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Cell-Cycle Phases and Genetic Profile of Bone Marrow-Derived Mesenchymal Stromal Cells Expanded In Vitro From Healthy Donors

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ABSTRACT

Human mesenchymal stromal cells (MSCs) expanded in vitro for cell therapy approaches need to be carefully investigated for genetic stability, by employing both molecular and conventional karyotyping. Reliability of cytogenetic analysis may be hampered in some MSC samples by the difficulty of obtaining an adequate number of metaphases. In an attempt to overcome this problem, a methodology apt to evaluate the cell-cycle structure on synchronous MSCs was optimised. Results obtained in five independent experiments by comparing cell-cycle analysis of synchronous and asynchronous MSC populations evaluated at early and late culture passages documented that in synchronous MSCs, 30% of cells entered G2/M phase after about 27–28 h of culture, while in asynchronous MSCs only 8% of cells in G2/M phase could be observed at the same time point. Cytogenetic analysis on synchronous MSCs allowed us to obtain 20–25 valuable metaphases/slide, whereas only 0–4 metaphases/slide were detectable in asynchronous preparations. J. Cell. Biochem. 112: 1817–1821, 2011.

KEY WORDS: MESENCHYMAL STROMAL CELLS; CELL CYCLE; KARYOTYPE

Mesenchymal stromal cells (MSCs) are an heterogeneous population of multipotent progenitors that can be detected and isolated from many adult and foetal tissues [Campagnoli et al., 2001; Da Silva et al., 2006]. MSCs propagated in vitro are characterized by plastic adherence, expression of a peculiar combination of cell surface markers and multilineage differentiation potential into osteoblasts, adipocytes, chondrocytes and other mesoderm-derived tissues [Dominici et al., 2006]. MSCs have recently gained wide interest in view of their therapeutic potential. However, cell expansion in vitro, which selects for rapidly dividing cells, may increase the risk of spontaneous malignant transformation [Burns et al., 2005; Bernardo et al., 2007a; Klopp et al., 2010; Wagner et al., 2010]. In particular, it has been reported that mouse MSCs show chromosomal abnormalities and are highly susceptible to transformation, while human MSCs are apparently more resistant to transformation in vitro than their murine counterparts, with no genomic instability detected so far. Even though no critical side effects, including tumour formation, have been described in patients receiving MSCs in clinical trials, the use of MSCs for clinical approaches requires that the bio-safety of these cells be carefully investigated by appropriate and sensitive tests. Indeed, the absence of genetic instability in MSCs propagated in vitro has to be documented before considering their clinical use, particularly in immune-compromised patients where failure of immune surveillance mechanisms might favour the development of tumours in vivo. Chromosomal stability of in vitro expanded MSCs can be at best evaluated by employing both molecular and conventional karyotyping. Comparative genomic hybridisation (CGH)-array is a

Abbreviations used: MSCs, mesenchymal stromal cells; BM, bone marrow; P, passages.

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rapid and high-resolution technique useful for the detection of both benign and disease-causing genomic copy-number variations in tumours and genetic disorders. However, this technique is unable to detect balanced chromosomal rearrangements that can be excluded by simultaneously performing conventional cytogenetic analysis. Reliability of cytogenetic analysis may be hampered in some MSC samples by the difficulty of obtaining an adequate number of metaphases. In an attempt to overcome this problem, a methodology apt to evaluate the cell-cycle structure on synchronous bone marrow (BM)-derived MSCs was optimised in order to identify the culture time point at which the highest number of metaphases is present.

RESULTS AND DISCUSSION

In the present study, the cell cycle of synchronous and asynchronous MSCs was evaluated. Synchrony is required to study the progression of cells through the cell cycle. Culture in the absence of growth factor supplements is one of the methods employed to synchronise cells at different stages of the cell cycle to the same phase [Merrill, 1998; Davis et al., 2001]. Short-term deprivation of growth factor supplements from the culture medium results in the accumulation of cells at the G0 phase. In this way, the subsequent addition of complete medium drives the great majority of cells to simultaneously start the duplication process.

