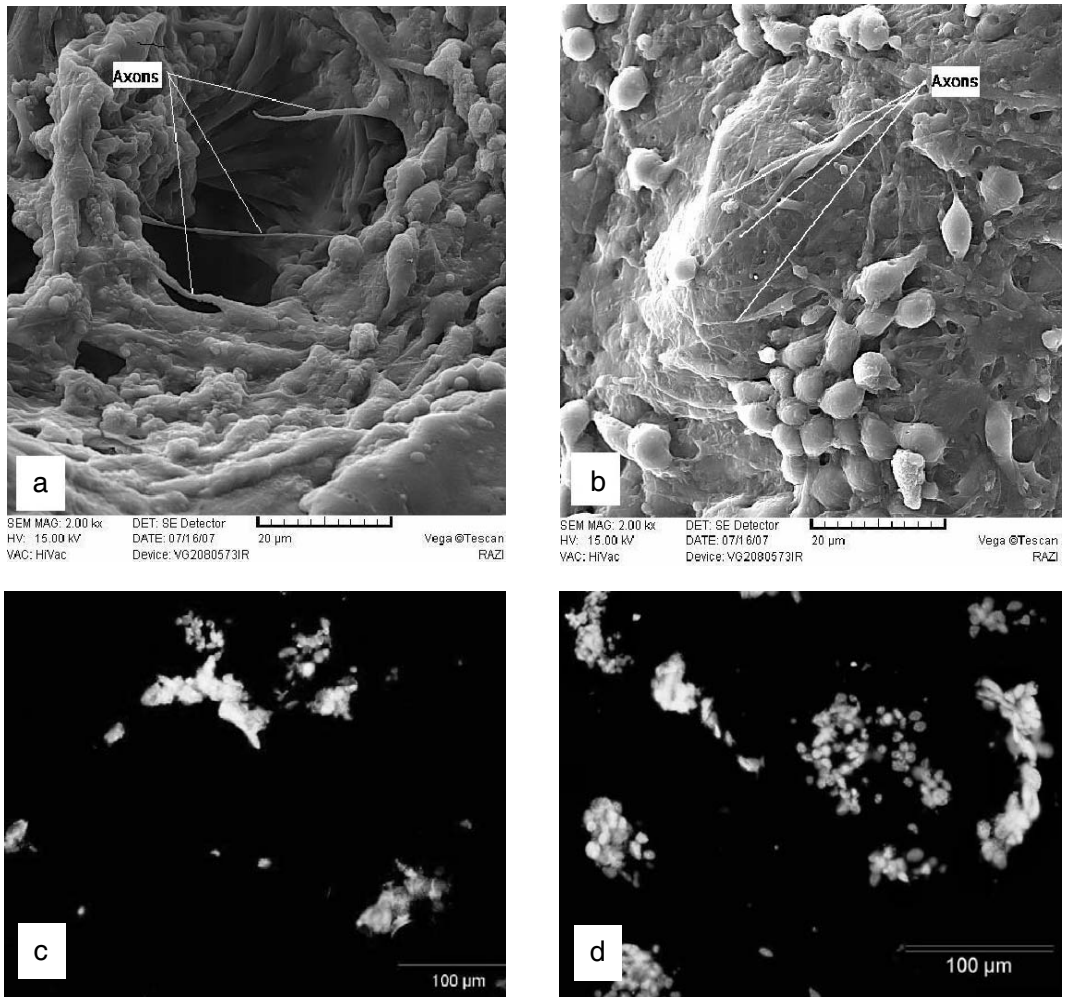

Figure 3. Scanning electron microscopy of attachment, expansion and cell differentiation on PLGA microspheres (a, b) and immunofluorescent staining for β-tubulinIII (c) and MapII (d) indicate neural differentiation and axons formation.

Institute, Tehran, Iran. The cells were cultured in Dulbecos modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin. For loading these cells on strilled microsphere, P19 cells were then trypsinised, centrifuged and resuspended in DMEM containing FBS. For seeding on microspheres, 1.5×10^5 cells/ml were loaded into each microsphere that previously were became wet with 70% ethanol and suspended in medium culture, containing DMEM and FBS by centrifuge at 1500 rpm for 5 min. After loading the cells, scaffold were suspended in bacterial grade Petri dishes for 6 days in the presence of RA, then the microspheres were plated onto tissue culture grade plastic Petri dishes. Aggregation, cell adhesion and expansion of P19 cells on the PLGA microspheres were examined by brightfield microscopy (Olympus IX51).

