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Differentiation of human bone marrow mesenchymal stem cells grown in terpolyesters of 3-hydroxyalkanoates scaffolds into nerve cells

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ABSTRACT

Polyhydroxyalkanoates, abbreviated as PHA, have been studied for medical applications due to their suitable mechanical properties, blood and tissue tolerance and in vivo biodegradability. As a new member of PHA family, terpolyester of 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate, abbreviated as PHBVHHx, was compared with polylactic acid (PLA), copolyester of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) for their respective functions leading to differentiation of human bone marrow mesenchymal stem cell (hBMSC) into nerve cells. Results indicated that 3D scaffolds promoted the differentiation of hBMSC into nerve cells more intensively compared with 2D films. Smaller pore sizes of scaffolds increased differentiation of hBMSC into nerve cells, whereas decreased cell proliferation. PHBVHHx scaffolds with pore sizes of 30–60 μm could be used in nerve tissue engineering for treatment of nerve injury. The above results were supported by scanning electron microscope (SEM) and confocal microscopy observation on attachment and growth of hBMSCs on PLA, PHBHHx and PHBVHHx, and by CCK-8 evaluation of cell proliferation. In addition, expressions of nerve markers nestin, GFAP and β-III tubulin of nerve cells differentiated from hBMSC grown in PHBVHHx scaffolds were confirmed by real-time PCR.

1. Introduction

The gold standard for treatment of nerve defects is to bridge the peripheral nerve gap with an autograft [1]. However, limitations still exist in these repairs of nerve injuries, partly because of the limited availability of donor tissues and partly due to the severity of the local pain suffered at the donor operative site [2]. Despite the good surgical advances, functional recovery was often poor [3,4]. The development of biomaterials for stem cell carriers has been regarded as a useful source of alternative tissue equivalents that provide a favorable microenvironment for tissue regeneration [5]. Tissue engineering techniques which enhance the beneficial endogenous responses to nerve injury could provide an alternative repair strategy [3].

Human bone marrow stromal cells (hBMSC) can continuously self-renew and differentiate into nerve cells in vitro under certain conditions [6]. There are also many evidences showing that hBMSC may be non-immunogenic or hypo-immunogenic [7]. hBMSC transplanted at sites of nerve injury are thought to promote functional recovery by producing trophic factors that induce survival and regeneration of host neurons [8]. Many researchers have attempted to regenerate nerve tissue by combining suitable biomaterials with hBMSC [9]. Artificial nerve scaffolds with cell and tissue compatibility have been used as carriers of cells to improve regeneration for nerve injury repair [10–15]. In the last few years, various biodegradable and non-biodegradable scaffolds have been tested using different experimental models of nerve injury [12,13]. In rat hemi-section spinal cord injury model, PLGA scaffolds with neural stem cell were implanted into the injured site, resulted in a functional improvement. Tissue loss and glial scarring were reduced by transplantation [14].

PHA, with their biocompatibility, biodegradability and strong mechanical properties, have been widely investigated for tissue engineering applications. Previous studies showed that PHB and PHBHHx could be used as potential candidate materials for peripheral nerve tissue engineering [15,16]. Novikova et al. demonstrated that a PHB scaffold promoted attachment, proliferation and survival of adult Schwann cells, and supported marked

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axonal regeneration within the graft [16]. An ideal scaffold should have a high affinity for cells to attach and proliferate, should be compatible to in vivo tissues and blood, should have durable strength. PHBHHx appears to be a material meeting these requirements [17]. Similar to PHBHHx, PHBVHHx is a new member of PHA family, our previous study showed that PHBVHHx had better biocompatibility compared with tissue cuture plates (TCP), PLA and PHBHHx [18]. Based on this finding, it had become interesting to investigate the possibility of PHBVHHx for hBMSC proliferation and differentiation.

Scaffold spatial structures have been shown to have effects on cell proliferation and differentiation in 3D directions [19]. In this study, porous PHBVHHx scaffolds intended for nerve tissue engineering were fabricated using thermally induced phase separation (TIPS), and these scaffolds with different pore sizes were studied for hBMSC proliferation and differentiation.

