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MESENCHYMAL STROMAL CELL

A Good Manufacturing Practice–grade standard protocol for exclusively human mesenchymal stromal cell–derived extracellular vesicles

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Abstract

Background aims. Extracellular vesicles (EVs) released by mesenchymal stromal cells (MSCs) may contribute to biological processes such as tissue regeneration, immunomodulation and neuroprotection. Evaluation of their therapeutic potential and application in future clinical trials demands thorough characterization of EV content and production under defined medium conditions, devoid of xenogenic substances and serum-derived vesicles. Addressing the apparent need for such a growth medium, we have developed a medium formulation based on pooled human platelet lysate (pHPL), free from animal-derived xenogenic additives and depleted of EVs. *Methods.* Depletion of EVs from complete growth medium was achieved by centrifugation at 120 000 *g* for 3 h, which reduced RNA-containing pHPL EVs to below the detection limit. *Results.* Bone marrow (BM)-derived MSCs propagated in this medium retained the characteristic surface marker expression, cell morphology, viability and *in vitro* osteogenic and adipogenic differentiation potential. The proliferation rate was not significantly affected after 48 h but was decreased by 13% after 96 h. EVs collected from BM-MSCs cultured in EV-depleted medium revealed a similar RNA pattern as EVs generated in standard pHPL EV-containing medium but displayed a more clearly defined pattern of proteins characteristic for EVs. Reduction of pHPL content from 10% to 2% or serum-/pHPL-free conditions strongly altered MSC characteristics and RNA content of released EV. *Conclusions.* The 10% pHPL-based EV-depleted medium is appropriate for purification of exclusively human MSC-derived EVs. With this Good Manufacturing Practice–grade protocol, characterization and establishment of protein and RNA profiles from MSC-derived EVs can now be achieved to identify active components in therapeutic EVs for future clinical application.

Key Words: *extracellular vesicles, exosomes, mesenchymal stromal cells, human platelet lysate, EV depletion, Good Manufacturing Practice*

Introduction

Extracellular vesicles (EVs) are released by many cell types and contribute to intercellular communication by the transfer of functional mRNA, miRNA, proteins and lipids [1–4]. EVs released by mesenchymal stromal cells (MSCs) mediate some biological functions attributed to MSCs, such as tissue regeneration, immunomodulation and neuroprotection [5]. MSC-derived EVs are therefore a promising alternative to cellular therapeutics.

On the basis of size and subcellular origin, EVs are currently divided into several subgroups, including exosomes (40–200 nm), which are considered of endosomal origin, and microvesicles (150–1000 nm), which bud directly from the cell membrane [6,7].

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Although minimal criteria for EV characterization have recently been defined [8], a precise nomenclature for the various EV categories is still missing, and specific markers to discriminate between the different vesicle subtypes are lacking [9,10]. In this article, we apply the term "EV" to refer to the vesicle species purified by sequential centrifugation and filtration.

Physicochemical characterization, assessment of the therapeutic potential and definition of a potential mode of action for future clinical application of MSC-EVs require defined culture conditions for the EV-releasing cells that meet Good Manufacturing Practice (GMP) standards. Commonly used medium formulations for MSC cultivation are based on the addition of fetal bovine serum (FBS) as a source of growth factors and cytokines but bear substantial disadvantages when used for the *ex vivo* expansion of cells for clinical application. First, contamination with animal pathogens (e.g., specific viruses, mycoplasma, prions) cannot be fully excluded [11–14]. Second, immune reactions to FBS proteins have been reported in patients receiving cell therapy [15–17]. Therefore, serum-free media formulations or supplements devoid of xenogenic substances, such as human platelet lysate (HPL), should be used to substitute for FBS in clinical applications. The risk of immunological reactions against serum antigens is largely reduced with HPL. Moreover, proliferation of MSCs is enhanced in HPL compared with FBS [18]. However, the high fibrinogen content present in HPL promotes the formation of fibrin gels when diluted into calcium-containing media. To prevent gel formation, porcine heparin is usually added to HPL-based growth media; however, this introduces xenogenic substances. Despite the addition of heparin, such media suffer from a short shelf life, and fibrin persistently precipitates during storage and cell culture.

To bypass these shortcomings, we recently developed a method for depleting fibrinogen by direct clotting of pooled HPL (pHPL) in the culture medium, followed by physical disruption of fibrin gels and removal of fibrin pellets by centrifugation to generate a growth medium suitable for MSC isolation and large-scale *ex vivo* expansion [19].

Culture media used for the production of MSC-derived EVs should also be devoid of serum-/HPL-derived EVs, however. Serum/HPL EVs co-purify with MSC-EVs and may not only interfere with the physicochemical characterization of MSC-EVs but also affect future therapeutic applications. Serum-/HPL-derived EVs are internalized into cells and influence cell migration, proliferation and differentiation [20,21]. One possibility to avoid serum/HPL EVs is the use of synthetic serum-free media for cell propagation and expansion. Commercially available synthetic serum-free medium formulations, which support cell proliferation, usually contain recombinant growth factors and/or other components of animal or bacterial origin or require culture flasks to be coated with adhesion-promoting factors for proper cell adhesion [22]. Such culture media are therefore rarely completely devoid of xenogenic substances and in most cases are costly. Moreover, serum-free culture conditions strongly influence the potential of MSCs to differentiate into osteogenic, adipogenic and chondrogenic cells *in vitro* [23,24]. Serum conditions also affect the number and content of released EVs. Notably, EVs from neuroblastoma cells cultured in serum-free medium were shown to be enriched in stress-related proteins [25].

As an alternative to serum-free cell culture conditions, serum/HPL depleted of its EVs can be used for investigation and production of cell-derived EVs. Several protocols for the depletion of FBS-derived EVs have been reported and applied to EV research in the past [20,26–28], but the use of EV-depleted HPL for MSC propagation has not been reported thus far. In the present study, we describe a protocol for producing a medium that is free of xenogenic substances and based on supplementation with EV-depleted pHPL. We evaluated the effect of this medium on bone marrow (BM)-MSC morphology, immunophenotype, proliferation and differentiation properties as well as on RNA and protein composition in the purified EVs. The data reveal that media produced under GMP-compliant conditions and supplemented with 10% EV-depleted pHPL are suitable for the production and characterization of BM-MSC-derived EVs.

Methods

Medium preparation

Heparin-free and fibrinogen-depleted medium (medium A) was prepared as follows: alpha-modified Minimum Essential Medium Eagle (alpha-MEM, M4526, Sigma-Aldrich) was supplemented with 5 mmol/L (N2)-L-alanyl-L-glutamine (Dipeptiven, 11051014, Fresenius Kabi) and 10% pHPL. pHPL was produced as described earlier [29]. The mixture was immediately divided into 14 aliquots (40 mL in 50-mL centrifuge tubes), maintained for 4–6 h at 20–24°C until biogel formation was completed and stored at 4°C for 12–18 h. To remove fibrin, the clotted medium was brought to 37°C. The fibrin gel was then physically collapsed, and the precipitated fibrin was pelleted at 2500 *g* at 20°C for 12 min [19]. The supernatant was then filtered (0.22 μm) to yield medium A. Heparin-free, fibrinogen-depleted and EV-depleted medium (medium B) was produced from medium A by a single ultracentrifugation step at 120 000 *g* for 3 h (using a Sorvall MX120 ultracentrifuge, fixed angle rotor S50-A, k-factor 60.7, Thermo Scientific). The