2.4. Histology and immunofluorescent staining

Microspheres were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Five micrometer-thick transverse sections were placed on silanized slides for immunohistochemistry or staining with hematoxylin and eosin (H & E). For immunocytochemistry, these slides were deparafinized in xylene and dehydrated in 100%, 96% and 70% ethanol for 5 min. and rinsed in ddH_2O. Then, slides should be retrival transveres by neutral buffered trypsin and calcium chloride. The sections were incubated in PBS containing 0.2% TritonX-100 for 15 min. and then incubated in blocking buffer (10% normal goat serum) for lh in 37 °C, thereafter in primary antibodies (β-TubulinIII and MapII) for 12h at 4 °C diluted in blocking buffers. After 12h, the slides were incubated with secondary antibody which was IgG FITC for 1h in 25 °C. Then the slides were washed 3 times with PBS at 5 min./wash and were also stained with PI, a nuclear stain, by a 10 sec at room temperature and three washes in PBS, then analyzed using the fluorescence microscope (Olympus, BX51, Japan).

2.5. RT-PCR

Total RNA was isolated by the NucleoSpin RNA II Kit (Macherey-Nagle, Duren, Germany). Prior to reverse transcription (RT), RNA samples were digested with DNase I (EN 0521; Fermentas) to remove

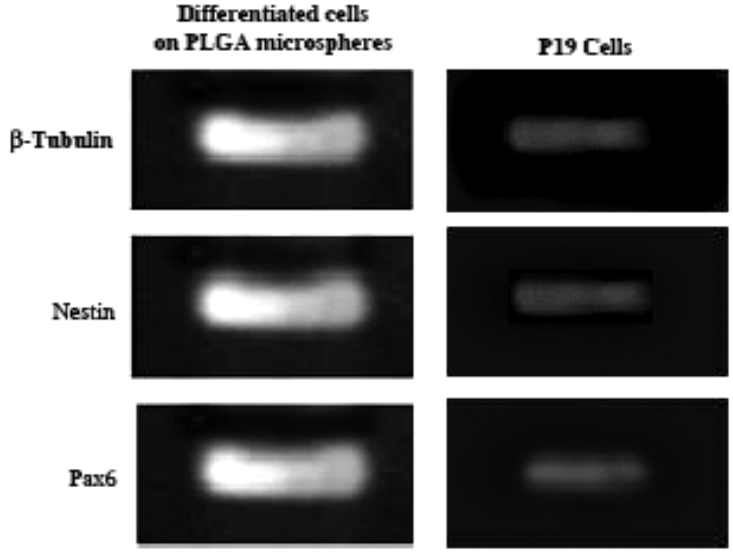

Figure 4. RT-PCR confirmed the neural differentiation of P19 carcinoma stem cells on PLGA microspheres.

contaminating genomic DNA. Extracted RNA was treated by DNase I (EN 0521; Fermentas) to avoid cross contamination of RNA by genomic DNA. Standard RT was performed using l µg total RNA, random hexamer primer and the RevertAid™ H Minus First Strand cDNA synthesis Kit (Kl622; Fermentas) according to the manufacturer's instructions. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using mouse specific primers designed using different exons (Table1). Amplification conditions were as follows: Initial denaturation at 94 °C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30s, annealing at 59-70 °C for 45s, extension for 45s at 72 °C and a final polymerization at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis on l.5% agarose and stained with ethidium bromide and visualized and photographed on a UV transilluminator (Uvidoc, UK).

3. Results

3.1. Characterization of microspheres

The particle size and surface morphology were determined by scanning electron microscopy (SEM). The average diameter of them was 13-100 µm and they had smooth outer surface (Figure 1).

3.2. Cell seeding

P19 cells adhesion and growth on the surface of PLGA microspheres on days 1-6 in culture in bacterial grade Petri dishes was observed using phase contrast micrographs (Figure 2a). Microspheres were totally surrounded with P19 cells.

3.3. Histology

H & E staining, on the slides, also demonstrated that P19 cells were placed on the surface of microspheres and surrounded them. The cells lined the outer surfaces of the scaffold, filling the area between microspheres, as demonstrated by histology and bright-field microscopy (Figure 2b).

3.4. Scanning electron microscopy (SEM)

SEM imaging was established to observe attachment and expansion of P19 cells on PLGA microspheres and differentiation of them to neural cells. Scanning electromicrographs shows adhesion of loaded cells on the surface of microspheres. As seen in the Figures 3a and 3b, attached cells were proliferated and made them seem to be axon and dendrites.

3.5. Immunoflourescent analysis

Immunoflourescent analysis using β-Tubulin III and Map II were performed on the PLGA microsphere that was loaded with P19 cells and PI used for staining of nuclei of cells. As is obsereved in Figures 3c and 3d, mature nervous structures (axons and dendrites) were stained by green dye of the β-Tubulin III and Map II.

