Are mesenchymal stromal cells from children resistant to apoptosis?

H. Dimitriou, Ch. Perdikogianni, G. Martimianaki, D. M. Choumerianou, J. Pelagiadis and M. Kalmanti

Department of Pediatric Hematology–Oncology, University Hospital of Heraklion, University of Crete Medical School, Heraklion, Crete, Greece

Received 25 February 2008; revision accepted 3 July 2008

Abstract

Objectives: Mesenchymal stromal cells (MSC) represent a novel cellular candidate in the field of transplantation and tissue regeneration. Their clinical application requires their *in vitro* expansion. The aim of this study was to assess the effect of conditions that would favour apoptosis, and of long-term expansion, on the characteristics of MSC from children.

Materials and methods: Bone marrow mononuclear cells were cultured for 10 passages (P1–P10). Expression of CD105, CD146, CD95 and apoptosis by 7-amino-actinomycin D staining were evaluated. CFU-F and cell doubling time (DT) were assessed in every passage. Cell-cycle study was performed at P2 and P6.

Results: CFU-F decreased from 38 ± 3.7 at P2 to 9.6 ± 3.2 per 10 MSC/cm² at P10 and DT increased from 1.93 ± 0.1 (P2) to 6.1 ± 2.45 days (P10). A low percentage of apoptotic (dead) cells was detected at P2 and this did not change until P10. Cells at P2 were at G₀/G₁ phase, but in advanced passages more cells were in an active state. Induction of apoptosis (addition of anti-Fas agonist antibody) using standard culture conditions, showed a minor effect on MSC survival. Serum deprivation of MSC (up to 72 h) revealed no substantial apoptotic effect while cells retained their tri-lineage differentiation capacity.

Conclusions: We conclude that MSC from children retain their functional characteristics throughout serial passages and remain stable under conditions that usually cause apoptosis. These features render MSC, especially those of early passages, optimal candidates for use in clinical applications.

Introduction

Human mesenchymal stromal cells (MSC) are pluripotent bone marrow cells that constitute principal elements of the bone marrow microenvironment. They expand *ex vivo* and give rise to cells of several mesodermal lineages, such as cartilage, bone and fat (1). It has been reported that MSC are even able to differentiate across the germ layer and into cells with neuroectoderm and endoderm characteristics (2). The fact that they easily expand *ex vivo* and have this extensive differentiation potential renders them very promising candidates for clinical applications in tissue engineering (3,4), repair of damaged organs (4,5) and correction of inherited disorders, such as osteogenesis imperfecta (5,6). In addition, it has been shown that they can accelerate haematopoietic engraftment following allogeneic transplantation (7,8), and have been administered to patients in an effort to prevent or treat graft-versus-host disease (9,10).

In order to achieve the required number of cells for clinical applications, *ex vivo* expansion has been applied. The features of these cells need to be thoroughly investigated to exclude any functional or immunophenotypic alterations as a result of cell aging after long-term *in vitro* culture. Concerns have also been raised about the possibility of spontaneous malignant transformation of adult tissue-derived MSC after long-term *in vitro* culture (11,12).

MSC are currently expanded *in vitro*, either for experimental purposes or for clinical applications, in the presence of 10–20% foetal calf serum (FCS), essential for effective culture of these cells (13). Although FCS is screened prior to use to ensure its biosafety, its utilization raises concerns in the clinical setting since it is an animal-derived product, potentially containing pathogens and can elicit immune responses (14). The effect of long-term culture on biological characteristics of MSC and their apoptotic behaviour through time must be extensively studied and documented before considering infusion of these cells to patients.

The aim of this study was to assess the effect of long-term expansion of bone marrow-derived MSC from children, through serial passages, on their biological

Correspondence: Helen Dimitriou, University of Crete, Medical School, Department of Pediatric Hematology–Oncology, PO Box 2208, GR 71003, Heraklion, Crete, Greece. Tel.: 3 0281 0394674; Fax: 3 0281 0392778; E-mail: lena@med.uoc.gr

characteristics and to study spontaneous apoptosis. We additionally studied the effect of known apoptosis-inducing conditions on the cells in respect to time in culture.