2. Materials and methods

2.1. Cell isolation and culture

Human bone marrow samples were acquired from donors (5–36 years old) without metabolic diseases at First Affiliated Hospital of Shantou University Medical College. The patients were informed and agreed on the sampling and the purposes, and local Ethical Committee approval was obtained for the use of the samples for this research. hBMSC cell isolation and culture were performed as previously described [20]. In brief, bone marrow samples were layered over a lymphoprep gradient and centrifuged at 2000 rpm for 15 min at room temperature. Mononuclear cells were washed twice with Hank's solution. Cells were resuspended in regular growth medium containing Dulbecco's modified Eagle's medium (low glucose content) (DMEM-LG, Invitrogen, California, USA) supplemented with 10 vol% fetal bovine serum (FBS, Hyclone, USA), penicillin/streptomycin, 2 mM glutamine, 5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, California, USA) and 10^3 U/ml leukemia inhibitory factor (LIF, Chemicon) to maintain their undifferentiated state. The cells were seeded in 6-well tissue culture plate (Corning Inc, Acton, Massachusetts, USA) at a density of 1×10^6 cells/cm^2. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. After 72 h, non-adherent cells were removed, and the medium was changed every other day until cells became 90% confluent. The cells were digested with 0.25% trypsin solution (GIBCO, USA) and seeded with a density of 1×10^4 cells/cm^2 in regular growth medium for expansion purpose.

For differentiation studies, cells were cultured in nerve induction medium containing Dulbecco's modified Eagle's medium, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, California, USA), 500 mM IBMX (sigma, USA), 200 mM, INDO (sigma, USA), and 5 mg/ml insulin (sigma, USA) without FBS.

2.2. Analysis of cell phenotype by flow cytometry

Flow cytometry study was performed similarly as described previously [21]. Cell surface antigen phenotyping study was carried out using the hBMSC. The following cell surface epitopes were detected with anti-human antibodies: CD73-phycoerythrin (PE), CD105-PE, CD45-fluorescein isothiocyanate (FITC), CD34-FITC, HAL-DR-PerCP, CD90-FITC. 5×10^5 cells were analyzed using a FACScan flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA).

2.3. Preparation of 2D membranes and 3D porous scaffolds

Films of PLA, PHBHHx and PHBVHHx were prepared as previously described [22], Briefly, 1 g material was dissolved in 50 ml chloroform under vigorous agitation for 45 min at 60 °C. Subsequently, the chloroform solution was poured into Petri dishes. The dishes were maintained at room temperature for 24 h to allow the complete evaporation of chloroform. Vacuum drying was applied to completely remove any possible solvent remaining in the films.

Scaffolds of PHBVHHx were prepared by thermally induced phase separation (TIPS) [23,24]. Briefly, 1 g of PHBVHHx was dissolved in 25 ml of 1,4-dioxane under vigorous agitation for 60 min at 65 °C. The clear polymer solution was frozen for 2 h under varied temperature conditions of −20, −80 and −196 °C. Solvent was removed by a freeze-drying process lasting 24 h.

2.4. Scanning electron microscopy (SEM) examination

Materials were treated for SEM as previously described [25]. Cell-seeded films were washed three times using phosphate buffered saline (PBS), and immersed in PBS containing 5% glutaraldehyde (pH 7.4) for 1 h. They were then dehydrated in increasing concentrations of ethanol (from 30%, 50%, 70%, 90%, and 95%–100%), followed by lyophilization. No cell-seeded scaffold did not go through the above treatments. All samples were mounted on aluminum stumps coated with gold in a sputtering device (JFC-1600, Japan) for 10 min, followed by examination under a SEM (JSM-6360LA, Nikon, Japan).