Table 1. Primers and the reaction conditions for RT-PCR.

Gene	Primer sequences (5´-3´)	Annealing temperature (°C)	Length (bp)
β-tubulin	F: 5' GGA ACA TAG CCG TAA ACT GC3' R:5' TCA CTG TGC CTG AAC TTA CC3'	58	317
Nestin	F: 5 TCGAGCAGGAAGTGGTAGG 3 R: 5 TTG GGA CCA GGG ACT GTT A 3	58	352
Pax-6	F: 5`- GAG AGG ACC CAT TAT CCA GAT G -3` R: 5`- GCT GAC TGT TCA TGT GTG TTT G -3`	63	466

3.6. RT-PCR analysis

RT-PCR analysis of the expression of Nestin, β-Tubulin and Pax6 in differentiated cells on PLGA microspheres has been shown in Figure 4.

4. Discussion

Tissue engineering of a relatively favorable niche for the regeneration and re-myelination of neurons emerges as a novel treatment modality. In order to create a 3-dimentional supportive environment to direct the differentiation and organization of carcinoma stem cells into nerve tissue reconstruction, we used biodegradable polyester scaffolds. Polyester family such as poly (lactic-co-glycolic) acid has been intensively investigated as a tissue engineering scaffold to provide a temporary substrate for proliferation and differentiation of cells to regenerate new tissues for subsequent implantation, because of good mechanical properties, non-toxicity, processability, biodegradability and their potential to provide cells with a suitable growth environment, optimal oxygen levels and effective nutrient transport as well as mechanical integrity. The aim is to provide 3-dimentional environments to bring cells in close proximity, so that they can differentiate into neural tissues [2, 13, 14].

We evaluated attachment and 10-days survival of differentiating carcinoma stem cells seeded into the PLGA microspheres.

An ideal injectable scaffold for nerve tissue engineering would be easily injectable, non-inflammatory, biocompatible and capable of inducing neural tissue formation. Therefore, these results demonstrate that the PLGA microspheres can be prepared as a favorable environment for cell proliferation and ECM formation.

It was inferred from the bright-field results that the cells started to attach and grow from day 1. It was found that there was an evidence of cell growth on the first day after cell culture. On day 1, the cells were randomly spread over the surface of the polymer scaffold. Then, cells progressively grew throughout the 3D scaffold. Cell attachment and growth of cells on the surface of microspheres were observed. The cells lined the outer surfaces of the scaffold, filling the area between microspheres, as demonstrated by histology and bright-field microscopy. It is believed that high surface area to volume ratio is favorable for better cell attachment and growth, in particular neuron cell growth.

The scaffolds with performed structures, such as fibrous meshes, porous foams and films are disadvantageous since they require invasive surgical procedures for implantation. In contrast, injectable microspheres for *in vivo* tissue regeneration have raised great interest because it allows cell implantation through minimally invasive surgical procedures. Gene expression analysis confirmed the induction of transcripts related to neuron precursor cells (β-tubulin, Pax6 and Nestin), also immunoflourescent studies on P19 confirmed the presence of β-tubulin and Map II, skeletal proteins of neural cells and microtubal associated proteins, respectively (Figures 3c and 3d).

Embryonic stem cells differentiate based on a series of spatially and temporally regulated signals. Therefore, given the proper series of signals, it may be possible to differentiate embryonic carcinoma stem cells into neural cells [14]. The morphologic and biochemical phenotype of differentiating P19 cells - derived 3D structures grown on PLGA microspheres and supplemented with representative retinoic acid (RA) inducer factor, one of the most important morphogens and its embryonic distribution correlates with neural differentiation and positional specification in the developing central nervous system [15], were performed.

PLGA microspheres in nerve tissue engineering have the potential to act as a cell transplantation scaffold. In addition, cell adhesion to the matrix may be necessary for

the survival and proliferation of implanted cells.

Studies have demonstrated that the fate of differentiating stem cells is strongly influenced by direct cell-to-cell and cell-to-extracellular matrix contacts involving a complex "cocktail" of growth factors, signaling molecules and ECM proteins [16]. Therefore, the microspheres with rough and porous surfaces coated by extracellular matrix proteins which contain cell adhesion motifs as cell and growth factor carrier is suggested for in vitro cell culture.

5. Conclusion

This study showed that the PLGA microspheres can produce the strong structure of scaffold that was favorable for cell carrier and support. We found that the biodegradable polymer, PLGA, could be used as a scaffold in tissue engineering, to support neuron differentiation and neurite outgrowth.

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