In vitro evaluation of mesenchymal stromal cell senescence

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Abstract

Mesenchymal stromal cells (MSCs) are adult multipotent stem cells that, for their immunomodulatory and reparative/anti-inflammatory properties, are a promising tool in cell therapy. For the treatment of patients, MSCs are in vitro stimulated to proliferate in a non-physiological condition and this situation could lead MSCs to malignant transformation. The clinical application of MSCs requires that the biosafety of these cells must be investigated. The cellular senescence is a crucial mechanism that prevents the growth of cells at risk for neoplastic transformation. The aim of this study was to investigate the capacity of healthy donor bone marrow derived MSCs to enter the senescence phase by evaluating the β-galactosidase activity. We tested 131 MSC batches after long-term in vitro culture, resulting positive for β-gal staining and showing a branched shape morphology typical of senescent cells. They displayed a progressive decline in proliferative capacity and none of them bypassed the senescence. In conclusions, our data indicate that MSCs are not inclined towards spontaneous neoplastic transformation. We believe that the control of MSC capacity to enter senescence is fundamental for quality and safety, considering the relevant interest in the MSC clinical applications.

Introduction

Mesenchymal stromal cells (MSCs) are adult multipotent stem cells, derived from mesoderm, the intermediate layer of the three embryonic tissue layers. Mesoderm is composed of an abundant extracellular matrix in which MSCs are absorbed [1]. Friedenstein and Petrakova first described in 1968 a population of self-renewal adherent cells in the rat bone marrow (BM) [2]. These cells showed fibroblast-like feature, high proliferate capacity, ability to differentiate into bone, muscle, cartilage and adipose tissue, as a hallmarks of their multipotency. In 1980, Castro-Malaspina and colleagues isolated MSCs from human BM [3]. In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria for defining MSCs, among these, it was emphasized their trilineage differentiation ability in vitro [4]. MSCs can be isolated from BM, adipose and other adult and fetal tissues [5]. The frequency of MSCs in human BM has been estimated to range from 0.001% to 0.01% of the total nucleated cells; however, they can be easily isolated and expanded ex vivo. Furthermore, the frequency of MSCs declines with age, from 1/104 nucleated marrow cells in newborns to 1/106 in elderly people [6]. MSCs have chemotactic ability and multiorgan homing capacity, migrating to the sites of inflammation and injury. They are capable of modulating the function of almost all the immune cells thanks to their immunosuppressive and regulatory properties [7]. MSCs also secrete paracrine mediators, such as cytokines, able to reverse acute organ failure and to stimulate hematopoiesis, creating a regenerative microenvironment [8]. For their immunomodulatory and reparative/anti-inflammatory properties, MSCs are a promising tool in cell therapy, regenerative medicine and tissue engineering, representing at the present the most clinically investigated advanced therapy medicinal product (ATMP). Due to their efficacy against a wide spectrum of human diseases and the established safety, more than 1000 clinical trials have been registered [8].

GMP facility: the Cell Factory

Following the regulatory agency requirements (EudraLex - Volume 4 –Good Manufacturing Practice guidelines), the ATMP must be manufactured in a controlled contamination room. In the clean room, the environmental parameters, such as temperature, humidity and pressure, are constantly monitored, and specific structural requirements are needed, for example: materials must not release particles and surfaces must be smooth and easy to clean [9]. ATMP must be manipulated in a class A laminar flow cabinet placed in grade B background with positive pressure. Clean room includes production area, quality control and research and development (R&D) laboratories. For ATMP production application of good manufacturing practice (GMP) is mandatory. GMP are European guidelines that define quality measures for both production and quality control, to ensure that all processes, personnel, rooms and materials are clearly defined, validated, reviewed and documented [9]. To minimize the risk of pharmaceutical production it is required to test

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the final product and cover all aspect of production, from the starting materials to the training of staff. Detailed, written procedures are essential for each process that could affect the quality of the ATMP [9]. In Italy, the compliance of Cell Factory (CF) to the GMP is assessed by Italian drug agency (AIFA) that release the production authorization following inspection visits. In our case, the conformity of the Cell Factory, in which we isolate and expand MSCs as ATMP, has been achieved by AIFA, obtaining production authorization (n. aM-209/2017).

