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Mineralization by mesenchymal stromal cells is variously modulated depending on commercial platelet lysate preparations

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Abstract

Background aims. Numerous cellular models have been developed to investigate calcification for regenerative medicine applications and for the identification of therapeutic targets in various complications associated with age-related diseases. However, results have often been contradictory due to specific culture conditions, cell type ontogeny and aging status. Human platelet lysate (hPL) has been recently investigated as valuable alternative to fetal bovine serum (FBS) in cell culture and bone regeneration. A parallel comparison of how all these multiple factors may converge to influence mineralization has yet to be reported. *Methods.* To compare mineralization of human mesenchymal cell types known to differ in extracellular matrix calcification potency, bone marrow–derived mesenchymal stromal cells and dermal fibroblasts from neonatal and adult donors, at both low and high passages, were investigated in an *ex vivo* experimental model by supplementing the osteogenic induction medium with FBS or with hPL. Four commercial hPL preparations were profiled by liquid chromatography/electrospray ionization quadrupole time-of-flight spectrometry, and mineralization was visualized by von Kossa staining and quantified by morphometric evaluations after 9, 14 and 21 days of culture. *Results.* Data demonstrate that (i) commercial hPL preparations differ according to mass spectra profiles, (ii) hPL variously influences mineral deposition depending on cell line and possibly on platelet product preparation methods, (iii) donor age modifies mineral deposition in the presence of the same hPL and (iv) reduced *in vitro* proliferative capacity affects osteogenic induction and response to hPL. *Conclusion.* Despite the standardized procedures applied to obtain commercial hPL, this study highlights the divergent effects of different preparations and emphasizes the importance of cellular ontology, donor age and cell proliferative capacity to optimize the osteogenic induction capabilities of mesenchymal stromal cells and design more effective cell-based therapeutic protocols.

Key Words: *aging, bone marrow stromal cells, calcification, fibroblasts, platelets, regenerative medicine*

Mineralization is a physiological process in hard connective tissues, but can also be considered pathological, when occurring in soft connective tissues. Although extensively characterized in *ex vivo*–expanded populations of mesenchymal stromal cells (MSCs), many facets related to osteogenic induction remain elusive [1,2]. Despite the ability to differentiate into calcifying osteoblasts *in vivo*, MSCs fail to differentiate and mineralize the extracellular matrix (ECM) in standard medium supplemented with fetal bovine serum (FBS). Therefore, to induce a temporal cascade of maturational stages toward the osteoblast phenotype that allows ECM mineralization [3], cells must be cultured in osteogenic induction medium (OIM, which contains β-glycerophosphate, ascorbic acid and dexamethasone) [4]. More recently, because platelets contribute to mineralization in both physiological and pathological microenvironments, influencing sites for mineral nucleation [5] and providing growth factors [6] as well as exosomal mediators [7], it has been shown that platelet-rich plasma can efficiently promote healing of hard and soft connective tissues, improving, for instance, bone regeneration in clinical trails [8]. Therefore, the fractured platelet derivative human platelet lysate (hPL) is increasingly favored as an FBS replacement for regenerative medicine clinical applications [9]. However, hPL sources and platelet product preparation methods can influence platelet number,

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growth factor concentration and, consequently, osteogenic induction/differentiation outcomes [10–12]. Moreover, because individual hPL effectiveness can differ according to donor-specific variability (i.e., age, gender), anticoagulant (i.e., heparin) and storage [11,13], commercial platelet lysates derived from pooled donors are frequently used to improve consistency and lower batch variability [14,15]; nevertheless, divergent results may be observed.

Therefore, we have investigated the effect of different commercial platelet lysates on *ex vivo* mineralization comparing various mesenchymal cell types (human bone marrow [hBM]-MSCs versus human dermal fibroblasts), each with contrasting ECM mineralization propensities. In particular, human MSCs are multipotent progenitors with enormous potential for repair and regeneration of bone and cartilage. Although these cells can be isolated from a variety of tissues, those from bone marrow, being the most widely investigated and characterized [16], were used in the present study as representative of cells positively associated with efficient mineralization. Conversely, human dermal fibroblasts (HDFs) are mesenchymal cells derived from tissues that typically mineralize in pathologic conditions [17].

