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AMNIOTIC FLUID CELLS

A humanized system to expand *in vitro* amniotic fluid-derived stem cells intended for clinical application

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Abstract

Background aims. The amniotic fluid is a new source of multipotent stem cells with therapeutic potential for human diseases. In agreement with the regulatory requirement to reduce and possibly to avoid animal-derived reagents in the culture of cells intended for cell therapy, bovine serum, the most common supplement in the culture medium, was replaced by human platelet-derived growth factors. *Methods.* We tested a new culture medium to expand monolayers of human amniotic fluid stem cells (hAFSC) for clinical use. The AFSC were isolated by c-Kit selection and expanded in media supplemented with either bovine serum or a human platelet lysate (Lyset). *Results.* We compared proliferation kinetics, colony-forming unit percentage, multilineage differentiation, immunophenotypic characterization and inhibition of peripheral blood mononuclear cell proliferation of the two AFSC cell cultures and we found no significant differences. Moreover, the karyotype analysis of the cells expanded in the presence of the platelet lysate did not present cytogenetic abnormalities and *in vitro* and *in vivo* studies revealed no cell tumorigenicity. *Conclusions.* Platelet derivatives represent a rich source of growth factors that can play a safety role in the homeostasis, proliferation and remodeling of tissue healing. We propose human platelet extracts as a preferential alternative to animal serum for the expansion of stem cells for clinical applications.

Key Words: *Amniotic Fluid Stem Cells (AFSC), fetal calf serum, platelet lysate, stem cells*

Introduction

Stem and progenitor cells are involved in the development, homeostasis and repair of tissues and organs [1–3]. Tissue damage triggers stem cell secretion of immunomodulatory and trophic factors, supporting the repair process [4]. Among several adult stem/progenitor cells, mesenchymal stem cells (MSC) show great therapeutic potential for numerous medical applications [5]. MSC can be derived from different adult tissues, such as fat, dermis, bone, synovium and bone marrow [6]. Because of the age-related decrease in the frequency and differentiating capacity of MSC, fetal tissues such as cord blood, Wharton's jelly, amniotic membrane and amniotic fluid are considered a valid alternative source of stem cells [7]. Moreover, fetal stem cells have the advantage, over their adult counterparts, of a greater differentiation, homing and engraftment potency and of a lower immunogenicity. Human amniotic fluid stem cells (hAFSC) appear as ideal candidates for stem cell therapy [8]. AFSC are multipotent cells that express early stem cell-specific markers such as Oct-4 and SSEA-4, but not SSEA-1and 3. Moreover, they present typical cell surface mesenchymal markers like CD73, CD90 and CD105, whereas they do not express markers of the hematopoietic lineage such as CD45, CD34, CD14 o CD11b, CD79 and the histocompatibility protein HLA-DR [9]. AFSC exhibit a significant plasticity and can be differentiated *in vitro* toward the mesodermal lineage [1]. An additional feature that makes AFSC good candidates for a potential therapeutic use is their ability to disseminate, supported by the

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expression of adhesion molecules shared with leukocytes such as CD44, CD24, CD29 and CD18. AFSC show also immunomodulatory properties and are not tumorigenic [10]. Therefore, AFSC, due to their plasticity, safety, easy accessibility and minor ethical requirement, represent a valid alternative to embryonic and adult human stem cells in clinical applications [11].

An appropriate *in vitro* propagation of these human fetal cells is propaedeutic for any clinical study. The expansion of a cell population requires the use of an adequate basal medium supplemented with growth factors, proteins and enzymes, supporting cell attachment and growth. The supplement commonly used is fetal bovine serum (FBS) [12,13]. Regulatory agencies strongly recommend reducing and possibly avoiding animal-derived reagents in the culture of cells intended to be use in in cellular therapy. In fact, the high lot-to-lot variability, as well as the risk of contamination (bacteria, viruses and prions) and xenoimmunization, makes mandatory the use of standardized and chemically defined media, approved for good manufacturing practice (GMP) and clinical use [14,15].