Materials and methods

Samples

Bone marrow mononuclear cell (MNC) cultures from children with benign haematological disorders ($n = 10$) and solid tumours without bone marrow involvement ($n = 6$) have been initiated and were maintained in culture for a number of passages (P1–P10). Mean age of the patients was 5.03 ± 1.1 years (median 2.5, range 0.25–15.5 years).

Bone marrow MNC following Ficoll-Hypaque (Lymphoprep-Nycomed $d = 1077$ g/ml) separation were cultured in α -MEM (Invitrogen, Carlsbad, CA, USA) in the presence of 10% lot selected FCS (HyClone Perbio Science, Belgium) and 200 mM L-glutamine and penicillin-streptomycin (10 000 U/ml and 10 mg/ml, respectively; Invitrogen) at cell concentration 50 000 cells/cm² in the presence of 1 ng/ml FGF2 (Abcys SA, Paris, France). Complete medium change was performed twice a week. When layers were > 85% confluent, cells were treated with 0.25% trypsin/1 mM EDTA (Invitrogen) and were replated at 1000 cells/cm² (P1).

Immunophenotypic analysis and assessment of functional characteristics

Flow cytometry. At day 0 of culture and at the different passages, 1×10^5 cells were stained for 20 min with CD45 fluorescein isothiocyanate (FITC), CD34 phycoerythrin (PE), CD146PE, CD95 FITC (BD Biosciences, San Jose, CA, USA), CD105PE (Caltag Laboratories Burlingame, CA, USA). Labelled cells were washed with phosphate-buffered saline (PBS) + 2% FCS + 0.05% sodium azide and analysed on an Epics-Coulter cytometer. In order to assess the proportion of apoptotic MSC, flow cytometry, after 7-amino-actinomycin D staining (7-AAD, Sigma, St Louis, MO, USA), was used. Cells were initially gated according to their morphology (FS/SSC), then a scattergram was generated by combining forward light scatter with 7-AAD fluorescence to quantitate 7-AAD negative (live), 7-AAD low (early apoptotic) and 7-AAD high (late apoptotic/dead) cells (15).

Proliferative capacity. For the CFU-F assay, 1×10^5 bone marrow MNC at day 0 of culture were seeded in each well of a 24-well plate in the culture medium without FGF2. At subsequent passages, MSC were plated at 10 cells/cm² in 20-cm² Petri plates. Following 14 days of culture at

37 °C and 5% CO₂, CFU-Fs were quantified after staining with Giemsa and were categorized according to their size as small CFU-F (S: 50 cells), medium CFU-F (M: 50–500 cells) and highly proliferating CFU-F (L: more than 500 cells). The sum of CFU-F of all sizes is denoted as CFU-F_{tot}. CFU-F results of MSC are presented as number of colonies resulting from 10 MSC/cm² plated as described above.

Cell doubling time (DT) was calculated using the formula:

$$DT = t \times \log(2) / \log(\text{cells harvested} / \text{cells inoculated}),$$

where t was time between initial plating and harvest, for the respective passage.

Multilineage differentiation capacity

Differentiation towards mesenchymal lineages (adipocytes, osteocytes, chondrocytes) – under appropriate stimuli – should be demonstrated in order to characterize any candidate cell as MSC.

MSC were induced to osteocyte differentiation by 3-week incubation in medium supplemented with ascorbic acid-2-phosphate, β -glycerophosphate, dexamethasone and foetal bovine serum. Under such conditions upregulation of alkaline phosphatase (ALP) activity and deposition of calcium-rich mineralized matrix are observed (16).

Adipogenic differentiation of MSC was induced by incubation of MSC in medium containing dexamethasone, isobutylmethylxanthine, and indomethacin. The result is production of cells with large lipid-filled vacuoles (17).

