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Research paper

Tridimensional configurations of human mesenchymal stem/stromal cells to enhance cell paracrine potential towards wound healing processes

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ABSTRACT

This study proposes to use alginate encapsulation as a strategy to assess the paracrine activity of 3D- and 2D-cultured human bone marrow mesenchymal stem/stromal cells (BM MSC) in the setting of wound repair and regeneration processes. A side-by-side comparison of MSC culture in three different 3D configurations (spheroids, encapsulated spheroids and encapsulated single cells) *versus* 2D monolayer cell culture is presented. The results reveal enhanced resistance to oxidative stress and paracrine potential of 3D spheroid-organized BM MSC. MSC spheroids (148 ± 2 μm diameter) encapsulated in alginate microbeads evidence increased angiogenic and chemotactic potential relatively to encapsulated single cells, as supported by higher secreted levels of angiogenic factors and by functional assays showing the capability of encapsulated MSC to promote formation of tubelike structures and migration of fibroblasts into a wounded area. In addition, a higher expression of the anti-inflammatory factor tumor necrosis factor alpha-induced protein 6 (TSG-6) was demonstrated by RT-PCR for encapsulated and non-encapsulated spheroids. Culture of spheroids within an alginate matrix maintains low aggregation levels below 5% and favors resistance to oxidative stress. These are important factors towards the establishment of more standardized and controlled systems, crucial to explore the paracrine effects of 3D-cultured MSC in therapeutic settings.

1. Introduction

Wound healing is a complex process mediated by several cytokines involved in inflammatory responses and tissue remodeling. The paracrine activity of mesenchymal stem/stromal cells (MSC) can stimulate tissue regeneration, namely promoting proangiogenic, immunomodulatory, antifibrotic and antioxidant cell activity (Caplan and Dennis, 2006). Several MSC-secreted bioactive factors have been suggested to be integrated in signaling networks that mediate wound healing response. Such factors include vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and hepatocyte growth factor (HGF), known for their role on angiogenesis (Ding et al., 2003; Ferrara, 2001; Gerritsen, 2005; Kwon et al., 2014); stem cell-derived factor-1α (SDF-1α, also known as CXCL12), a chemotactic factor involved in cellular migration (Liu et al., 2012); tumor necrosis factor alpha-induced protein 6 (TSG-6), an important cytokine for the regulation of immunomodulatory processes (Lee et al., 2014), similarly to HGF and IL-6 (Di Nicola et al., 2002; Janssens et al., 2015), which are involved in inflammatory responses (in addition to their role on angiogenesis).

Although the potential benefits of MSC trophic activity have been well described, lack of MSC retention in targeted tissues decreases their impact once delivered *in vivo*. Typical ischemic wounded tissues present high levels of local reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), severe hypoxia and inflammation, which dramatically decrease cell viability in wounded sites (Khanna et al., 2010; Potier et al., 2007; Song et al., 2010). In response to the alarm signals triggered in a wounded area, MSC secrete a series of trophic factors involved in the wound healing process. A hypoxic environment, for instance, characteristic of ischemic wounds, leads to activation of hypoxia-inducible factor-1α (HIF-1α), promoting angiogenesis (namely through secretion of VEGF, fibroblast growth factor-2 (FGF-2), HGF, insulin-like growth factor-1 (IGF-1) and thymosin beta-4 (Gnecchi,

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2006)) and inducing migration of fibroblasts and keratinocytes (Tandara and Mustoe, 2004). Particularly, when cultured as 3D spheroids, MSC evidence increased survival in ischemic tissues, while simultaneously present a higher expression of anti-inflammatory and proangiogenic factors with a key role on wound healing mechanisms (Bhang et al., 2011). In a pro-inflammatory environment, MSC were also shown to alter their secretome, in complex feedback mechanisms with surrounding immune cells (Yagi et al., 2010). A stress response, namely through a caspase-dependent mechanism, is thought to be involved on the spheroids upregulated expression of anti-inflammatory cytokines, such as TSG-6, prostaglandin E2 and stanniocalcin-1 (Bartosh et al., 2013; Tsai et al., 2015; Zimmermann and Mcdevitt, 2014). Human bone marrow (hBM) MSC could contribute to restore the oxygen levels and function of wounded tissues through their capability to promote angiogenesis, regulate inflammation and recruit native cells involved in the wound healing process, particularly when organized as spheroids. Nevertheless, the benefits associated with this 3D cell culture might be lost when cells are delivered *in vivo* into the target tissue, as cells tend to migrate from the spheroid to the surrounding extracellular matrix (ECM) as it has been observed in both *in vitro* (Blacher et al., 2014) and *in vivo* settings (Bhang et al., 2012).

To potentiate the response of MSC in regenerative processes, it is crucial to maximize cell survival, retention and function at the injured site. In an oxidative stress environment, matrices such as a pullulan-collagen hydrogel could improve MSC delivery, quenching ROS within ischemic wounds (Wong et al., 2011), an ability that has also been attributed to alginate hydrogels (Eftekharzadeh et al., 2010; Matyash et al., 2012). Encapsulation of BM MSC within a biocompatible and inert biomaterial, such as alginate microbeads, able to mediate cellular paracrine activity can be exploited as a MSC delivery system to study and modulate 3D cellular configurations towards improved wound healing processes. Alginate is an inert, European Medicines Agency (EMA)- and Food and Drug Administration (FDA)-approved material that has been used as a wound healing dressing and cell carrier (Hashimoto et al., 2004; Lee et al., 2010). This polysaccharide can be easily crosslinked with divalent cations (such as Ca^{2+}), resulting in formation of a hydrogel that allows diffusion of growth factors and nutrients/metabolites while simultaneously excludes host immune cells (Strand et al., 2002). Previous studies showed that alginate microcapsules can maintain their integrity for several months once implanted in mice (Landázuri et al., 2012; Safley et al., 2008), therefore retaining cells whose secretome can favor biological processes that take days to months, such as vascularization and tissue repair (Broughton et al., 2006).

