Biochemical and Biophysical Research Communications 430 (2013) 325–330

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Good manufacturing practice-compliant animal-free expansion of human bone marrow derived mesenchymal stroma cells in a closed hollow-fiber-based bioreactor

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ARTICLE INFO

Article history:
Received 28 October 2012
Available online 9 November 2012

Keywords:
Mesenchymal stem cells
Bioreactor
Expansion

ABSTRACT

Mesenchymal stroma cells (MSC) are increasingly recognized for various applications of cell-based therapies such as regenerative medicine or immunomodulatory treatment strategies. Standardized large-scale expansions of MSC under good manufacturing practice (GMP)-compliant conditions avoiding animal derived components are mandatory for further evaluation of these novel therapeutic approaches in clinical trials.

We applied a novel automated hollow fiber cell expansion system (CES) for in vitro expansion of human bone marrow derived MSC employing a GMP-compliant culture medium with human platelet lysate (HPL). Between 8 and 32 ml primary bone marrow aspirate were loaded into the hollow fiber CES and cultured for 15–27 days. 2–58 million MSC were harvested after primary culture. Further GMP-compliant cultivation of second passage MSC for 13 days led to further 10–20-fold enrichment. Viability, surface antigen expression, differentiation capacity and immunosuppressive function of MSC cultured in the hollow fiber CES were in line with standard criteria for MSC definition. We conclude that MSC can be enriched from primary bone marrow aspirate in a GMP-conform manner within a closed hollow fiber bioreactor and maintain their T lymphocyte inhibitory capacity. Standardized and reliable conditions for large scale MSC expansion pave the way for safe applications in humans in different therapeutic approaches.

1. Introduction

Mesenchymal stroma cells (MSC) are immature fibroblasts possessing an enormous growth capacity in vitro and the inherent capability to differentiate into a variety of tissues [1]. MSC harbor a distinct antigen expression profile [1,2] and exhibit immunomodulatory function [3–5]. Their ability to migrate to sites of tissue injury [6,7] make MSC useful in tissue repair [8–10] or treatment of autoimmune disorders [11,12], steroid refractory graft versus host disease (GvHD) [13,14] or chronic allograft rejection [15]. MSC can be derived from various human tissues like fat [16], umbilical cord [17] or cord blood [18]. The use of bone marrow as a source of human MSC has been first described by Friedenstein et al. [19,20] and represents still the most reliable source for adult MSC until today. Recently, a hollow fiber bioreactor system (Quantum® Cell Expansion System, Terumo BCT) has been developed providing an expansion surface for adherent cells [21]. In order to move MSC transplantation into properly controlled clinical studies, safe and standardized ex vivo expansion protocols in a GMP-compliant manner are required. Efforts have been made to avoid fetal calf serum for MSC cultivation [22–24] because of the risk of transmission of pathogens as well as xenoimmunization against bovine antigens [25–28]. In addition, growth of MSC in conventional cell culture monolayer requires large surfaces for sufficient cell expansion in a sterile GMP-compliant microenvironment. Therefore, cell-stack chambers placed into incubators [29] or rotating bioreactor devices [30,31] have been used for clinical-grade MSC expansion. We have applied a GMP-compliant culture medium based on human platelet lysate (HPL) for MSC expansion from primary adult human bone marrow in a novel closed hollow fiber cell expansion system (CES) with a fibronectin-coated surface of 2.1 m in order to establish a safe, efficacious and standardized protocol according to the guidelines of the European Medicines Agency (EMA).

Abbreviation: CES, cell expansion system.

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http://dx.doi.org/10.1016/j.bbrc.2012.11.001

2. Materials and methods

2.1. Collection of bone marrow and pre-enrichment of MSC

Bone marrow was aspirated from the iliac crest of volunteer healthy donors. For pre-enrichment of MSC, spongiform bone fragments were obtained from hip replacement surgery as described previously [32]. The procedures had been approved by the local ethics committee at the Philipps-University Marburg (study no. 64/01 and 25/10) and patients had been given written informed consent. 10 ml syringes prefilled with 160 I.U. of heparin anticoagulant (Sarstedt, Nuembrecht, Germany) were used for bone marrow collection.

In brief bone marrow fragments were subjected to density gradient centrifugation, washed with PBS (PAA, Linz, Austria) and resuspended in Dulbecco's modified medium (DMEM) with low glucose (Biochrom, Berlin, Germany) containing 1% N(2)-L-alanyl-L-glutamine (Dipeptamin, Fresenius Kabi, Germany), and 10% HPL, manufactured at the Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany in a GMP-compliant manner as described [23,33]. Preparation of pooled HPL is an efficient replacement for animal serum-free human stem cell cultures. For expansion of MSC in plastic flasks, cells were plated at a concentration of 5000 cell/cm^2 (Greiner Bio-One, Frickenhausen, Germany). Initially medium was replaced after the first 24 h, afterward medium was exchanged every 3–4 days until the cells reached ~80% confluence. With every splitting procedure the passage number was increased. The pre-enriched MSC of passages 2–3 were loaded into the Quantum cell expansion system (TerumoBCT). For primary MSC expansion, unprocessed BM aspirates were directly transferred into a Quantum system cell inlet bag (TerumoBCT, Lakewood, USA) under clean room conditions.

