Toxicology in Vitro 29 (2015) 1254–1262

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Evaluation of a new standardized enzymatic isolation protocol for human umbilical cord-derived stem cells

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ARTICLE INFO

Article history:
Received 20 June 2014
Accepted 3 December 2014
Available online 23 December 2014

Keywords:
Isolation
Umbilical cord
Mesenchymal stem cell
Liver development
Hepatocyte

ABSTRACT

The umbilical cord (UC) represents an important source of mesenchymal stem cells (MSC). These human UC-derived MSC (UC–MSC) have already been isolated using a protocol based on the migratory and plastic adhesive properties of MSC (UC–MSC–Mig). The UC–MSC–Mig isolation method, however, is difficult to standardize. Therefore, we developed an enzymatic isolation protocol (UC–MSC–Enz) to overcome the above mentioned disadvantages. First, we investigated the UC–MSC–Enz for their MSC properties. We found that UC–MSC–Enz express the MSC markers CD73, CD90 and CD105 and are able to differentiate into osteoblasts, adipocytes and chondroblasts fulfilling the MSC criteria of the International Society for Cellular Therapy. Previously we found that UC–MSC–Mig are unique among MSCs due to their significant expression of several hepatic (progenitor) markers. Therefore, we also investigated the expression of hepatic transcription factors and other hepatic markers in UC–MSC–Enz at both the mRNA and protein level. We found that the expression of hepatic transcription factors (GATA4, GATA6, SOX9 and SOX17) and hepatic markers (AFP, DPP4, CX43, DKK1, DSG2, KRT18 and KRT19) in UC–MSC–Enz was not significantly different from those of UC–MSC–Mig. Consequently, this optimized enzyme-based method represents a fast, robust and standardized way to isolate UC–MSC for a broad range of applications.

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1. Introduction

Mesenchymal stem cells (MSC) are multipotent stromal cells that can be isolated from different tissues (Sharma et al., 2014). They do not raise the ethical issues that are linked to the use of human embryonic stem cells (Leeb et al., 2011). Moreover, MSC possess remarkable properties, including immunomodulation and regeneration (Sharma et al., 2014) and they can differentiate towards cell types different from their own tissue of origin (Sousa et al., 2014). Consequently, MSC are an interesting stem cell source for both *in vivo* and *in vitro* applications. Yet, from previous work we know that one of the parameters to be considered to efficiently generate multipotent MSC is the age of the donor (Gago et al., 2009). It is therefore essential to explore the youngest postnatal source that is rich in non-hematopoietic stem cells, namely the umbilical cord (UC) (Weiss and Troyer, 2006). In earlier work, we isolated UC-derived MSC using a migration method (UC–MSC–Mig) (De Bruyn et al., 2011). This methodology was based on the migratory and plastic adhesive properties of MSC and took approximately 5 days. Furthermore, it was difficult to standardize, a property which is of key importance for further development of *in vitro* and *in vivo* applications (Ball et al., 1995; LeCluyse et al., 2012). Consequently, we developed an optimized enzymatic isolation protocol that allows to isolate human UC-derived stem cells (UC–MSC–Enz) within 3 h using a procedure that is easy to

Abbreviations: ACTB, beta-actin; AFP, α-foetoprotein; ALB, albumin; B2M, beta-2-microglobulin; CD, cluster of differentiation; CX, connexin; CYP, cytochrome P450; DAPI, 4′,6-diamidino-2-phenylindole; DKK, dickkopf; DPP, dipeptidyl-peptidase; DSG, desmoglein; DT, doubling time; Enz, enzymatic; FOX, forkhead box; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA, gata-motif binding protein; GJ, gap junction; HLA, human leukocyte antigen; HMBS, hydroxy-methylbilane synthase; HNF, hepatocyte nuclear factor; ISCT, International Society for Cellular Therapy; KRT, keratin; Mig, migration; MSC, mesenchymal stem cells; NH, cell harvest number; NI, inoculum cell number; PBS, phosphate buffered saline; ND, not significantly detected; P, passage; PFA, paraformaldehyde; qPCR, quantitative real-time reverse transcriptase polymerase chain reaction; SD, standard deviation; SOX, sex-determining region Y box; TP, time period; UBC, ubiquitin C; UC, umbilical cord.

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http://dx.doi.org/10.1016/j.tiv.2014.12.008
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standardize. In this study, we investigated whether the thus obtained UC–MSC–Enz can indeed be classified as MSC according to the criteria set by the International Society for Cellular Therapy (ISCT), defining the expression of specific MSC surface markers. Next, we evaluated the ability of the cells to differentiate into chondro-, osteo- and adipocytes and determined their doubling time. In previous work, we could show that UC–MSC–Mig are a unique population among MSC due to the expression of key hepatogenic transcription factors, including GATA motif binding protein (GATA)4, GATA6, sex-determining region Y box (SOX)9 and SOX17, and liver progenitor markers, including dickkopf (DKK)1, dipeptidyl-peptidase (DPP)4, desmoglein (DSG)2, connexin (CX)43, keratin (KRT)18 and KRT19 (Si-Tayeb et al., 2010; De Kock et al., 2012; Buyl et al., 2014). Consequently, we investigated whether the expression of these markers, at both the mRNA and protein expression level, is affected by the newly developed isolation method.

2. Materials and methods

2.1. Isolation and cultivation of stem cells according to both a migration (UC–MSC–Mig) and an enzymatic (UC–MSC–Enz) protocol

After receiving informed consent from the mothers of the neonates involved, umbilical cords were collected and processed. Only full-term deliveries were included in this study.

