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ADIPOSE-DERIVED STROMAL CELLS

Senescence and quiescence in adipose-derived stromal cells: Effects of human platelet lysate, fetal bovine serum and hypoxia

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Abstract

Background aims. Adipose-derived stromal cells (ASCs) are attractive sources for cell-based therapies. The hypoxic niche of ASCs *in vivo* implies that cells will benefit from hypoxia during *in vitro* expansion. Human platelet lysate (hPL) enhances ASC proliferation rates, compared with fetal bovine serum (FBS) at normoxia. However, the low proliferation rates of FBS-expanded ASCs could be signs of senescence or quiescence. We aimed to determine the effects of hypoxia and hPL on the expansion of ASCs and whether FBS-expanded ASCs are senescent or quiescent. *Methods.* ASCs expanded in FBS or hPL at normoxia or hypoxia until passage 7 (P7), or in FBS until P5 followed by culture in hPL until P7, were evaluated by proliferation rates, cell cycle analyses, gene expression and β-galactosidase activity. *Results.* hPL at normoxia and hypoxia enhanced proliferation rates and expression of cyclins, and decreased G0/G1 fractions and expression of p21 and p27, compared with FBS. The shift from FBS to hPL enhanced cyclin levels, decreased p21 and p27 levels and tended to decrease G0/G1 fractions. *Conclusion.* Hypoxia does not add to the effect of hPL during ASC expansion with regard to proliferation, cell cycle regulation and expression of cyclins, p21 and p27. hPL rejuvenates FBS-expanded ASCs with regard to cell cycle regulation and expression of cyclins, p21 and p27. This indicates a reversible arrest. Therefore, we conclude that ASCs expanded until P7 are not senescent regardless of culture conditions.

Key Words: *adipose-derived stromal cells, clinical therapy, fetal bovine serum, human platelet lysate, hypoxia, quiescence, senescence*

Introduction

Over the years, adipose-derived stromal cells (ASCs) have gained increasing attention because of their relatively high abundance, easier accessibility and higher proliferation rates *in vitro*, compared with mesenchymal stromal cells from bone marrow (BMSCs) [1].

ASCs reside in a hypoxic niche *in vivo*, which suggests that hypoxic conditions during *in vitro* expansion might be beneficial [2]. Indeed, hypoxia has been shown to enhance *in vitro* proliferation rates of ASCs cultured in fetal bovine serum (FBS) [3–5]. Human platelet lysate (hPL) enhances ASC proliferation rates, compared with FBS. Thus, a combination of hypoxia and hPL might enhance ASC proliferation rates and prevent senescence during culture expansion [2,4–6]. However, the low proliferation rates of ASCs expanded in FBS at normoxia could potentially be due to the presence of quiescent or slowly proliferating cells, rather than senescent cells.

Regulation of ASC proliferation is mediated by cyclins, which drive progression through the cell cycle. Of these, cyclin E has been shown to be essential and rate-limiting for transition through the first checkpoint, known as G1/S [7,8]. Quiescent and slowly proliferating cells share a number of common features including large proportions of cells in G0/G1 phase, high expression levels of p21 and p27, and reversibility. This means that quiescent and slowly proliferating cells will reinitiate or increase proliferation and decrease the proportions of cells in G0/G1

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phase in response to mitogenic stimulation [9,10]. In the case of quiescent cells, reversibility is dependent on transcription factor HES1, which suppresses senescence and terminal differentiation [11].

In contrast to the reversible cell states, the cell cycle arrest of senescence is essentially irreversible. Once senescent, cells will not re-enter the cell cycle if the senescence-inducing stimuli are terminated [12], nor will senescent cells respond to mitogenic stimulation [13]. The features of senescent cells include high expression levels of p21 and $p16^{INK4A}$, and increased senescence-associated β-galactosidase activity [9,14,15].

Because senescent cells acquire functional abnormalities, their presence in a clinical cell product could compromise efficacy [16,17]. Therefore, we have, with modifications, adapted the excellent study design of Griffiths *et al.* to distinguish reversible cell states from irreversible senescence in our FBS cultures [18]. Griffiths *et al.* demonstrated that hPL completely rejuvenates middle passage (P6–P8) BMSCs expanded in FBS, whereas late passage (P15-16) BMSCs only respond transiently to a shift to hPL before their proliferation is significantly impaired [18]. This suggests that BMSCs P6–P8 are reversibly arrested, while BMSCs P15–16 are irreversibly arrested, and hence senescent.

