Biomaterials 32 (2011) 6006–6016

Contents lists available at ScienceDirect

Biomaterials

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

Large-scale production of murine embryonic stem cell-derived osteoblasts and chondrocytes on microcarriers in serum-free media

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ARTICLE INFO

Article history:
Received 21 March 2011
Accepted 5 April 2011
Available online 26 May 2011

Keywords:
Osteoblasts
Chondrocytes
Stirred suspension bioreactors
Microcarriers
Serum-free cultivation

ABSTRACT

The generation of tissue-engineered constructs from stem cells for the treatment of musculoskeletal diseases may have immense impact in regenerative medicine, but there are difficulties associated with stem cell culture and differentiation, including the use of serum. Here we present serum-free protocols for the successful production of murine embryonic stem cell (mESC) derived osteoblasts and chondrocytes on CultiSpher S macroporous microcarriers in stirred suspension bioreactors. Various inoculum forms and agitation rates were investigated. Produced osteogenic cells were implanted ectopically into SCID mice and orthotopically into a murine burr-hole fracture model. Osterix, osteocalcin and collagen type I were upregulated in osteogenic cultures, while aggrecan and collagen type II were upregulated in chondrogenic cultures. Histological analysis using alizarin red S, von Kossa and alcian blue staining confirmed the presence of osteoblasts and chondrocytes, respectively in cultured microcarriers and excised tissue. Finally, implantation of derived cells into a mouse fracture model revealed cellular integration without any tumor formation. Overall, microcarriers may provide a supportive scaffold for ESC expansion and differentiation in a serum-free bioprocess for *in vivo* implantation. These findings lay the groundwork for the development of clinical therapies for musculoskeletal injuries and diseases using hESCs and iPS cells.

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1. Introduction

The growing demand for organ replacements coupled with a severe shortage of donors has led to the emergence of alternative therapies [1–3], including tissue engineering, which involves the production of cells, tissues or organs to replace or restore function in damaged or injured tissue. Cellular therapies, including therapies for musculoskeletal diseases, will require at least 10^7–10^9 cells per treatment [4]. The demand for clinically relevant numbers of cells necessitates the development of a robust, controllable system to scale-up the expansion of stem cells and their derived progeny.

Current therapies for musculoskeletal diseases, including osteoporosis, osteoarthritis and non-union fractures, commonly focus on the use of palliatives, which only serve to alleviate pain [5]. Other treatment protocols involve the use of autograft and allograft transplantations for skeletal defects, which are effective but have several limitations, including tissue availability, donor site morbidity and host immune rejection. There are currently no cures for these diseases. Although the benefits of cell-based bone tissue engineering has been known for some time [6–10], several issues are yet to be resolved before clinical applications of this technology can be realized. One of these issues is the production of relevant numbers of the correct type of cells.

The unlimited self-renewal capabilities of embryonic stem cells (ESCs) coupled with their ability to differentiate into every cell type in the body makes them attractive for use in regenerative medicine therapies [11]. However, successful downstream clinical use of these cells will require large-scale generation in a robust and tightly regulated fashion [12]. Produced cells may eventually be transplanted as aggregates, dissociated single cells or incorporated into a scaffold.

Conventional methods for production of osteoblasts and chondrocytes from embryonic stem cells involve the use of static tissue cultures (e.g., dishes, cell factories) [13–21]. The available surface area for propagation of cells in static tissue culture is limited and

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doi:10.1016/j.biomaterials.2011.04.015

passaging and harvesting protocols are time consuming and labor-intensive. Static tissue culture systems also lack continuous monitoring and tight control of the culture microenvironment, which could lead to spontaneous stem cell differentiation and/or culture heterogeneity, and typically employ the use of animal-derived products [13–21]. Furthermore, the production of mineralized extracellular matrix from ESC-derived osteogenic cells creates tremendous difficulties when attempting to harvest the cells from adherent static cultures, and leads to extensive cell loss.

Exposure of ESCs to serum-containing media poses a major concern for downstream clinical applications because of the effects on ESC immunogenicity and potential pathogenicity. Serum is subject to considerable lot-to-lot variability and may contain unidentified pathogens, prions and viruses [22]. Moreover, for clinical applications, it will be important to avoid immune-based rejection responses including xenogeneic responses induced by the presence of animal-derived components [23].