2.2. Expansion of MSC within the hollow fiber cell expansion system

One day prior loading of cells the expansion tubing set was placed into the Quantum system (provided by TerumoBCT) and coated with 10 mg fibronectin (BD Biosciences, Heidelberg, Germany) according to manufacturers instructions. The Cell Inlet Bag (CIB) containing bone marrow aspirate or pre-enriched MSC was then filled with medium (DMEM + 10% HPL + 1% N(2)-L-alanyl-L-glutamine) up to a total volume of 100 ml, connected to the cell expansion system via tubing welder (TSCDII, Terumo, Tokyo, Japan) and loaded into the cell expansion system using the automated functionality of the system. After 24 h, a complete medium exchange was performed in order to remove unattached cells (e.g. red blood cells or non-viable MSC). Cells were continuously fed with medium during the expansion. Loading, washing and feeding tasks were performed by the Quantum system following a program sequence according to manufacturer's instructions. Medium samples were collected regularly for determination of glucose and lactate levels. The inlet rate was started with 0.1 ml/min and was increased 2-fold when either glucose levels dropped below 70 mg/dl or lactate levels rose above 4 mmol/l. Cell harvest was initiated when glucose levels dropped below 70 mg/dl at a flow rate of 48 ml/h. For MSC harvest a CIB pre-filled with 180 ml of 0.25% trypsin/EDTA solution (PAA) was sterilely welded to the CES tubing and introduced into the hollow-fiber-system via automated tasks. After an incubation time of 4–6 min the cells were released into the cell harvest bag (TerumoBCT) by flushing with 500 ml medium. Cell count and viability were assessed using trypan blue staining and a Neubauer counter chamber.

Table 1
MSC yield after primary expansion of unprocessed bone marrow from 5 healthy volunteers in the hollow fiber cell expansion system (A). Subsequent cultivation of primary culture MSC from donor no. 5 and early passage MSC pre-cultured in plastic flasks led to further propagation of cells (B).

Donor	1	2	3	4	5
(*A*) *MSC expansion from primary bone marrow aspirate*					
Bone marrow aspirate (ml)	30	8	30	26	32
Expansion time (days)	15	19	15	18	27
Harvest (million cells)	2	6	7	9	58
(*B*) *Expansion from preselected MSC*					
	A	B	C	5	
No. of seeded cells (million)	2	3.5	5	7.6	
Expansion time (days)	6	11	13	13	
Harvest (million cells)	18.7	50	98	17.2	
Fold expansion	9.4	14.2	20	2.3	

2.3. Immunophenotyping of bioreactor-derived MSC by flow cytometry

The surface marker expression of MSC expanded in HPL-supplemented medium was analyzed with a four-color flow cytometer (FACSCalibur, BD Biosciences). In brief, MSC were stained for 15 min at 4 °C with fluorochrome-labeled monoclonal antibodies CD14, CD45, CD34, CD73, CD105, CD90, CD44 (all obtained from BD Biosciences) and HLA-DR (Beckman Coulter GmbH, Krefeld, Germany), washed with PBS and resuspended in FACSFlow™ (BD Biosciences) with 3% formaldehyde (Merck, Darmstadt, Germany). The samples were measured with a four-color flow cytometer

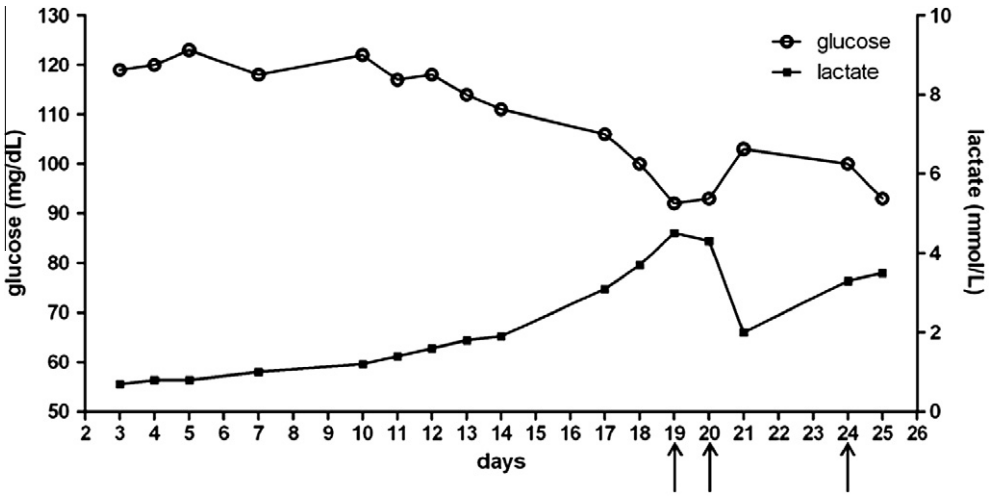

Fig. 1. Determination of glucose and lactate levels during MSC expansion within the bioreactor as indicators of cell growth. The medium flow rate was increased (→) at latest when lactate values rose above 4 mmol/l.