MSC differentiation towards chondrocytes was achieved in serum free medium with the addition of TGF- β 1 in a three-dimensional culture format. Cells rapidly lose their fibroblastic morphology and begin to express a number of cartilage-specific extracellular matrix components (18).

Differentiation was performed at P2 or P3 and verified by histochemical staining with Oil Red O for adipocytes, von Kossa reagent for osteocytes, and alcian blue for chondrocytes. RT-PCR analysis was performed for assessment of various lineage-specific genes (lipoprotein lipase expression for adipocytes; ALP and osteoprotegerin expression for osteocytes; and aggrecan and collagen II expression for chondrocytes). A complete list of primer sequences and their final concentration, expected PCR product sizes and annealing temperatures is given in Table 1.

Cell-cycle analysis

Cell-cycle characteristics were assessed by propidium iodide staining at P2 and P6. MSC were detached by

Table 1. Primer pair sequences for RT-PCR and final concentrations, annealing temperatures and expected product sizes in bp

Gene	Forward primer	Reverse primer	Conc (mM)	Site	Annealing temperature
GAPDH	GGGCTGCTTTTAACTCTGGT	TGGCAGGTTTTTCTAGACGG	50	710	58
Collagen II	AACTGGCAAGCAAGGAGAC	AGTCTCAGGTCTCTGCAGGTG	200	621	58
ALP	CTGGACCTCGTTGACACCTG	GACATTCTCTCGTTCACCGC	100	526	58
LPL	AAAGCCCTGCTCGTGCTGAC	TAAACCGGGCCACATCCTGT	160	406	58
Osteoprotegerin	GACAATTTGCCTGGCACCAA	GTGCGTGCATTAGGCCCTTC	130	373	58
Osteocalcin	CTCCTCGCCCTATTGGCC	CCAACCTCGTCACAGTCCGG	1200	218	58
Aggrecan	ATGCGTGGGTGACAAGGACA	CCTGGAGAAGGAACCGCTGA	200	416	58
PPARgamma2	TTCTCCTATTGACCCAGAAAG	CTCCACTTTGATTGCACTTTG	1600	307	58

enzymatic treatment (trypsin/EDTA 0.25%), centrifuged at 150 *g* for 10 min at 4 °C and washed with PBS. Cells at 1×10^6 were stained with 1 ml propidium iodide staining solution (50 µg/ml propidium iodide, 1 mg/ml RNase in PBS without $\text{Ca}^{++}/\text{Mg}^{++}$, pH 7.4) overnight at 4 °C and DNA flow cytometry was performed. Cells were initially gated according to forward vs. side scatter (FS/SSC) characteristics in order to exclude doublets from the G_2/M region. For each sample, 10 000 events were acquired. Cell-cycle analysis was performed using the WinMDI software version 2.8 (19).

Induction of apoptosis

Addition of anti-Fas antibody. In order to test whether CD95 was functional, induction of apoptosis was triggered ($n = 3$) by addition of an agonistic antibody (anti-Fas clone CH-11, Upstate Biotechnology, Milton Keynes, UK) at concentration of 1 µg/ml under standard MSC culture conditions as described above. Incubation of cells with the antibody lasted for 24 and 48 h. Apoptosis was assessed by 7-AAD staining at these time points in culture supplemented with anti-Fas antibody.

Serum deprivation. Another technique for apoptosis induction was serum deprivation for up to 72 h ($n = 10$). When MSC of either P2 (P2-MSC) or P4 (P4-MSC) were 60–70% confluent, culture medium was changed to either standard or to a medium identical to the standard, except lacking FCS. Cells were trypsinized after 24, 48 and 72 h exposure to either standard or serum deprivation conditions and assessed for apoptosis after 7-AAD staining on an Epics-Coulter cytometer.

