Biomaterials 35 (2014) 8566–8575

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Retention of stemness and vasculogenic potential of human umbilical cord blood stem cells after repeated expansions on PES-nanofiber matrices

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ARTICLE INFO

Article history:
Received 14 May 2014
Accepted 19 June 2014
Available online 4 July 2014

Keywords:
PES-nanofiber
Re-expansion
CD34$^+$ stem cells
Proangiogenic growth factors
NOD/SCID mice
Limb ischemia

ABSTRACT

Despite recent advances in cardiovascular medicine, ischemic diseases remain a major cause of morbidity and mortality. Although stem cell-based therapies for the treatment of ischemic diseases show great promise, limited availability of biologically functional stem cells mired the application of stem cell-based therapies. Previously, we reported a PES-nanofiber based *ex vivo* stem cell expansion technology, which supports expansion of human umbilical cord blood (UCB)-derived CD133$^+$/CD34$^+$ progenitor cells ~225 fold. Herein, we show that using similar technology and subsequent re-expansion methods, we can achieve ~5 million-fold yields within 24 days of the initial seeding. Interestingly, stem cell phenotype was preserved during the course of the multiple expansions. The high level of the stem cell homing receptor, CXCR4 was expressed in the primary expansion cells, and was maintained throughout the course of re-expansions. In addition, re-expanded cells preserved their multi-potential differential capabilities *in vitro*, such as, endothelial and smooth muscle lineages. Moreover, biological functionality of the re-expanded cells was preserved and was confirmed by a murine hind limb ischemia model for revascularization. These cells could also be genetically modified for enhanced vasculogenesis. Immunohistochemical evidences support enhanced expression of angiogenic factors responsible for this enhanced neovascularization. These data further confirms that nanofiber-based *ex-vivo* expansion technology can generate sufficient numbers of biologically functional stem cells for potential clinical applications.

1. Introduction

Hematopoietic stem cells (HSC) are multipotent in nature [1]. During the past several decades, HSC transplantation has been used as standard treatment for various hematological disorders [2,3]. Even though HSC transplantation has been applied in the clinics, regulation of HSC self-renewal and differentiation remain a major challenge that hampers ex-vivo expansion of HSCs, and limits generation of sufficient number of biologically functional HSCs for routine clinical use from a single unit of human umbilical cord blood (UCB). Furthermore, the maintenance, proliferation and differentiation of HSCs are mainly regulated by the microenvironment in bone marrow or stem cell niche that contains niche cells and extracellular matrix (ECM). While ECM provides the basic physical and chemical support for stem cell function, niche cells control stem cell quiescence, proliferation and differentiation [4–6]. Many of the current *ex vivo* expansion technologies are being developed mimicking bone marrow microenvironment to acquire optimum condition for survival and proliferation of HSCs with limited differentiation [5].

ECM plays very important role in stem cell regulation, survival and differentiation by supporting mechanical ultra-structure of the microenvironment present in the bone marrow. ECM interacts with stem cells through adhesion molecules, control cell

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http://dx.doi.org/10.1016/j.biomaterials.2014.06.037
0142-9612/

geometry, mechanical property and nanotopography [7]. As for example, adhesive segments of an ECM protein fibronectin were able to enhance growth and proliferation of HSCs [8]. Mechanical signals developed within the microenvironment also alter the cytoskeletal tensions of ECM and regulate the fate of HSCs, enabling them to proliferate, differentiate, migrate or undergo apoptosis [9]. Osteoblasts residing within the bone marrow niche are the most important cells that support maintenance of HSCs by secreting various cytokines and growth factors [10]. Osteoblasts also secrete chemo-attractant, stromal cell-derived factor (SDF)-1, which binds to CXC chemokine receptor 4 (CXCR4) expressed on HSCs [11]. SDF-1 also stimulates the growth and survival of CD34+ progenitor cells [12,13].