Cell-cycle analysis, carried out before growth factor supplement restoration on five synchronised MSC samples evaluated at different culture passages, showed that 86 ± 6% of cells were in the G0 phase. The evaluation of cell cycle at different time points after restoration of growth factor supplements, documented that the G1 phase takes 14–15 h, S phase 8–9 h and G2/M phase 3–4 h of culture (Fig. 1). At these time points, in synchronous MSCs, median and range of cells in G1, in S and in G2/M phase were 85% (81–90%), 34% (29–62%) and 30% (18–47%), respectively (Fig. 2A–C). The same kind of analysis carried out on asynchronous MSC samples documented that, at the same time points, 75% (72–76%) of cells were in G1 phase, 15% (11–18%) in S phase and 8% (4–11%) in G2/M phase ($P = 0.04$, respectively, as compared to synchronous MSCs) (Fig. 2D–F). No significant differences in either the cell percentage or in duration of different cell-cycle phases were observed in both synchronous and asynchronous MSCs evaluated at different passages (data not showed).

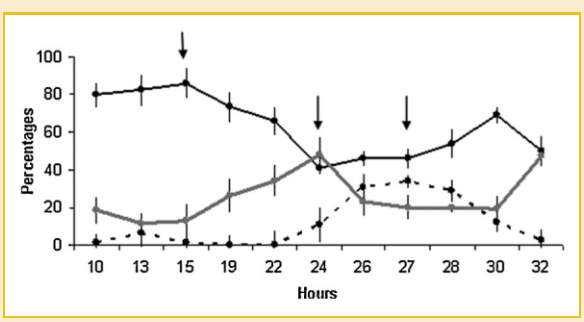

Fig. 1. MSCs cell-cycle kinetics. Duration of the cell-cycle phases are reported after synchronisation by growth factor supplement deprivation. The mean and SD of cell percentages in different cell-cycle phases evaluated at all time points in the five MSC preparations are reported. Black continuous line represents G1 phase, grey continuous line represents S phase and black broken line G2/M phase. Arrows show time points in which the maximum percentage of cells are in the respective cell-cycle phases.

Altogether our results demonstrate that in vitro expanded BM-derived MSCs exhibit a peculiar cell-cycle structure, intermediate between differentiated and pluripotent cells. It has been reported that in human somatic cells this process takes about 16–24 h, with G1 phase of 6–12 h, S phase of 6–8 h, G2/M of 3–4 h, while pluripotent cells need about 32–38 h to complete the cycle [Ohtsuka and Dalton, 2008; Dalton, 2009]. We observed that the major difference between differentiated cells and MSCs consists in the duration of the G1 and S phases. In agreement with previously reported studies, it is conceivable to hypothesise that an extended G1 phase may predispose MSCs to be especially reactive to differentiation signals, while a long S phase may allow the maintenance of an higher proportion of cells in the euchromatic rather than heterochromatic state [Herrera et al., 1996; Singh and Dalton, 2009].

The definition of the extent of MSC cell cycle has allowed us to identify the time for colcemid addition at 27–28 h of culture. Colchicine inhibits microtubule polymerisation by binding to tubulin, thus blocking mitosis. In this way, by synchronizing MSCs and adding colcemid when a higher percentage of cells are entering mitosis, we were able to obtain 20–25 valuable metaphases/slide in all synchronous MSC samples, as compared with 0–4 metaphases/slide detectable in asynchronous MSC samples. Present results obtained by analysing a sizable number of mitosis confirmed previously reported data documenting that BM-derived MSCs propagated in vitro display a normal karyotype (Fig. 3) [Barry and Murphy, 2004; Bernardo et al., 2007a,b]. On the other hand, Tarte et al. [2010] have recently demonstrated the presence of chromosomal aberration, in the absence of malignant transformation, in a proportion of human MSC lots propagated in vitro under clinical grade conditions. These data, obtained in MSCs at early culture passages, further emphasize the importance of implementing sensitive and informative quality control assays able to carefully investigate the bio-safety and functional capacity of MSCs propagated in vitro for cell therapy approaches. Indeed, chromosomal instability may not only increase the risk of spontaneous malignant transformation of MSCs but also hinder their biological properties that are necessary to develop the immune suppressive or reparative/regenerative in vivo effect [Jorgensen et al., 2004; Le Blanc et al., 2008; Bernardo et al., 2011].

