Cytotherapy, 2010; 12: 466–477

informa
healthcare

GMP-manufactured density gradient media for optimized mesenchymal stromal/stem cell isolation and expansion

GIULIA GRISENDI[1], CECILIA ANNERÉN[2], LUIGI CAFARELLI[1], RITA STERNIERI[1], ELENA VERONESI[1], GIAN LUCA CERVO[1], STEFANO LUMINARI[1], MICHELA MAUR[1], ANTONIO FRASSOLDATI[1], GIOVANNI PALAZZI[3], SATORU OTSURU[4], FRANCO BAMBI[5], PAOLO PAOLUCCI[3], CONTE PIERFRANCO[1], EDWIN HORWITZ[4] & MASSIMO DOMINICI[1]

[1]*Department of Oncology, Hematology and Respiratory Diseases, University Hospital of Modena and Reggio Emilia, Modena, Italy,* [2]*GE Healthcare Bio-Sciences AB, Uppsala, Sweden,* [3]*Department of Mother and Child, University Hospital of Modena and Reggio Emilia, Modena, Italy,* [4]*Division of Oncology, Children's Hospital of Philadelphia, Abramson Research Center, Philadelphia, USA, and* [5]*Blood Bank and Cell Therapy Unit, Meyer Hospital, Firenze, Italy*

Abstract

Background aims. Bone marrow (BM) mesenchymal stromal/stem cells (MSC) are therapeutic tools in regenerative medicine and oncology. MSC isolation is often performed starting from a separation step based on research-grade 1.077 g/mL density gradient media (DGM). However, MSC clinical application should require the introduction of good manufacturing practice (GMP) reagents. We took advantage of two novel GMP DGM with densities of 1.077 and 1.073 g/mL (Ficoll-Paque™ PREMIUM and Ficoll-Paque PREMIUM 1.073, respectively) to test whether these reagents could isolate MSC efficiently while simultaneously comparing their performance. *Methods*. BM samples were processed using either 1.077 or 1.073 g/mL GMP DGM. BM mononucleated cell (MNC) fractions were analyzed for viability, immunophenotype, clonogenic potential, *ex vivo* expansion and differentiation potential. *Results*. No differences were noticed in cell recovery and viability between the groups. Fluorescence-activated cell-sorting (FACS) analyzes on freshly isolated cells indicated that the 1.073 g/mL GMP DGM more efficiently depleted the $CD45^+$ fraction in comparison with 1.077 GMP DGM. Moreover, in the 1.073 group, fibroblastic colony-forming units (CFU-F) were 1.5 times higher and the final MSC yield 1.8 times increased after four passages. Both reagents isolated MSC with the expected phenotype; however, 1.073-isolated MSC showed a higher expression of CD90, CD146 and GD2. Additionally, MSC from both groups were capable of fully differentiating into bone, adipose cells and cartilage. *Conclusions*. Both GMP DGM enriched MSC from BM samples, suggesting that these reagents would be suitable for clinical-grade expansions. In addition, the density of 1.073 g/mL provides a significant advantage over 1.077 g/mL GMP DGM, impacting the quantity of MSC obtained and reducing the *ex vivo* expansion time for optimized cell-based clinical applications.

Key Words: *cell expansion, density gradient media, good manufacturing practice, 1.073 g/mL, Ficoll-Paque PREMIUM, mesenchymal stromal/stem cells*

Introduction

Human mesenchymal stromal/stem cells (MSC) were first identified in the bone marrow (BM) by Friedenstein *et al.* in 1974 by their plastic-adherence (1). More recently, the International Society for Cellular Therapy (ISCT) provided additional criteria for defining MSC, including specific surface antigen expression and multipotent differentiation potentials (2). According to these reports and more recent studies, adherent MSC express several specific antigens, such as CD90, CD73, CD105, CD146 and GD2, and lack the expression of hematopoietic markers (2–5). Moreover, they should have the ability to differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* conditions. Based on this knowledge, MSC have been considered attractive candidates for cell therapies, and their relatively easy harvesting procedure allows their use in several autologous and allogeneic transplantation settings (6,7). So far, MSC have been introduced successfully

Correspondence: **Massimo Dominici,** MD, Division of Oncology, Department of Oncology, Hematology and Respiratory Diseases, University Hospital of Modena and Reggio Emilia, Via del Pozzo, 71, 41100, Modena, Italy. E-mail massimo.dominici@unimore.it

(Received 18 September 2009; accepted 20 January 2010)

ISSN 1465-3249 print/ISSN 1477-2566 online
DOI: 10.3109/14653241003649510

to improve neurologic, cardiovascular, blood-related, musculoskeletal and immunologic disorders (8–11). More recently, evidence has supported the concept that MSC can be used as gene vehicles for cancer therapy, because these cells show a preferential migration towards malignant cells, where they can deliver cytotoxic molecules (12–14).