Moreover, calcification can be also influenced by functional changes related to donor age or reduced cell proliferation capability. For instance, replicative senescence in hBM-MSC cultures can impair bone progenitor osteogenic differentiation and, consequently, matrix mineralization [18]. Yet replicative senescence can enhance ECM calcification in soft connective tissue mesenchymal cells (i.e., smooth muscle cells, fibroblasts) by increasing the response to pro-mineralization stimuli. By contrast, studies on HDFs isolated from neonatal donors demonstrated that these cells are significantly less responsive to pro-osteogenic factors [19]. Higher incidence of mineralization-associated diseases, including either osteoporotic paucity of bone mineralization or aberrant calcification in soft connective tissues and atherosclerotic vasculature [20], is consistently associated with age.

The aim of this study was to evaluate whether (i) different commercial hPLs have the same influence on mineral deposition in different cell lines, (ii) reduced *in vitro* proliferative capacity affects mineral deposition upon hPL supplementation and (iii) donor age modifies mineral deposition in the presence of the same hPL.

Methods

Liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry analysis

Electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) accurate mass spectrometer (G6520A, Agilent Technologies), controlled by MassHunter (v. B.04.00) and interfaced with an HPLC-Chip Cube to an Agilent 1200 nano-pump, was used for analysis.

Chromatographic separation was performed on an integrated HPLC-Chip (Agilent Technologies) with a 75-μm ID, 43 mm, 300 Å C18 column, before a desalting step through a 40-nL trap column. The injected sample (1 μL) was loaded onto the trap column with a 4 μL/min 0.1% FA:ACN (98:2) phase flow, and after 3 min, the pre-column was switched inline with the nanoflow pump (400 nL/min, phase A: water:ACN:FA 96.9:3:0.1, phase B: ACN:water:FA 94.5:5:0.1), equilibrated in 10% mobile phase B. Proteins were eluted from the reverse phase column through the following gradient: 10–90% mobile phase B for 5 min, held in 90% mobile phase B for 5 min and switched back to 10% mobile phase B for 3 min, for a total runtime of 40 min, including a 10-min post-run reconditioning step.

Mass spectra were recorded from 350 to 3200 m/z at scan rates of 1 Hz; the detector was operated at 2 GHz in extended dynamic range mode. Mass spectra were automatically recalibrated with two reference mass ions. Spectra were displayed and processed by the software MassHunter Qualitative Analysis (B05.00, Agilent Technologies).

Mass spectra across the whole chromatogram (0–26 min) were averaged and, after subtracting the background (obtained by averaging the mass spectra at the end of the run), the spectrum was deconvoluted by using the maximum entropy algorithm in the range of 10 000–150 000 Da.

Cell culture

hBM-MSCs were harvested from a 42-year-old male donor after informed consent, according to the Declaration of Helsinki and local ethical committee–approved procedures for isolation and immunephenotypic characterization, as previously described [16,21]. Briefly, cells were routinely grown in α-minimum essential medium (MEM) without nucleosides (Gibco Invitrogen), supplemented with 8% hPL (obtained from pooled batches of 50 donors), 1% L-glutamine (Gibco Invitrogen), 1 UI/mL heparin (Sigma-Aldrich), and 10 mg/mL ciprofloxacin (HIKMA) [16]. In this study, hBM-MSCs were selected among those obtained from six independent donors as representative of the behavior of hBM-MSCs. HDFs from adult tissue (aHDFs; Thermo Fisher Scientific, cat. # C-013-5C). To evaluate whether hPL can reverse the "resistant phenotype" of cells considered to be "low responders" to pro-osteogenic stimuli, we also used HDFs derived from neonatal tissue (nHDFs; Thermo Fisher Scientific, cat. # C-004-5C) [19].