The *ex vivo* expansion of human stem cells has been studied extensively using biological or biomaterial approaches. In a biological approach, stromal layers were used for expansion of stem cells, however, secretory products from these methods are not clearly defined and additionally, anti-proliferative signals are also generated from these methods that limits proliferation of HSCs [14]. To mimic *in vivo* ECM structure, numerous synthetic polymeric biomaterial substrates such as polyethylene terephthalate (PET), tissue culture polystyrene (TCPS), maleic anhydride, and polyether sulfone (PES) fibers are being extensively studied for *ex vivo* expansion of HSCs [15]. These materials have advantages because of their well-defined composition, reproducibility of surface chemistry topography, toxicity profile, and degradation rates. Therefore, several biomaterials have been used without modifications for the *ex vivo* expansion of HSCs with limited success [16,17]. Thus, modifications of base materials with ECM molecules or chemical moieties and topographical patterns were applied for effective HSC expansion. Studies support that the surface chemistry and topography affect the rate of HSC proliferation and expansion [18–21]. Human UCB-derived CD34+ cells were *ex vivo* expanded on chemically modified PES substrate. PES that conjugated with amine group has shown to have different patterns of focal adhesion and supports highest expansion of HSCs compared to other chemically modified PES or unmodified PES [19].

One of the major causes of human mortality and morbidity in the world are ischemic diseases [22]. Ischemia is generally caused by occlusion of artery due to cholesterol deposition into the arterial lumen resulting in reduction of oxygen supply and nutrition leading to cellular death. Although advancement in traditional therapy in the last decade, improved life expectancy, however, a significant number of patients are not suitable for the common therapeutic approaches [23]. Thus new strategies for revascularization would be beneficial to increase blood flow via an alternative stem cell therapeutic approach for these patients. Herein, we explore the concept of therapeutic angiogenesis in which neovascularization is induced in ischemic tissues to improve blood flow and subsequently, reduce symptoms of these suboptimal patients [24]. In this study, we assess the biological functionality of re-expanded cells in a hind limb ischemic model.

2. Materials and methods

2.1. CD133+ cell isolation

Fresh human umbilical cord bloods (70–100 ml) were obtained from The Wexner Medical Center at The Ohio State University after IRB approval and written consent from donors. Blood samples were processed following a similar protocol earlier published [20,25–28]. In brief, the citrate phosphate dextrose-adenine 1 (CPDA-1) anti-coagulated blood was diluted with PBS and 10 ml of Ficoll-Paque plus (GE Healthcare, Piscataway, NJ) was carefully under layered. After 30 min centrifugation in a swinging bucket rotor at 14000 rpm, the upper layer was aspirated and the mononuclear cell layer was collected. Furthermore, following labeling with magnetic bead conjugated anti-CD133 (CD133) monoclonal antibody (Miltenyi Biotec Inc, Bergisch Gladbach, Germany), two cell separation cycles (with different columns) were performed using the AutoMACS cell sorter (Miltenyi Biotec) according to the manufacturer's protocol and reagents. After separation, periodic purity of the cell product was determined by flowcytometry.

2.2. Electrospinning of PES nanofiber mesh

Electrospinning, PAAc grafting and amination of PES nanofibers were performed following earlier described protocol [19]. All reagents were purchased from Sigma–Aldrich (USA) except PES granules (MW: 55,000), which were purchased from Goodfellow Cambridge Limited. In brief, PES granules were dissolved in DMSO at 20% w/v concentration to pass through a plastic syringe with 27G needle. A pump (KD Scientific, USA) with fixed speed (0.3 ml/h) was used to feed the polymer solution into the syringe. Electrospinning was performed at 13 kV with a high voltage power supply (Gamma High Voltage Research, USA). Nanofibers were collected directly onto grounded 15 mm diameter glass coverslips (Paul Marienfeld, Germany) located at a fixed distance of 160 mm from the needle tip, over a collection time of 25 min. PES films were fabricated by dip-coating glass in 10% PES in DMSO. The deposited nanofiber and film samples were washed thoroughly in distilled water and ethanol to remove any residue of DMSO, and subsequently dried and stored in a desiccator.