Long term in vitro MSCs cultures and the spectrum of malignant transformation

The clinical application of MSCs requires that the biosafety of these cells must be carefully investigated through appropriate and sensitive tests [10]. They should be tested for sterility, morphology, immunophenotype, identity and potency. However, in vitro MSCs are stimulated to proliferate in a non-physiological condition in order to obtain a sufficient number of cells for the treatment of pediatric and adult patients. This situation could lead MSCs to malignant transformation due to a progressive accumulation of DNA damage that, until now, is never been described in literature [10]. Conversely, it has been observed that long-term in vitro MSC cultures maintain their morphologic, phenotypical and functional characteristics, showing a progressive decline in their proliferative capacity reaching a senescence phase [10]. This observation is fundamental to be sure, that malignant transformation did not occur, since it is known that cancer cells exhibit uncontrollable proliferation.

Cellular senescence

Cellular senescence is a crucial anticancer mechanism that prevents the growth of cells at risk for neoplastic transformation [11]. Cellular senescence was described more than 40 years ago as a process of a stable and irreversible growth arrest of cells. Genomic changes, including DNA damage, oncogene activation, oxidative stress, mitochondrial dysfunction, irradiation and exposure to chemotherapeutics can lead to senescence. The senescent cells undergo characteristic morphological changes, exhibit the activation of the p53 and/or p16 pathways leading to permanent growth arrest of the G1 cell cycle phase, chromatin changes and nuclear envelope disturbance [11, 12]. Regarding MSCs, the senescence phase is characterized by changes in morphology, becoming flat and hypertrophic with constrained nuclei and granular cytoplasm. They show an excess of actin fibers, a decreased adherence to plastic surface, a reduced number of colony-forming unit-fibroblast (CFU-F) and the loss of proliferative and differentiation ability [4]. In 1995, the cell senescence was associated to intracellular -galactosidase activity, not detectable in quiescent or terminally differentiated cells [13, 14]. These enzymes are known to induce hydrolysis of lactose into glucose and galactose and their activity is now used to study cellular senescence of in vitro cultured cell lines [13, 14].

The aim of this study was to evaluate the capacity of healthy donor BM derived MSCs, expanded as ATMP, to enter the senescence phase after long-term in vitro culture to avoid the risk of spontaneous malignant transformation.

Materials and Methods

In CF, we in vitro isolated and expanded MSCs from 8 BM of hematopoietic stem cell healthy donors (HD) after informed consensus was obtained. Mononuclear cells were isolated from BM aspirates by density gradient centrifugation (Ficoll 1.077 g/ml; Lympholyte, Cedarlane) and plated in non-coated 175 cm2 polystyrene culture flasks (Corning Costar) at a density of 160.000/cm2 in complete culture medium: Dulbecco's Modified Eagle Medium low glucose (D-MEM, Gibco) + 5% MultiPL human platelet lysate (Macopharma) + Heparin (PharmaTex) 2 U/ml. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Culture medium was replaced twice a week and MSCs were harvested after reaching more than 80% confluence, using TrypLETM Select CTSTM (Gibco). A maximum of 14x106 MSCs was re-plated at 4.000 cells/cm2, expanded until passage (P)4 and cryopreserved as final product for clinical applications. The MSCs intermediate passages were instead cryopreserved in liquid nitrogen vapors (-196°C) in controlled temperature dewars for further expansions. At P4, 1x105 cells were assigned to the R&D laboratory and cultured in non-coated 25 cm2 polystyrene culture flasks (Corning Costar) in complete culture medium until senescence, characterized by a non-homogeneous morphology, a slow growth rate and a cell recovery lower than the number of cells initially plated. MSCs in the senescence phase were closely monitored for an additional 8 weeks before interrupting the cultures, to be sure that cells did not start again to proliferate, escaping senescence phase. At this time, senescence β-galactosidase staining (Cell Signaling Technology) was setting up following the manufacturing instructions. It is appropriate to use for each experiment a negative control represented by MSCs at early passage to guarantee more reliable results.