Different studies have been exploring differences in the wound healing potential of cell culture performed in 2D monolayer configurations in comparison to 3D-organized cells seeded on gelatin microbeads (Zhao et al., 2015a,b), encapsulated in hydrogel microcapsules (Bussche et al., 2015), aggregated in spheroids cultured under static (Santos et al., 2015) or bioreactor platforms (Kwon et al., 2015). Nonetheless, our study presents, at the best of our knowledge, the first side-by-side comparison of the paracrine potential of BM MSC cultured in three different 3D configurations (spheroids, encapsulated spheroids and encapsulated single cells) against monolayer culture, focusing wound healing and angiogenesis. Functional assays aiming to unravel several aspects of wound healing, including the chemotactic, wound closure and angiogenic potential of MSC were performed. The advantages of a 3D cell culture of non-encapsulated MSC spheroids were assessed, particularly regarding their protection against an oxidative stress environment and compared with the most conventional 2D culture of MSC grown as monolayer adherent cells to tissue culture polystyrene (TCPS). Importantly, the use of alginate to promote encapsulation of either single MSC or 3D-organized MSC spheroids (Fig. 1) facilitated a direct comparative approach to assess the enhanced trophic properties of 3D spheroids over single-cultured MSC. This could therefore favor a more straight evaluation of the role played by cellular aggregation on the paracrine potential of MSC, not limiting the study to comparison with TCPS-adherent cells, and further allowing us to evaluate the advantages of using biomaterial vehicles to culture MSC spheroids.

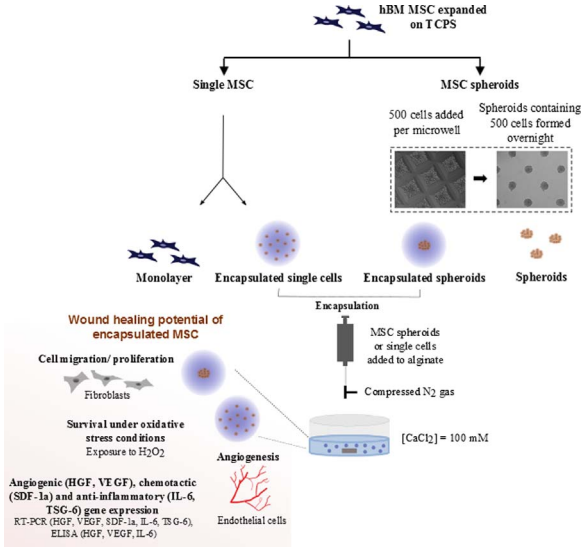

Fig. 1. Schematic representation of the experimental setup. BM MSC were expanded on TCPS and cultured as monolayer adherent cells, encapsulated single cells, non-encapsulated spheroids or encapsulated spheroids. Spheroids incorporating 500 cells were formed overnight in microwells and either cultured as non-encapsulated cells or encapsulated in alginate. Both spheroids and single MSC were embedded in alginate and extruded through a syringe needle into a calcium bath. The wound healing potential of encapsulated spheroids, encapsulated single cells, non-encapsulated spheroids and monolayer adherent MSC was studied through evaluation of survival under oxidative stress conditions, expression of relevant genes for the wound healing response, assessment of their angiogenic potential and involvement on the migration/proliferation of surrounding cells.

2. Materials & methods

2.1. Cell culture

hBM MSC (from at least 3 different individual donors, passage 4–6), isolated and immunophenotypically characterized as previously described (dos Santos et al., 2010), were cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (MSC qualified), 1% Antibiotic-Antimycotic (A/A) (all from Life Technologies). Cells were seeded at a density of 2–3 × 10^3 cells/cm^2 on 75 cm^2 Falcon® T-flasks in 10 mL of medium, which was exchanged every three to four days. After reaching approximately 70% confluence, cells were harvested with 0.05% (w/v) trypsin (Life Technologies) − 1 mM Ethylenediaminetetraacetic acid solution (EDTA) (Sigma). MSC were then cultured as monolayer cells, encapsulated as single cells or used to preform spheroids, which were cultured either as non-encapsulated or alginate-encapsulated spheroids for 7 days (Fig. 1). Non-encapsulated spheroids were cultured in suspension on a rotary orbital shaker (Rotamax 120, Heidolph) at 65 rpm whereas encapsulated single cells and encapsulated spheroids (all added at a ratio of 5 × 10^4 cells/mL of expansion medium) and monolayer cells (plated at an initial density of 3 × 10^3 cells/cm^2) were cultured under static conditions in Falcon® 6-well plates. Non-encapsulated spheroids were cultured on Ultra-Low Attachment plates (Corning) and culture medium was changed at day 4 of culture.

2.2. Formation of spheroids and spheroid size measurement

MSC spheroids with 500 cells per spheroid were formed overnight in an array of agarose (SeaKem® LE Agarose) 400 × 400 μm-sized