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Influence of culture parameters on ear mesenchymal stem cells expanded on microcarriers

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ABSTRACT

Mesenchymal stem cells (MSCs) have an accrued potential as a tool for cell-based therapies, thanks largely to their trophic properties. The significant amounts of cells needed for this goal should be attainable through optimized bioreactor expansion of MSCs. However, because of the specific properties of these cell populations, there is a need to investigate novel cell culture strategies adapted from established bioreactor cultivation practices. Among these, stirred culture on microcarriers appears as an appropriate approach for the expansion of MSCs but its optimization requires the identification of key limiting parameters to achieve a further increase in growth span. In this work, among the physico-chemical and physiological parameters affecting the expansion of ear-derived MSCs (E-MSCs) on porous microcarriers, supply of growth factors was important in controlling their growth span. The apparent growth rate of E-MSCs was found to be correlated with the percentage of cells in the S phase of the cell cycle. Moreover, this percentage was directly linked with the fraction of growth factor/receptor complexes. Thus, controlling the percentage of E-MSCs in S phase with suitable growth factor feeds led to an increase of their growth span. Finally, in response to these adapted feeds the cells maintained the key properties defining their MSC phenotype in terms of expression of markers and *in vitro* differentiation potential.

1. Introduction

In recent years, stem cell-based therapies have been attracting a lot of attention because of their potential applications in regenerative medicine. More specifically, the use of adult stem cells has been hailed as an efficient tool in this domain and as an attractive alternative to the use of embryonic stem cells (ESCs), despite their lower versatility. Mesenchymal stem cells (MCSs) display *in vitro* features of mesenchymal progenitor cells. MSCs are commonly characterized by expression of fibroblastic markers and by their *in vitro* differentiation potential into, at a minimum, adipocytes, chondrocytes and osteoblasts (Dominici et al., 2006, 2009). Moreover, MSCs have been found to display the general features of primary and non-oncogenic (=non-transformed) cell type: serum dependency, limited growth rate and limited life span during *in vitro* culture. Nevertheless, some kinetic data from mouse MSCs have contributed to a biased interpretation of the growth properties of MSCs: they have been reported to grow slowly for many weeks, after which they suddenly multiply rapidly, developing a genomic instability that enables them to proliferate and become tumorigenic: a "multistage carcinogenesis in cell culture" (Prockop, 2009). This apparent tumorigenesis has been also associated with unexpected cell functions of MSCs due to gene activation and dedifferentiation (Dominici et al., 2009). Tumoral transformation involves abnormal glucose metabolism (Macheda et al., 2005) as well as receptor signaling and trafficking, especially involving growth factors (Haglund et al., 2007). These are typical features of well established cell lines used for production of biologicals, i.e. hybridomas (resulting from the fusion of B-cells and myeloma tumor cells), Chinese hamster ovary (CHO) cells (Yang et al., 1999), VERO (Manohar et al., 2008) etc. Such cells are cultured in suspension with a drastic reduction of serum and growth factors (Burteau et al., 2003), harboring a typical highly oncogenic transformed phenotype (O'Callaghan and James, 2008): anchorage-independence, growth factor-independence, uncontrolled cell proliferation and aberrant apoptosis, as defined by Zhang et al. (2003). Conversely, due to their non-tumoral phenotype, MSCs are anchorage-, growth factor- and serum-dependent for their growth and survival (Pal et al., 2009).

Use of MSCs for therapeutic purposes necessitates large amounts of cells (Sensebe and Bourin, 2009). Moreover, given the

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low efficiency of cell recovery mainly due to a lack of specific markers, extensive *ex vivo* expansion is needed. To achieve this goal, various bioreactor strategies (in particular perfusion modes (King and Miller, 2007; Pankaj Godara et al., 2008)) and vessel types (spinner flasks, rotating wall vessels (Chen et al., 2006), Wave™ bioreactors etc. (Pankaj Godara et al., 2008)) have been tested. Expansion protocols making use of spinner flasks with microcarriers (MCs) have been shown to be particularly suitable for the propagation of MSCs (Frauenschuh et al., 2007; Rubin et al., 2007; Sart et al., 2009; Schop et al., 2008; Yang et al., 2007). However, among the panoply of bioreactor designs established for the production of biopharmaceuticals, reliable monitoring of the cellular microenvironment is essential for MSC expansion and differentiation, in terms of specific physiological parameters (e.g. oxygen requirements), mass transfer and mechanical environment (Pankaj Godara et al., 2008). Thus, a better understanding of MSC metabolism is required for subsequent optimization of the process of expansion of MSCs *in vitro* (Higuera et al., 2009). Due to the anchorage-dependent nature of MSCs, MC-based strategies are important for these cells' three-dimensional expansion. In addition to monitoring of the culture system, space saving might also be facilitated by using MCs, that could additionally serve as a delivery system for tissue engineering purposes.