Finally, a three dimensional (3D) culture system more closely resembles the physiological environment and has been shown to promote lineage-specific differentiation of several types of cells [24]. In considering the high demand for alternative treatments for musculoskeletal diseases and the limited supply of cells, as well as concerns surrounding differentiation efficiencies, it is clear that the development of a robust, reproducible bioprocesses for the production of clinically relevant quantities of osteoblasts and chondrocytes is necessary.

First introduced by van Wezel in 1967 to mass produce viral vaccines and biological cell products using mammalian cells [25], microcarriers have been successfully used for the large-scale production of a variety of biological products [26]. Microcarriers provide immense surface areas for attachment and expansion of anchorage-dependent cells in stirred tank reactors. The use of microcarriers offers a number of advantages including lower total costs, ease of harvesting and downstream processing procedures, ease of scale-up, and an overall reduction in the space required for a given-sized operation due to the high growth surface area per unit volume of reactor [27].

Microcarriers have typically been used in the production of viral vaccines using VERO cells, and the production of recombinant therapeutics using Chinese Hamster Ovary (CHO) and Baby Hamster Kidney (BHK) cells [28–30]. In addition to their capacity to serve as scaffolds for the propagation of anchorage-dependent and anchorage-preferred cells, microcarriers are also efficacious to employ as delivery vehicles for implantation of the cells *in vivo*. They have been used for the culture of pancreatic islet cells [31] mesenchymal stem cells [32–34] and fibroblast cells [35–37] amongst others.

Despite the potential benefits of producing clinically relevant quantities of osteoblasts and chondrocytes for cellular therapies, to date however, there have been no studies on the production of ESC-derived osteoblasts or chondrocytes in a feeder-free, serum-free system using microcarrier scaffolds. To this end, we set out to develop an effective system for the large-scale production of ESC-derived cells on microcarriers under feeder-free and serum-free conditions in stirred suspension bioreactors.

2. Materials and methods

2.1. Mouse embryonic stem cell line

Unless otherwise stated, all cell-handling procedures were conducted in a sterile laminar flow hood using aseptic techniques and all ESC cultures were carried out in a humidified, 37 °C and 5% CO_2 incubator. Murine D3 ESCs (ATCC, American Type Culture Collection, Rockville, MD, USA) were used in all cultures. All bioreactor cultures were performed in duplicate and all cell counts were performed on duplicate samples.

2.2. Culture medium

2.2.1. Maintenance medium

Murine ESC maintenance medium consisted of Dulbecco's Modified Eagle Medium with high glucose (DMEM, Invitrogen, Carlsbad, California USA. Cat# 10569-010) supplemented with 15% Knockout-Serum Replacement (KSR, Invitrogen, Cat# 10828-018), 1% v/v non-essential amino acids (NEAA, Invitrogen, Carlsbad, California USA Cat# 11140-050), 0.2% (v/v) β-mercaptoethanol (Invitrogen, Carlsbad, California USA Cat# 21985-023) and 1% v/v penicillin/streptomycin (Pen/Strep, Invitrogen, Cat# 15140-122).

2.2.2. Osteogenic medium

Osteogenic medium (OM) consisted of maintenance medium supplemented with 10^{-5} M dexamethasone (Sigma–Aldrich, Ontario Canada. Cat# D4902), 50 μg/mL ascorbic acid (Sigma–Aldrich, Ontario Canada. Cat# A4403), and 5 mM β-glycerophosphate (Sigma–Aldrich, Ontario Canada. Cat# G9891).