Functional characteristics and immunophenotypic analysis after serum-free culture. MSCs cultured under serum-free conditions were sequentially set in standard culture conditions, as described above, for two additional passages and their functional and cell-cycle characteristics and

spontaneous apoptotic events in two experiments, as well as their differentiation potential after exposure to serum-deprived conditions for 72 h, were tested.

MTT assay. MTT assay was performed for assessment of growth inhibition in serum-free culture. MSCs were seeded in 24-well-plates at initial concentration of 3.3×10^3 cells/well and incubated for 72–96 h. Medium was removed and 0.5 ml of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) was added to each well. After incubation for 4 h at 37 °C in a humidified incubator, 0.5 ml of propanol-1 was added to each well and 200 µl were transferred to a 96 well-plate in order to read absorbance at 570 nm, using a microplate reader (20).

Statistical analysis

Statistical analysis was performed using SPSS software (version 13.0) for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables are presented as mean $\pm$ standard error.

Differences between groups were assessed using the non-parametric Mann–Whitney *U*-test. Effect of passage on CFU-F total count, when this parameter was adjusted for age, was evaluated with a general linear model. Correlation between two continuous variables was evaluated using Spearman's rank correlation test. Analysis results were considered significant if probability values were lower than 0.05.

Results

Clonogenicity

Mononuclear cells under standard culture conditions developed 6.24 ± 0.4 total CFU-F colonies/ 10^5 MNCs (d0). P2-MSC formed 38 ± 3.7 CFU-Ftot, but decreased with sequential passages and was found to be 9.6 ± 3.2 at P10 ($p = 0.001$). When evaluating colony growth in different

Figure 1. CFU-F development according to size and passage. Statistical difference compared to P2 was evident for medium- and large-sized CFU-F colonies from P4 and all later passages.

passages, it was observed that the number of small-sized colonies did not vary among the different passages (P2: 7.5 ± 0.99 and P10: 4 ± 1.4 ; $p = 0.07$), in contrast to counts of large- (P2: 3.2 ± 1.0 and P10: 0.2 ± 0.1 ; $p = 0.01$) and medium-sized ones (P2: 27.3 ± 3.2 and P10: 5.4 ± 1.9 ; $p = 0.002$) that declined with progression of passages. This decrease was statistically significant from P4 and onwards, in comparison to P2 (Fig. 1). Thus, medium- and large-sized colonies contributed to final countdown of CFU-F numbers from P4 and thereafter. It should be noted that colony development was heterogeneous among the samples in the same passage.

Apart from clonogenicity and its development with progression of culture, we also examined if and how age of the patient would correlate with size of derived colonies. Negative correlation was observed between age of patients and development of CFU-F_{large} up to P4 (for P4 $p = 0.01$ $r_s = -0.457$), indicating that MSC from younger patients were more clonogenic.

When CFU-F_{tot} was adjusted for age, it was observed that effect of passage on CFU-F number was highly significant ($p = 0.0001$, $r^2 = 0.279$). Same analysis on large, medium and small CFU-Fs provided similar results only for large- ($p = 0.04$, $r^2 = 0.14$) and medium-sized colonies ($p = 0.001$, $r^2 = 0.312$). Finally, when colony growth was correlated to proliferation capacity (DT), it was shown that slowly progressing samples produced less CFU-F_{tot} (for P2 $p = 0.041$, $r_s = -0.375$).

Proliferative rate (doubling time)

Time required by MSC present in the MNC fraction to reach confluency was 17.25 ± 0.82 days, with a range of 11–26 days. Doubling time of MSC increased with progression of passages (P2: 1.93 ± 0.1 , P10: 6.1 ± 2.45 days; $p = 0.01$) and great variability of growth rate was observed in the latest passages. Noteworthy, MSC were

Figure 2. Doubling time and population doublings per passage. Histograms correspond to DT values (in days) and dots to population doublings.

Figure 3. Age of patients from each passage.

found to grow slower from P6 and thereafter, compared to earlier passages and concurrently population doublings decreased in the course of passing (Fig. 2).