MSCs were plated in 96-well flat bottom plate (Corning Costar) at 2.500 MSCs/well. Culture was maintained at 37°C in a humidified atmosphere containing 5% CO2 overnight. This step allow MSCs to adhere to plastic.

The next day, the culture medium was removed and the plate rinsed once with 200 µl phosphate buffer saline 1x (PBS, Euroclone). Then 200 µl of 1x Fixative solution (formaldehyde/glutaraldehyde 2% v/v) were added to each well and the plate incubated for 10 minutes at room temperature. After fixation,

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the plate was rinsed twice with PBS, and 200 µl/well of staining solution were added. The staining solution should be prepared in polypropylene tubes just prior to use. We prepare the stock solution of β-galactosidase substrate, dissolving 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 1 ml dimethylformamide (Sigma). For the staining solution 50 µl X-gal stock solution were added to 930 µl 1x trisodium orthophosphate/acid citric, 10 µl 100x tetrapotassium iron/hexacyanide trihydrate (Solution A), 10 µl 100x tripotassium hexacyanoferrate (Solution B). The pH solution must be 6.0. The plate was incubated at 37°C overnight in a dry incubator without CO2.

The cells were checked under a phase-contrast microscope (4x magnification) for the development of blue color. For long-term storage the staining solution was removed, 200 µl of 70% glycerol were added and the plate stored at + 4°C.

Results

From the 8 HD BM- MSCs we obtained 131 lots of MSCs ready for clinical application. All of them have been evaluated in the R&D laboratory, propagated and monitored in long-term in vitro culture until the senescence. At this stage, all of them resulted positive for β-galactosidase staining (Figure 1). All the 131 MSC batches showed growth arrest and progressively entered the senescence phase (median passage P11, range 6-16), in particular 116/131 (88.5%) reached senescence at passages ranging between P8-P13 (Table 1). As expected, all MSCs showed a progressive decline in their proliferative/expansion capacity (Table 2). None of our MSC samples bypassed the senescence by developing a crisis phase, characterized by a restart of cell proliferation. Senescent MSCs appeared smaller, flat and hypertrophic, characterized by a branched shape morphology known as "fried egg appearance" (Figure 2). Moreover, they showed a decreased adherence capacity to plastic surface.

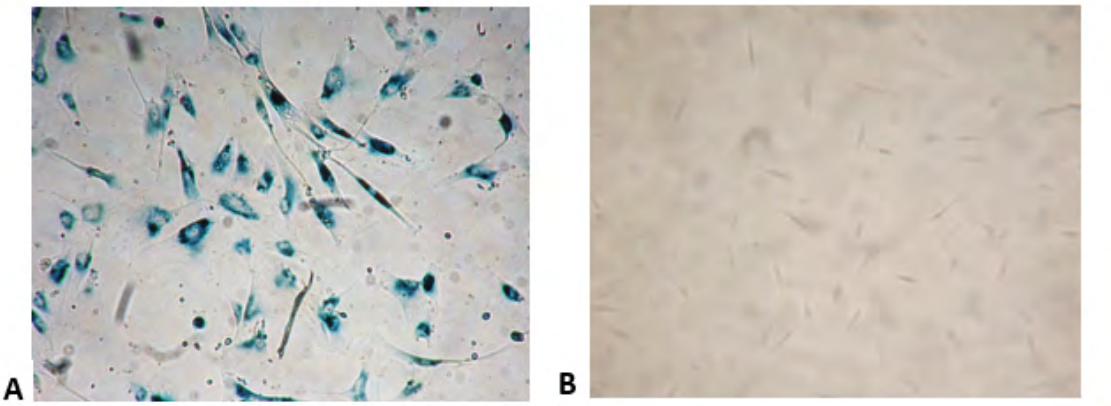

Figure 1. *β-gal staining of MSCs at senescence and at early passage. Senescent MSCs stained for β-gal resulted colored in blue (A) while MSCs at early passage do not show blue staining (B) (4x magnification).*