MSC represent a small fraction (0.001–0.01%) in the BM population *in vivo*; for this reason they have to be expanded extensively *ex vivo* to reach a therapeutic dose and be used efficiently in clinical trials. The discovery that MSC can be enriched substantially by a density gradient separation step has allowed the introduction of density gradient media (DGM) into current laboratory practice (15,16). However, the use of MSC as cellular therapies should require the development and introduction of good manufacturing practice (GMP) reagents and protocols for their isolation and culture. In this study, we compared two novel GMP DGM (Ficoll-Paque™ PREMIUM and Ficoll-Paque PREMIUM 1.073; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with different densities (1.077 and 1.073 g/mL respectively) to verify the efficiency of these GMP DGM for mesenchymal progenitor isolation. In addition, we tested whether the use of the lower-density Ficoll-Paque PREMIUM 1.073 was associated with a further enrichment of MSC with distinct physical and biologic properties. The data demonstrated that both GMP DGM tested are suitable for MSC expansion and that Ficoll-Paque PREMIUM 1.073 is able to isolate more and better-performing MSC, even starting with cancer patients' BM.

Methods

Patients

BM harvests were performed from patients ($n=13$) referred to the Department of Oncology and Hematology at the University Hospital of Modena (Modena, Italy), with informed consent. The protocol was approved by local ethics committee. At the time of recruitment, the mean patient age was 48 years ($\pm$19.04; 6–62), with nine males and four females.

BM processing and ex vivo *expansion*

BM specimen harvests were performed on the posterior iliac crest following standard procedures (17). The samples were aspirated with a luer-lock 10-mL syringe containing 0.5–1 mL Na citrate (38 mg/mL), and processed as follows. BM was diluted 1:1 (v:v) with sterile Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) (PAA, Pasching, Austria) and passed 20 times through a sterile 10-mL syringe (Becton Dickinson Plastipak, Drogheda, Ireland) with a 19-G needle. Small aliquots (50 μL) were processed with Türk staining solution (*in vitro* diagnostic medical device, Merk KGaA, Darmstadt, Germany) for total nucleated cell count with a Neubauer cytometer, Axiovert 40C (Zeiss, Oberkochen, Germany) inverted microscope. Subsequently, the BM was divided into three fractions. A small aliquot (500 μL) was treated with lysis buffer (BD Pharmingen, San Jose, CA, USA) for immunophenotypical analyzes. The remaining volume was divided into two equal fractions of 2.3 mL (on average) and processed for clonogenic assay and *ex vivo* expansion using Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences AB), with a density of 1.077 g/mL, and Ficoll-Paque PREMIUM 1.073 (GE Healthcare Bio-Sciences AB), with a density of 1.073 g/mL, respectively. In both cases, cell suspensions were layered on top of DGM and centrifuged at 623 ***g*** for 20 min at room temperature (RT). The BM mononucleated cell (BM MNC) fractions were collected and washed twice with PBS. Cells were seeded *in vitro* in a serum-free medium, Quantum 333 (PAA), with the addition of glutamine (2 mM) (Euroclone, Padmington, UK) and 1% penicillin–streptomycin (Euroclone, Milan, Italy) at a density of 800 000 cells/cm^2 in 6-well plates (Corning, New York, NY, USA). The cells were kept in incubators with a controlled atmosphere (5% CO_2, 37°C). The medium was replaced every 2–3 days, discarding non-adherent cells. Once 80–90% confluence was reached, cells were detached with trypsin 0.05%/EDTA 0.02% (Euroclone), counted and seeded at 5000 cells/cm^2. Extended cultures were also protracted until reaching confluence at passage (P)4. At each passage, cells were counted using 0.5% trypan blue (Biochrom AG, Berlin, Germany) exclusion, to evaluate viable cell expansion. All experiments were performed in duplicate.

MSC clonogenic and proliferation potential

To assess the clonogenic potential of cultured MSC, the BM MNC fraction was additionally seeded for a fibroblastic colony-forming unit (CFU-F) assay (15). Cells were seeded at a density of 800 000/cm^2 and the medium changed weekly, discarding non-adherent cells. MSC clonogenic precursors (CFU-F) were quantified after 15 days with a inverted microscope, and clones of more than 50 cells were considered to be colonies. Colonies were scored by two independent investigators and all experiments were performed in duplicate. To assess population doubling (PD), the following formula was used: $PD=\log(N/N_0)/\log_2$, where N_0 is the seeded cell number and N the harvested cell number (18). The cell doubling time calculation was performed as reported previously (19).