The effect of patient age on capacity of the samples to expand was evident from the finding that the younger the patient, the greater expansion capacity was observed (maintenance in culture for up to passage 10) (Fig. 3).

Cell-cycle analysis

Cell-cycle analysis was performed at P2 and P6 at confluence of ~80%. The majority of P2-MSC was in G_0/G_1 phase (G_0/G_1 , $86.7 \pm 1.9\%$; S, $9.4 \pm 1.6\%$; G_2/M , $3.8 \pm 0.3\%$) while at P6 a higher percentage seemed to be in an active state (G_0/G_1 , $80.9 \pm 2.6\%$; S, $12.8 \pm 2.1\%$; G_2/M , $6.4 \pm 0.7\%$; $p = 0.004$). We examined cell-cycle pattern during development of stroma for one passage. Cell-cycle analysis was performed at different time points and more specifically when cells were 40%, 60% and

Table 2. Induction of apoptosis by anti-Fas antibody

	With anti-Fas antibody			Without anti-Fas antibody		
	CD95 (%)	Apoptotic cells (%)	Dead cells (%)	CD95 (%)	Apoptotic cells (%)	Dead cells (%)
24 h	62.0 ± 3.9	4.45 ± 0.55	1.75 ± 0.7	93.9 ± 3.2	3.35 ± 1.1	1.15 ± 0.60
48 h	49.8 ± 2.7	1.90 ± 1.00	3.65 ± 1.4	91.6 ± 1.9	0.95 ± 0.3	2.15 ± 1.05

Table 3. Cell cycles in standard and serum deprived culture conditions

	G ₀ /G ₁		S		G ₂ /M	
	With FCS	Without FCS	With FCS	Without FCS	With FCS	Without FCS
24 h	75.4 ± 3.1	82.4 ± 2.0	16.8 ± 2.0	12.5 ± 1.6	7.0 ± 1.4	4.6 ± 1.1
48 h	84.3 ± 1.4	83.6 ± 2.0	9.6 ± 1.2	9.7 ± 1.4	5.6 ± 0.8	4.3 ± 0.7
72 h	86.3 ± 0.2	83.7 ± 2.7	8.0 ± 0.8	12.4 ± 3.3	5.7 ± 0.7	3.9 ± 0.5

100% confluent. At 40% confluence, 82.6% of cells were in G₀/G₁ phase, 7% in S phase and 10.4%, in G₂/M phase. At 60% confluence 82.2% of cells were in G₀/G₁ phase, 9.9% in S phase and 7.9%, in G₂/M. Finally, when cells were 100% confluent, 92.4% of them were in G₀/G₁ phase, 4.3% at S phase and 3.1% at G₂/M, showing that with progression of stroma formation more MSC entered G₀/G₁ phase.

Spontaneous apoptosis-surface marker expression

A low percentage of spontaneous apoptosis, expressed by assessment of apoptotic (A) and dead (D) cells, was detected by flow cytometry after 7-AAD staining at P2 (A: 2.14 ± 0.5%; D: 1.38 ± 0.44%); this did not change until P10 (A: 1.67 ± 0.52%; D: 2.80 ± 0.95%; $p = 0.3$). A similar pattern was observed in the CD105⁺CD95⁺ population (data not shown).

MSC expressed markers CD105 and CD146 at percentage of >95% as early as P1, while they lacked expression of haematopoietic ones CD34 and CD45 (<2%). This remained constant through all passages examined. MSC also constantly highly expressed CD95 (Fas) molecule through all passages studied (95.2 ± 1.07% at P2 and 90.6 ± 5.6.0% at P10; $p = 0.43$).