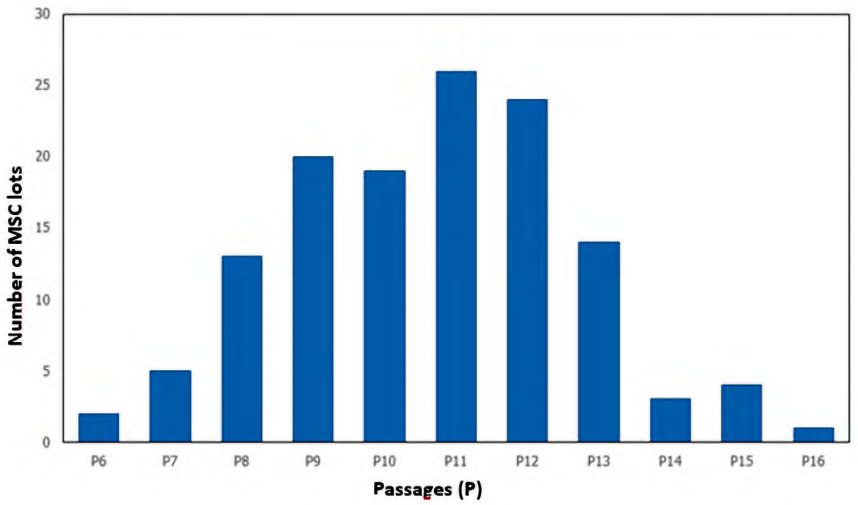

Table 1. *Number of lots entered senescence at different passages, defined in the 131 MSC lots after long-term culture following β-gal staining.*

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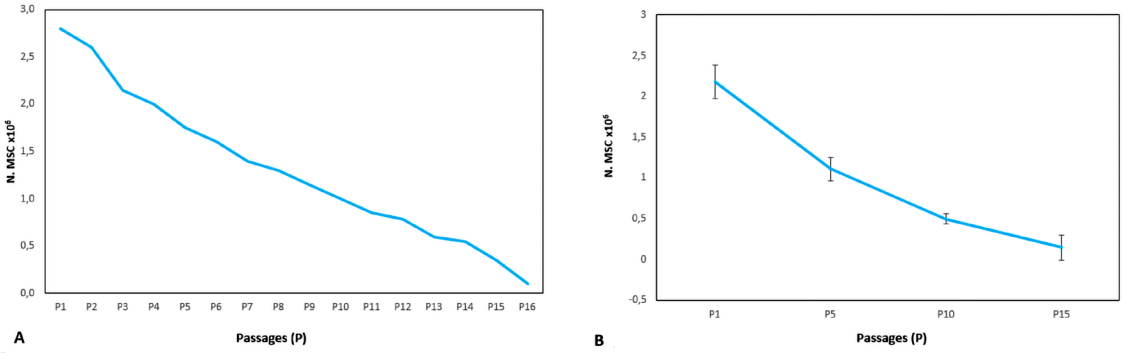

Table 2. *Curve of a representative MSC lot proliferation from passage P1 to senescence passage (A). The growth curve of all the 131 MSC lots tested (B); results are reports as mean ±SE.*