2.2.3. Chondrogenic medium

There were two types of chondrogenic media which were used in separate cultures; (i) chondrogenic medium 1 (CM1) consisted of DMEM (Invitrogen, Carlsbad, California USA. Cat# 12100-061), supplemented with 15% Knockout-Serum Replacement (KSR, Invitrogen, Cat# 10828-018), 1% non-essential amino acids (NEAA, Invitrogen, Carlsbad, California USA. Cat# 11140-050), 1% penicillin/streptomycin (Pen/Strep, Invitrogen, Cat# 15140-122), 1 mM Sodium Pyruvate (100×, Gibco, Carlsbad, California USA. #11140-050), 100 mM β-mercaptoethanol (550×, Gibco #21985-0230), 50 μg/mL Ascorbic acid (Sigma–Aldrich, Ontario Canada), 10 ng/mL BMP-2 (BMP-2, PeproTech # 120-02), 10 ng/mL TGFβ1 (TGFβ1, PeproTech Rocky Hill, NJ, USA. Cat# 100-21C) and 1% of an insulin–transferrin–selenium complex (ITS, Invitrogen, Ontario Canada #51500-056), and (ii) chondrogenic medium 2 (CM2) consisted of all of the components in CM1 minus the TGFβ1.

2.3. Mouse embryonic stem cell cultivation

2.3.1. Microcarrier preparation and cell expansion

For microcarrier preparation, 0.1 g CultiSpher S (Percell Biolytica, Åstorp, Sweden), a highly cross-linked type A porcine-derived gelatin macroporous microcarrier was weighed dry and initially hydrated separately in 50 mL of Ca^{2+}, Mg^{2+} – free phosphate buffer saline (PBS, Invitrogen) overnight at room temperature. The supernatant was then removed and the microcarriers were washed twice in fresh PBS and sterilized by autoclaving at 120 °C for 30 min. After sterilization, microcarriers were equilibrated in 20 mL of culture medium with LIF in NDS 125 mL bioreactors (NDS Technologies, Palo Alto, California USA, Cat# 264501-125) at 37 °C and 5% CO_2. After 1 h, medium volume was increased to 50 mL and cultivation in bioreactors was performed at 60 rpm, 5% CO_2 and 37 °C. ESCs previously thawed and expanded in T-75 tissue culture vessels were used to seed sterilized microcarriers at a 5:1 cell to bead ratio. Cultures were then intermittently stirred (3 min of stirring at 60 rpm followed by 27 min off) during the first 8 h of culture. The medium was topped-up to a final volume of 100 mL and stirred continuously at 60 rpm for 6 days. Medium was changed every second day during the expansion phase.

2.3.2. Differentiation to osteoblasts and chondrocytes

Osteogenic cultures consisted of maintenance medium replaced with osteogenic medium; (i) a single cell inoculum without microcarriers (SC-MCs), agitated at 100 rpm, (ii) single cell inoculum plus empty sterilized microcarriers (SC + MCs) agitated at 60 rpm, (iii) cell-loaded inoculum microcarriers from day 6 expansion, induced into differentiation without dissociation (CL + MCs) agitated at 60 and 100 rpm. For all conditions, differentiation was carried out for 30 days.

For chondrogenic differentiation, maintenance medium was replaced with CM1 and bioreactors were seeded with day 6 cell-loaded microcarriers (CL + MCs) and agitated at 60 or 100 rpm. On day 5, CM1 was replaced with CM2 for the remainder of the differentiation. For all conditions, differentiation proceeded for a total of 30 days.

2.4. Cell harvest

To harvest cells from microcarrier cultures, 2.0 mL duplicate samples were retrieved from spinners and placed in 15 mL centrifuge tubes. The supernatant was removed and the microcarriers rinsed twice with PBS and trypsinized with 0.25% Trypsin-EDTA (Invitrogen, Cat# 25200-056) for 15 min, with mechanical dissociation every 5 min. CultiSpher S microcarriers completely degraded upon trypsinization. The resulting cell suspension was preserved for later analyses.

2.5. Immunocytochemistry and flow cytometry

For immunocytochemistry, cells were washed twice with PBS and fixed with 4% Paraformaldehyde (Sigma) in PBS (pH 7.4) for 15 min at room temperature. After washing 3× with PBS, cells were permeabilized with 0.5% Saponin (Sigma, G-7900) in 1× PBS/1% BSA for 15 min at room temperature and then blocked with 3% bovine serum albumin (Sigma–Aldrich, Ontario, Canada. Cat.# A9418) in 1× PBS for 30 min. The cells

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