At our stem cell centre, FBS-expanded ASCs and BMSCs used for clinical trials do not exceed P3 [19–22]. We have chosen to expand ASCs cultured in FBS for two additional passages (until P5) before subjecting them to our test for reversibility. In addition, we have included the combination of hypoxia and clinically approved hPL to determine the optimal culture conditions for clinical ASC production.

The aim of this study was to investigate whether our ASC cell product is devoid of senescent cells, regardless of the media supplements FBS and hPL and oxygen tension. This was investigated based on proliferation rates, cell cycle analysis, gene expression and senescence-associated β-galactosidase activity.

Methods

Experimental design

ASCs isolated from the stromal vascular fraction (SVF) of adipose tissue were cultured in two clinically approved complete media containing either hPL or FBS, at normoxic or hypoxic (5% O_2) conditions. ASCs were expanded until P7 at the following conditions: (i) FBS, (ii) FBS hypoxia, (iii) hPL, (iv) hPL hypoxia, (v) P5 FBS shifted to hPL and (vi) P5 FBS hypoxia shifted to hPL hypoxia (Figure 1).

To avoid contact inhibition-induced quiescence, cell cycle analysis was performed and RNA was extracted from a pool of sub-confluent ASCs.

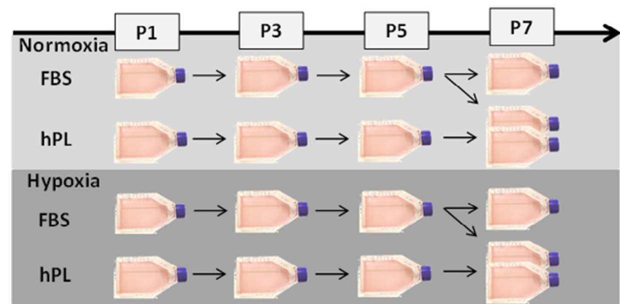

Figure 1. Flow chart of the experimental design.

Lipoaspirate preparation and SVF isolation

Lipoaspirate was obtained from three healthy donors (one male, two females, aged 23–55 years, mean age 35 years). The use of lipoaspirate from healthy volunteers was approved by the National Ethical Committee protocol no. H-3-2009-119. All donors agreed to and signed the informed written consent.

Approximately 100 mL lipoaspirate was obtained from liposuction of subcutaneous abdominal fat performed under local anaesthesia. The lipoaspirate was washed 2–4 times with phosphate buffered saline (PBS) pH 7.4 (Gibco, Life Technologies) to remove residual blood. The adipose tissue was digested by incubation with 0.6 PZ U/mL collagenase NB 4 (Serva) dissolved in HBSS (2 mmol/L Ca^{2+}, Life Technologies) at 37°C for 45–55 min, at constant rotation. The collagenase was inactivated with complete medium containing 10% FBS (Irradiated, Gibco, Life Technologies), followed by filtration through a 100-µm mesh (Steriflip Filtration System, Millipore). Cells were centrifuged for 10 min at 1200*g*, resuspended in complete medium containing FBS or hPL and cell number and viability were measured using NucleoCounter NC-100 (Chemometec) according to manufacturer's instructions.

Cell culture

SVF cells were seeded in T-75 flasks (Thermo Fisher Scientific) at a density of 4.5×10^6 cells/flask in complete medium containing Minimum Essential Medium alpha (αMEM) (Gibco, Life Technologies) with 1% penicillin/streptomycin (Gibco, Life Technologies) and one of the following supplements: 10% FBS or 5% hPL (Stemulate, Cook Regentec). The FBS batch has previously been used in our clinical studies [19–22], and thus it has been pre-qualified for ASC culture.

SVF cells were incubated at normoxic conditions (37°C, 5% CO_2, 21% O_2, humidified air). On the third day of culture, cells were washed twice with PBS to remove non-adherent cells. Subsequently, medium was changed every 3–4 days.

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