2.3. Surface grafting of PES nanofiber mesh with poly acrylic acid (PAAc)

Acrylic acid (AAc) (Merck, Germany) was distilled and stored at −20 °C prior to use. PAAc was grafted onto the PES nanofiber mesh surface by photo-polymerization [18]. Briefly, samples were immersed in aqueous solution containing 3% AAc solution and 0.5 mM $NaIO_4$ in a flat-bottom glass container. The temperature of the solution was maintained at 8 °C. The samples were then exposed to UV from a 400 W mercury lamp (5000-EC, Dymax, Germany) for 2 min at a fixed distance of 25 cm. The PAAc-grafted meshes were then thoroughly washed with deionized water at 37 °C for over 36 h to remove any ungrafted PAAc from the surface of the scaffold and dried in a storage desiccator.

2.4. Amination of PAAc-grafted PES nanofiber mesh

Following cross-linking method the PAAc-grafted PES nanofiber mesh was conjugated with ethylene diamine (EtDA). Briefly, each scaffold was first gently shaken in 2 ml acetonitrile containing 50 mM N-hydroxysuccinimide (NHS) and 50 mM dicyclohexylcarbodiimide (DCC). After 6 h, the reaction solution was carefully aspirated and each scaffold was immediately immersed into 2 ml acetonitrile containing 0.03 mmol EtDA. After 12 h, the reaction solution was carefully aspirated and each scaffold was thoroughly washed in absolute ethanol to remove any dicyclohexyl urea (DCU), a byproduct of the reaction. All substrates were subsequently sterilized in 70% ethanol, then loaded into 24-well tissue culture plates (Nunc) and stored in sterile PBS until use. Surface amine density was quantified according to the method described earlier [29].

2.5. CD133+ hematopoietic stem cell expansion and re-expansion cultures

The StemSpan SFEM medium was purchased from StemCell Technologies (Vancouver, BC, Canada). Purified recombinant human stem cell factor (SCF), Flt-3 ligand (Flt3), TPO and IL-3 were purchased from Peprotech Inc. (Rocky Hill, NJ, USA), and low-density lipoprotein was purchased from Athens Research and Technology Inc., Athens, GA, USA. Nanofiber meshes were securely glued to the bottoms of wells of a 24-well tissue culture plate. Eight hundred CD133+ cells were seeded onto each scaffold in 0.6 ml StemSpan™ serum-free expansion medium, which consists of 1% BSA, 0.01 mg/ml recombinant human insulin, 0.2 mg/ml human transferrin, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine in Iscove's MDM, supplemented with 0.04 mg/ml low-density lipoprotein, 100 ng/ml SCF, 100 ng/ml Flt3, 50 ng/ml TPO and 20 ng/ml IL-3. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2 for 10 days without medium change. Cells were harvested after 10 days of expansion. All substrates were washed once with non-trypsin cell dissociation solution and twice with 2% FBS Hanks' buffer at 5–10 min intervals between each wash. The cell suspensions collected were then concentrated through centrifugation at 500× *g* for 10 min. Aliquots of the concentrated cells were then used for cell counting by a hemocytometer, flow cytometric analysis, as well as for further studies. Primary expanded cells were re-expanded at least for four more times by following the same expansion methods and the same 24-well nanofiber plates.

2.6. Flowcytometry

Flow cytometric analysis for cell surface markers were performed by blocking expanded cells first with FCR Blocking Reagent (1:5; Miltenyi Biotec Inc) and followed by incubating for 20 min at 4 °C with the following antibodies: anti-CD34-PE, and anti-CD133/2 FITC (all from Miltenyi Biotec Inc), CXCR4, CD31, CD14, CD161, MHC Class-I, MHC Class-II, LFA-1, VCAM-1, CD45, CD69, CD18, CD36, CD3, CXCR2, CD71, CD45R and Isotype controls (IgG1 and IgG2a) were purchased from BD Biosciences, (USA). After incubation, cells were washed with MACS sorting buffer and analyzed using a FACS Calibur flowcytometer (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded via propidium iodide staining. At least 20,000 events were acquired. Data analysis was performed with BD Cell Quest software.