Blood platelets are a reservoir of different growth factors and cytokines playing a major role in cell proliferation and tissue regeneration [16,17]. The platelet-rich plasma (PRP) is a platelet concentrate that could represent a valuable, non-xenogenic alternative to FBS in cell culture. The PRP formulation mostly used for *in vitro* studies is the platelet-released supernatant derived from PRP after activation by thrombin with or without calcium gluconate [18]. The high degree of variability between the different platelet preparations obtained from a single blood donor or from small pools of blood units and the lack of standardized procedures made difficult the achievement of a consensus on the applicability of this novel cell culture medium supplement. Recently, a "ready to use" platelet derivative was developed starting from large pools of human-certified buffy coat samples (Lyset, http://www.lyset.it). Lyset is a dual-component product consisting of a platelet lysate (PL) derived from a blood fraction with a defined amount of platelet bioactive factors [19] and a platelet poor plasma (PPP) derivative. These two components are combined in different percentages to optimize the PL concentration for each different cell type.

In the present study, the PL/PPP supplement was used to expand AFSC in monolayer culture [20,21]. We describe the isolation, expansion *in vitro* and characterization of AFSC cultured in a xeno-free medium, compared with parallel cultures supplemented with FBS. AFSC expanded with the platelet derivative presented excellent stability in morphology, proliferation, karyotype, clonogenicity, pluripotency, mesenchymal stem marker expression and immunomodulatory properties. Therefore, AFSC expanded in Lyset-supplemented medium met all required criteria to be used in clinical studies.

Methods

Cell culture and AFSC growth kinetics

Human amniotic fluids were obtained by amniocentesis performed at the Cytogenetic Laboratory of Galliera Hospital (Genoa, Italy) for fetal karyotyping, between 15 and 17 weeks of gestation. Six different samples were transferred to our laboratory, after obtaining written informed consent from the donors. Primary cells were isolated from amniotic fluid by centrifugation and suspended in Chang Medium (Chang B + Chang C, Irvine Scientific, CA, USA). After 5–6 days, non-adherent cells and debris were discarded and adherent cells were c-Kit selected on a Mini-MACS apparatus (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described. AFSC were then expanded in α-MEM (Gibco, Invitrogen) with 20% of Chang medium plus (Irvine Scientific, Irvine, CA, USA) supplemented with the following: (i) 15% FBS (Euroclone) for standard culture condition; (ii) 5% platelet-derived components (1% Lyset + 4% Lyset Diluent [Sclavo Diagnostic International, Siena, Italy]). Lyset is a commercially available freeze-dried platelet lysate obtained starting from a very large pool of blood samples with standardized platelet concentration. Both cell cultures were maintained at 37°C with 5% CO_2 atmosphere. Adherent cells were detached from the plastic plate using a trypsin-Ethylenediaminetetracetic acid (EDTA) solution (Sigma, Milano, Italy) and re-plated. At each culture passage the cells were detached, pooled, counted and re-plated. The cumulative cell doublings of each cell population were plotted against time in culture to determine the growth kinetics. Cell morphology was monitored using optical microscope observation.

Colony forming unit fibroblast (CFU-F) assay

To test the clonogenic potential, cells were plated at low density (10 cells/cm^2) and cultured for 2 weeks. Medium was changed twice a week. CFU-F analysis and cell counting were performed after 2 weeks of culture. Cells were washed with phosphate-buffered saline (PBS) pH 7.2, fixed with 3.7% formaldehyde in PBS, stained with 1% methylene blue in borate buffer (10 nm, pH 8.8) for 30 minutes and the colonies counted. Colony counting was performed in duplicate in all six primary cultures.

AFSC immunofluorescence staining and immunophenotypic characterization by flow cytometry

For immunofluorescence experiments AFSC were grown on coverslips. Cells were fixed with