UC–MSC–Mig were isolated from the umbilical cord based on the migratory and plastic adhesive properties of MSCs, thus omitting enzymatic digestion or dissection. Briefly, umbilical cord segments of 5–10 cm were cut longitudinally and plated for 5 days in Dulbecco's modified eagle medium–low glucose (Lonza, Braine-l'Alleud, Belgium) supplemented with 18% (v/v) heat-inactivated fetal bovine serum (Sigma, Diegem, Belgium), 2 mM L-glutamine and 0.5% (v/v) antibiotic/antimycotic solution containing penicillin, streptomycin and fungizone (all from Lonza, Braine-l'Alleud, Belgium). Afterwards, the cord segments were removed (De Bruyn et al., 2011).

UC–MSC–Enz were isolated from the umbilical cord using enzymatic digestion. Briefly, umbilical cord segments of approximately 10 cm were disinfected with 70% (v/v) alcohol during 30 s followed by rinsing with phosphate buffered saline (PBS). The blood vessels were flushed with PBS to remove the blood. Next, the umbilical cord was minced into small pieces of 1–3 mm^2, which were consequently incubated in 0.05% (m/v) collagenase type II solution at 37 °C during 1 h. The tissue suspension was, every 10 min, shaken vigorously and crushed for 2 min before being filtered through a 63 μm mesh. The obtained suspension was centrifugated at 931g for 10 min and the supernatant was discarded. The cell pellet was resuspended in Dulbecco's modified eagle medium–low glucose (Lonza, Braine-l'Alleud, Belgium) supplemented with 18% (v/v) heat-inactivated fetal bovine serum (Sigma, Diegem, Belgium), 2 mM L-glutamine and 0.5% (v/v) antibiotic/antimycotic solution containing penicillin, streptomycin and fungizone (all from Lonza, Braine-l'Alleud, Belgium). Both, UC–MSC–Mig and UC–MSC–Enz, were cultured at 37 °C in a 5% (v/v) CO_2 containing humidified atmosphere until subconfluency. After 48 h, non-adherent cells were removed by changing the medium. The time lines for both isolation protocols are shown in Fig. 1.

Medium changes were subsequently performed twice a week. When subconfluency (90%) was achieved, adherent cells were harvested after detachment by incubation in a TrypLE® solution (Life Technologies) for 10 min. For all subsequent passages, the cells were expanded by replating them at a lower density [1×10^3 cells/cm^2 (UC–MSC–Mig) and 4×10^3 cells/cm^2 (UC–MSC–Enz)].

2.2. Morphology

The morphology of UC–MSC–Enz (passage (P)3, $n = 3$) and UC–MSC–Mig (P3, $n = 3$) was examined by phase-contrast light microscopy (Nikon).

2.3. Doubling time of UC–MSC–Enz

When 80%-90% confluency was reached, the population doubling time (DT) between P1/P2 was determined by the following formula where the time period (TP) represents the time of the culture in hours, NI is the inoculum cell number and NH the cell harvest number.

$$DT = TP\ \log 2 / (\log NH - \log NI)$$

2.4. Flow cytometry analysis of UC–MSC–Enz

Freshly isolated UC–MSC–Enz were examined for P0–P3 ($n = 3$) for their expression of mesenchymal and hematopoietic cell surface markers using flow cytometry. The fluorochrome-conjugated monoclonal antibodies used in this study are listed in Supplementary Table S1. Adherent cells were harvested with the TrypLE Select (Lonza) solution, washed by centrifugation in PBS (Lonza) and finally resuspended in Miltenyi Biotec buffer. Then, harvested cells were incubated for 30 min at room temperature with fluorochrome-conjugated monoclonal antibodies directed against cluster of differentiation (CD) 90 (R&D Systems, United Kingdom), CD73 (Miltenyi Biotec, The Netherlands), CD105 (Ancell Corporation, USA), CD19, CD14 (Immuno Tools, Germany), HLA-DR, CD34 and CD45 (BD Biosciences, USA). After cell labeling, the cells were again washed, resuspended in PBS and fixed with 4% (w/v) paraformaldehyde (PFA). The data were acquired and analyzed using a MacsQuant analyzer (Miltenyi Biotec, The Netherlands).

2.5. Differentiation into adipocyte-like cells

UC–MSC–Enz (P3, $n = 3$) were cultivated at 100% confluency in adipogenesis differentiation medium consisting of 90% (v/v) STEMPRO® Adipocyte Differentiation Basal Medium and 10% (v/v) STEMPRO® Adipogenesis Supplement (all from Gibco, Life Technologies). The medium was changed every 2 days. After 2 weeks of differentiation, cells were fixated with 4% (w/v) PFA during 30 min. Following fixation, the wells were rinsed twice with PBS and the cells were incubated during 30 min with a 1:100 dilution of LipidTOX™ Green. Finally, the wells were rinsed twice with PBS. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Labconsult, Brussels, Belgium) and the cells were visualized using a fluorescence microscope (Nikon).

2.6. Differentiation into osteocyte-like cells

UC–MSC–Enz (P3, $n = 3$) were cultivated in osteogenesis differentiation medium consisting of 90% (v/v) STEMPRO® Osteocyte/Chondrocyte Differentiation Basal Medium and 10% (v/v) STEMPRO® Osteogenesis Supplement (all from Gibco, Life Technologies). The medium was changed every 2 days. After 3 weeks, cells were fixated with 4% (w/v) PFA during 30 min. After fixation, the wells were rinsed twice with distilled water. Next, the cells were stained with 2% (w/v) Alizarin Red S solution (pH 4.2) for 3 min. Finally, wells were rinsed three times with distilled water and visualized with a phase contrast microscope (Nikon).

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