In conclusion, results of our study document that reliable and informative evaluations can be obtained from cytogenetic analysis of human MSCs propagated in vitro by analysing synchronous cells. Consequently, conventional karyotyping associated with genomic molecular analysis of MSCs can be considered a suitable quality control assay for the release of human MSCs for clinical use.

MATERIALS AND METHODS

MSC EXPANSION

BM-derived MSC samples derived from five healthy donors, which had been employed in previously reported studies [Bernardo et al.,

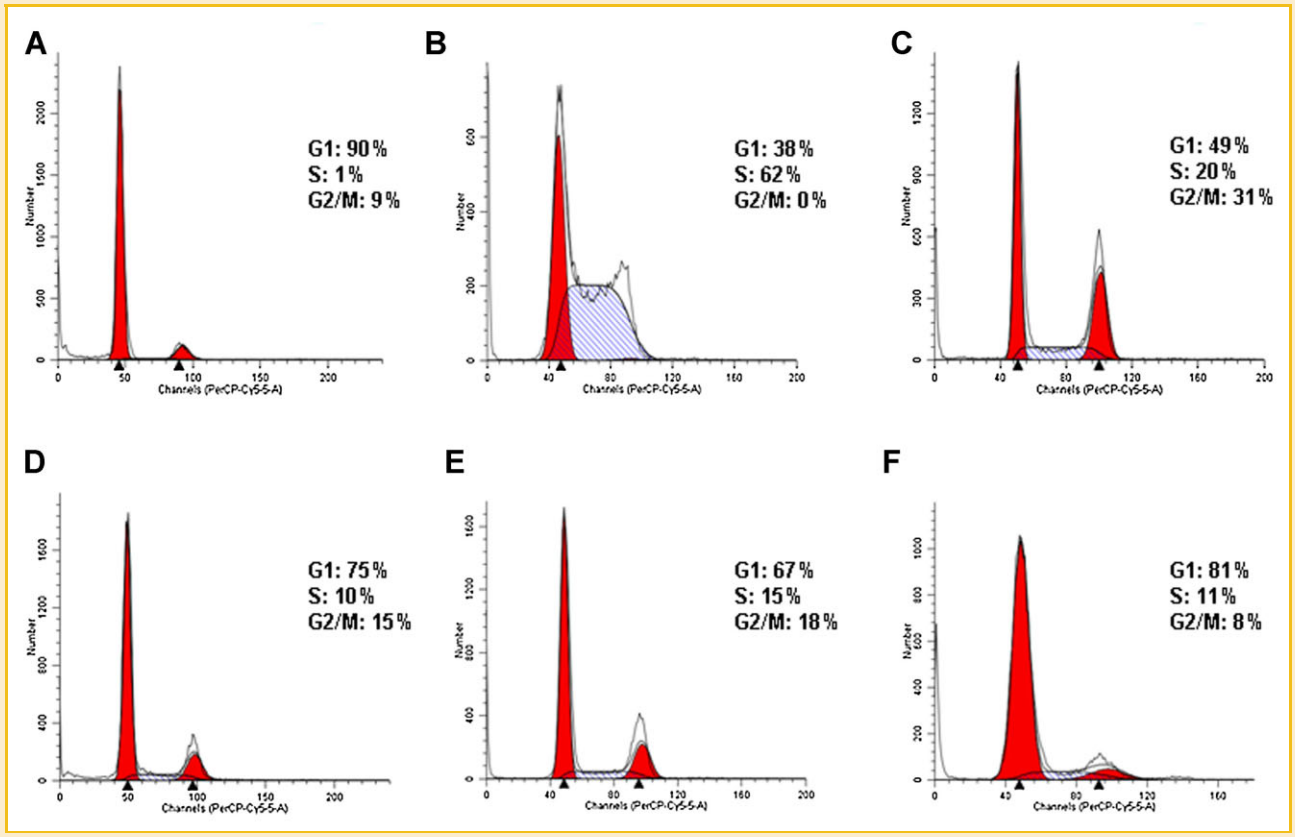

Fig. 2. Representative histograms of cell cycle in synchronous (A–C) and asynchronous (D–F) MSCs. In the five synchronous MSCs preparations analysed at 15 h (A,D), 24 h (B,E) and 27 h (C,F), median and range of cells in G1, S and G2/M phase was 85% (81–90%), 34% (29–62%) and 30% (18–47%), respectively. The same kind of analysis carried out on asynchronous MSC lots documented that, at the same time points 75% (72–76%) of cells were in G1 phase, 15% (11–18%) in S and 8% (4–11%) in G2/M. These percentages of cells in synchronous and asynchronous MSCs resulted statistically different ($P = 0.04$, respectively).

2007a,b], were propagated in vitro following a previously described method [Bernardo et al., 2007b]. Briefly, mononuclear cells were isolated from BM aspirates by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) and plated in non-coated 75–175 cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm^2 in complete culture medium: LG-DMEM (Invitrogen, Paisley, PENN) supplemented growth factors (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies, Vancouver, Canada) and gentamycin 50 μg/ml (Gibco-BRL, Life Technologies, Paisely, UK). Cultures were maintained at 37°C in a humidified atmosphere, containing 5% CO_2. After 48-h adhesion, non-adherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥80% confluence, using trypsin (Sigma–Aldrich, Milano, Italy), and replated at 4,000 cells/cm^2.

CELL-CYCLE ANALYSIS