2.5. Cell viability study

A cell count kit-8 (CCK-8, Beyotime, China) was employed in this experiment to quantitatively evaluate hBMSC viability [26]. Briefly, approximately 1×10^4 cells were seeded on each film placed in the 24-well plates for 72 h, the culture medium was removed and the cultures were washed with PBS twice. Approximately 900 μl serum-free DMEM medium and 100 μl CCK-8 solution were added to each sample, followed by incubation at 37 °C for 3 h. Supernatant was transferred to 96-well plate, the optical density (OD) at 450 nm was determined using a microplate reader (Multiskan MK33, Thermolabsystems, Finland). Six parallel experiments in each sample were used to assess the cell viability.

2.6. Cell seeding in PHBVHHx 3D scaffolds

PHBVHHx 3D scaffolds fabricated under different temperature gradients were evaluated for cell differentiation and proliferation [27]. Briefly, 3D scaffolds were pre-sterilized with 75% (v/v) ethanol followed by repeated washing with PBS to remove any residual alcohol. Scaffolds were first conditioned with DMEM for 2 h before cell seeding. Scaffolds were placed in 6-well culture plates (1 scaffold/well), each scaffold was seeded with 3×10^5 cells in 20 μl of the cell suspension. Seeded scaffolds were incubated for 2 h in a humidified atmosphere under 37 °C and 5% CO_2. Then, 3 ml DMEM was added to each well and scaffolds were incubated for 72 h. Half of the scaffolds were moved and transferred to nerve induction medium for differentiation study and then real-time PCR analysis, the other halfs were used to determine cell proliferation and distribution on scaffolds.

2.7. Study of cell proliferation and distribution in scaffolds using confocal microscopy

hBMSC proliferation and distribution on PHBVHHx scaffolds were studied using confocal microscopy. Briefly, scaffolds with cells were washed with phosphate buffered saline, fixed for 5 min in 3.7% formaldehyde in PBS. They were then immersed in 0.1% Triton X-100 for 3 min, followed by washing again in PBS. The so treated samples were taken for phalloidin-FITC (Sigma, USA) staining for 40 min under room temperature. Imaging experiments were conducted under laser scanning confocal microscope (LSM 510 Meta, Zeiss, Germany).

2.8. Real-time PCR analysis of gene expression

Total cellular RNA was extracted from cells grown on films of PLA, PHBHHx, PHBVHHx and in three independent PHBVHHx scaffolds immersed in nerve induction medium, hBMSC cultured in standard medium was used as a control. RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) was employed in this process following manufacturer's instructions with DNase I (Qiagen) treatment. Complementary DNA synthesis was performed with 1 μg total RNA using PrimeScript RTase (TaKaRa, Japan). Real-time PCR study was conducted as described previously [28]. SybrGreen PCR MasterMix (Toyobo, Japan) was used in each reaction. PCR reaction was carried out under conditions of 95 °C, 15 s; 60 °C, 15 s; 72 °C, 30 s for 40 cycles using ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression level of GAPDH gene was used as an internal control. The PCR primers and probes were as synthesized with following sequences:

GAPDH forward primer: 5′-GCACCGTCAAGGCTGAGAAC-3′;
GAPDH reverse primer: 5′-TGGTGAAGACGCCAGTGGA-3′.
Nestin forward primer: 5′-CTCCAAGAATGGAGGCTGTAGGAA-3′;
Nestin reverse primer: 5′-CCTATGAGATGGAGCAGGCAAGA-3′.
GFAP forward primer: 5′-TGGCAGAGCTTGTTAGTGGTAAAGG-3′;
GFAP reverse primer: 5′-GTGAGACAGAGGCTGCTGCTTG-3′.
β-III tubulin forward primer: 5′-GAACCCGGAACCATGGACAG-3′;
β-III tubulin reverse primer: 5′-GACCCTTGGCCCAGTTGTTG-3′.

The expression level of GAPDH [GAPDH(ct) = 18] was used for normalization. A Ct of 38 was designated arbitrarily as 1 [29].

2.9. Statistical analysis

All data were calculated with the mean and standard deviation. The statistical significance of the data obtained was analyzed by the Student's *t*-test. Probability values of $p < 0.05$ were interpreted as denoting statistical significance.