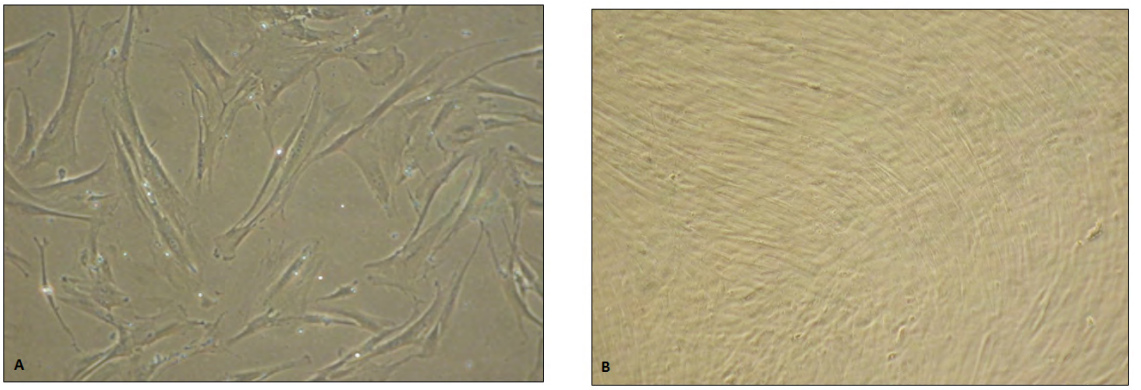

Figure 2. *MSC branched shape morphology known as "fried egg appearance "of cells in senescence phase (A) compared to MSC spindle-shaped morphology at early passage (B). (4x magnification).*

Discussion

In the last years, the MSCs have been used in cell-based therapy for several diseases, due to their immunomodulatory, inflammatory and regenerative ability. Since they are present in bone marrow and other tissue in very low rate, in order to reach adequate number for the treatment of pediatric and adult patients, they are extensive stimulated to in vitro proliferate in a non-physiological condition that could lead to malignant transformation.

For this reason, the biosafety features of these cells need to be carefully investigated with appropriate and sensitive tests to exclude any functional or genetic alterations before their release for clinical use [10]. Standardized protocols for quality controls of therapeutic cell preparations are a prerequisite for reliable and reproducible cellular therapy and we believe, also supported by literature, that senescence assessment should be included [15]. Different methods have been tested to predict the passage at which MSCs are approaching a senescent state. One of the most convenient predictive indicators of senescence in vitro could be the number of passages. However, as there is great variation in both seeding densities and time of harvesting, passage numbers may lead to deceptive results under non-standardized conditions. These limitations could be overcome by evaluating the number of cumulative population doublings (cPD), however, analysis of cPD does not consider the probable cell lost for apoptosis, necrosis or during passaging [15]. Taken together, these data suggest that it is hard to define MSCs entering in the senescent phase. A recognized approach is to stain the senescent cells based on the accumulation of senescence-associated -galactosidase activity (SA--gal) [15]. SA--gal is detectable by a chromogenic substrate, which yields an insoluble blue compound when cleaved by the enzyme and this staining is presently the most widely used test for senescent cells [14]. However, this assay, even if simple and fast, shows some critical steps that could compromise the outcome of the staining. First, the assay must be carried out on sub-confluent cell, as it was reported that confluence could give nonspecific positive staining. The fixation time is also critical since if longer than 10 minutes may give nonspecific negative staining. Color detection required incubation with the substrate at a final pH of 6.0; a higher pH can result in false negative, while a lower pH in false positive. It has been observed that at pH 4.0, -gal enzyme is also expressed in lysosomes of proliferating cells. Moreover, the staining development must be carried out at 37°C in absence of CO2, which induces changes of the pH [16].

Our results show that all the BM-derived MSC batches showed a progressive decline in their proliferative capacity reaching the senescence phase, resulting positive for -gal staining. Our data

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indicating that in vitro cultured MSCs do not display the aptitude towards spontaneous transformation, suggest important information also regarding the passage that could be used for clinical application. It is advised to use MSCs at early passages, far from senescence, to preserve their functionality, fundamental for the development of efficient cell-based therapeutic approaches. We believe that the control of MSC capacity to entered senescence is essential for quality and safety of MSCs when used as ATMP, considering the relevant interest in their clinical applications.

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Conceptualization, E.L.; methodology, E.L.; formal analysis, E.L.; writing- original draft preparation, E.L.; writing-review and editing, M.A.A, P.C, A.M, M.Z.; supervision, M.A.A, P.C, A.M, M.Z.
All authors have read and agreed to the published version of the manuscript. Authorship must be limited to those who have contributed substantially to the work reported.

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***Conflicts of Interest*:**
The authors declare no conflict of interest.

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