In this work, we used cartilage-derived MSCs (C-MSCs) as an alternative model to bone marrow-derived MSCs (BM-MSCs). C-MSCs emerge as a powerful tool for cartilage tissue regeneration compared to BM-MSCs (Peng et al., 2008). Indeed, *in vitro* as well as *in vivo* chondrogenic differentiation of auricular (ear-derived) and articular (joint-derived) C-MSCs has been shown to be more efficient compared to BM-MSCs (Gawronska-Kozak, 2004; Peng et al., 2008; Togo et al., 2006). Ear progenitor cells in this differentiation path did not present signs of calcification, a bottleneck in the use of BM-MSCs for the treatment of cartilage defects (Hardingham et al., 2006). Moreover, other *in vivo* beneficial effects of MSCs are related to secretion of trophic factors (Peng et al., 2008): C-MSCs presented angiogenic potential (expression of vascular endothelial growth factor (VEGF), insulin like growth factor (IGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGFβ1), angiopoetin-1 (Ang-1), and stem cell factor (SCF)). Moreover, C-MSCs displayed a higher resistance to superoxide stress compared to BM-MSCs, rendering them suitable for transplantation into ischemic tissues (Peng et al., 2008). C-MSCs from ear (thereafter referred to as E-MSCs) are easy to harvest, and the techniques used for collecting them from a living animal are noninvasive (Staszkiewicz et al., 2010). E-MSCs have been extensively characterized on the basis of their *in vitro* expression of MSC-related markers and *in vitro* differentiation potential into adipogenic, chondrogenic, osteogenic (Rim et al., 2005) and myogenic pathways (Gawronska-Kozak et al., 2007). This cell population responds to the same signalling molecules as BM-MSCs toward these *in vitro* differentiation pathways, indicating that E-MSCs is a valuable model for studying adult mesenchymal stem cell activity and differentiation (Staszkiewicz et al., 2010). Finally, using rat-derived E-MSCs, we have shown previously in a 1-L bioreactor setting that this cell model displays a threefold higher growth rate *in vitro* compared to BM-MSCs, a clear advantage in our quest for rapid testing of bioprocess parameters affecting MSCs (Sart et al., 2009).

Our overall interest is to understand the impact of key culture parameters with potential to improve MSC proliferation in stirred porous MC culture, while at the same time validating that cells keep their main *in vitro* features. The specific purpose of this work was to investigate the influence of culture parameters on the yield of E-MSCs and to demonstrate the link between growth factors and increased growth span.

2. Materials and methods

2.1. Primary ear mesenchymal stem cell culture and characterization

E-MSCs were extracted as previously described (Sart et al., 2009). Briefly, external ears of Wistar rats (*Rattus norvegicus*) were cut and minced. The tissue was digested using a 2 mg/mL collagenase type II (Sigma–Aldrich) solution in DMEM (Lonza) plus 10% Fetal Bovine Serum (FBS) (HyClone). E-MSCs were selected by the plastic anchorage methodology (Gawronska-Kozak, 2004), and cultivated in DMEM plus 10% FBS (referred to as expansion medium).

E-MSCs were characterized by semi-quantitative RT-PCR analysis of the expression of the markers Notch-1, Sca-1 and CD73, as in Sart et al. (2009). Sequences of the corresponding primers used are presented in Table 1.

2.2. Microcarrier culture, bead-to-bead transfer and cell counting

A gelatin porous MC (Cultispher-S) (from 1 to 3 g/L) (Percell-Biolytica) and a collagen coated solid MC (Cytodex-3) (5 g/L) (Sigma–Aldrich) were prepared and cells were seeded as described previously (Sart et al., 2009). Briefly, in a 250-mL spinner flask (ABS), E-MSCs were seeded on MCs using an intermittent stirring (3 min agitated at 70 rpm, 30 min static) for 4 hours in 50 mL of expansion medium. After the seeding period, the medium volume was adjusted to 100 mL in a continuous stirring mode (70 rpm). Bead-to-bead transfer was carried out as in Sart et al. (2009). Briefly, on day 4 fresh beads were added into the vessel at a ratio of 1:1. Intermittent stirring was applied as above. As mentioned in Sart et al. (2009), due to the low amount of cells initially attached on the new beads added, the cells displayed an extensive lag phase of about 4–6 days. After this period, cell growth was monitored until proliferation ceased.

Cell counting on Cultispher-S was carried out as described previously (Sart et al., 2009). Dispase grade II (Roche) was replaced by trypsin 10× (PAA). For the comparison of E-MSC behavior on Cytodex-3 versus Cultispher-S, cell counting was performed using the crystal violet method. Briefly, MCs were allowed to settle and the expansion medium was replaced by a solution of 0.1% crystal violet in 1 M citric acid plus 0.1% Triton X100. After one hour of incubation at 37 °C, the stained nuclei were counted using a haemacytometer.

Table 1
Primer sequences for RT-PCR analysis.

Name	Orientation	Sequence	Product size (pb)
C/EBP α	5′ 3′	GCCAAGAAGTCGGTGGATAA CCTTGACCAAGGAGCTCTCA	238
Col2a	5′ 3′	TCCCTCTGGTTCTGATGGTC CTCTGTCTCCAGATGCACCA	161
Osteocalcin	5′ 3′	AGCTCAACCCCAATTGTGAC AGCTGTGCCGTCCATACTTT	190
Notch-1	5′ 3′	GCAGAACAACAAGGAGGAGA CCTTGAGGTCCTTAGCTTCC	365
CD73	5′ 3′	TCTGCAGCAAGTCATTACCA TTCCCCTACCCACTACCTTC	305
Sca-1	5′ 3′	CCTGGGTACTGTTGTCCAAG TGGCATTCCAGGAAGTAGAG	354
β actin	5′ 3′	CTCGTCATACTCCTGCTTGC CCATCCTGCGTCTGGACCTG	574