Cell-cycle analysis was performed on synchronous and asynchronous MSCs, evaluated at different culture passages (P) (three lots were at P3, which we consider early P and the other two were at P6, which we consider late P). In order to obtain synchronization, MSCs at >80% confluence are detached, replated at 7,000 cells/cm^2 and maintained in culture without growth factor supplements (namely without the Mesenchymal Stem Cell Stimulatory Supplements) for 20 h. At this time, MSCs were evaluated to document the percentage of cells in G0/G1 phase. After the 20 h of growth factor deprivation, complete medium was restored into the culture and cell cycle was analysed at different time points (10, 13, 15, 19, 22, 24, 26, 28, 30, 32 h). Asynchronous MSCs, simultaneously obtained by culturing the cells in complete medium, were evaluated at the same time points. MSCs (5×10^5) were then collected and, after addition of 500 μl of a commercial solution containing propidium iodide and RNAse (DNA QC particles, BD, San Diego, CA), were incubated in the dark for 30 min. The cells' DNA content was then measured using a FACSCanto flow cytometer (BD). At least 20,000 events were acquired. The distribution of cells at different cell-cycle phases was analysed using the model included in the ModFit LT™ (Verity Software House, Inc., Maine, MI) software program.

CYTOGENETIC ANALYSIS

After 27–28 h of culture in complete medium, MSCs were incubated at 37°C with 0.1 μM Colcemid solution (Irvine Scientific, Santa Ana, CA). After 4 h, cells were harvested, treated with 0.56 mM KCl and fixed in methanol/acetic acid (3:1). Metaphases of cells were Q-banded and karyotyped in accordance with the International System for Human Cytogenetic Nomenclature recommendations.

Fig. 3. Normal Q-banding karyotype (360–400 band) of one out of five synchronous MSC preparations analysed in accordance with the International System for Human Cytogenetic Nomenclature recommendations.

STATISTICAL ANALYSIS

Statistical analysis was performed using Wilcoxon non-parametric test for paired samples. *P*-values <0.05 were considered to be statistically significant.

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