Induction of apoptosis

Addition of the agonistic antibody anti-Fas. In order to test whether Fas antigen expression on MSC surface was functional, we assessed the effect of addition of anti-Fas, CH-11 the agonistic antibody, on MSC survival under

standard culture conditions. CD95 expression was 93.9% in the absence of anti-Fas at 24 h and 91.6% at 48 h; the corresponding values after incubation with anti-Fas were 62% and 49.8% at 24 and 48 h, respectively. The proportion of apoptotic cells did not increase following addition of anti-Fas (Table 2) irrespective of duration of incubation of MSC with it (6.2% apoptotic and dead cells in total at 24 h and 5.55% at 48 h).

Serum deprivation. For the next step, since no spontaneous apoptosis had been detected either after a long-term culture for 10 consecutive passages or after induction with anti-Fas agonistic antibody, a further protocol of induction of apoptosis was designed. After 24 h serum deprivation, apoptotic cells were 4.4 ± 1.71%, and the corresponding values for 48 and 72 h were 4.36 ± 1.15% and 5.38 ± 1.0%, respectively ($p = 0.631$, $p = 0.775$, and $p = 0.142$ compared to serum supplemented culture). No statistically significant difference was observed when apoptotic cells after 24 h were compared to those of 48 and 72 h in culture in the absence of serum.

Although apoptosis was not detected in serum-deprived conditions, MTT assay was performed in order to assess if any cell growth-inhibition effect was induced by this condition. The MTT assay revealed 5.5 ± 0.6% at 24 h and 29.4 ± 10.4% at 48 h growth inhibition.

Cell-cycle analysis showed no statistically significant difference between serum-deprived and serum-supplemented cultures (Table 3).

MSC cultured under serum-free conditions for 24, 48 and 72 h were sequentially set in standard culture medium for two additional passages and were tested for their functional and cell-cycle characteristics, and spontaneous

Table 4. DT and CFU-F after serum deprivation and in standard culture conditions following incubation in culture medium without serum

	DT	DT c't 1	CFU-F	CFU-F c't 1
+ FCS	3.7 ± 1.30	4.50 ± 0.96	32.3 ± 5.7	24.8 ± 4.30
– FCS 24 h	3.2 ± 0.57	3.17 ± 1.03	34.7 ± 3.2	27.0 ± 5.70
– FCS 48 h	3.2 ± 0.51	4.80 ± 2.90	19.5 ± 4.6	27.7 ± 10.4
– FCS 72 h	3.4 ± 0.66	2.52 ± 0.81	13.7 ± 4.9	27.0 ± 11.97

c't 1 and c't 2: P1 and P2 cells reset in standard culture conditions after 24, 48 and 72 h incubation in culture medium without serum.

Figure 4. Mesenchymal stromal cells (MSC) deprived of serum for 72 h successfully differentiated (first row), and MSC cultured under standard conditions, used as controls (second row). Undifferentiated MSC (a, e) and MSC differentiated towards adipocytes stained with Oil Red O (b, f), osteocytes (c, g) stained with von Kossa reagent and chondrocytes stained with alcian blue (d, h) are shown.

Figure 5. Molecular analysis of lineage-specific markers in mesenchymal stromal cells (MSC) differentiated after 72 h serum deprivation (w/o), and control MSC cultured in the presence of serum (w). Undifferentiated (U) MSC, adipocytes (A), osteocytes (O) and chondrocytes (C) were analysed. No significant differences were observed in gene expression between cells cultured without serum, compared to those cultured with serum.

apoptotic events, in three experiments. They were also evaluated for their trilineage differentiation ability after culture without serum for 72 h. No difference in growth rate (DT) and proliferative capacity (CFU-F) as well as in spontaneous apoptosis compared to MSC continuously grown in serum-supplemented medium, was observed (Table 4). Cells were also successfully differentiated into adipocytes, osteocytes and chondrocytes (Fig. 4) and expressed lineage-specific markers (Fig. 5).

Cell-cycle pattern was similar to that of cells cultured under standard conditions.

Discussion

Clinical applications of MSCs require their *ex vivo* expansion. This further postulates long-term culture that could probably lead to functional impairment of MSC or to apoptosis. Based on our previous study regarding characteristics of MSC from children, such as clonogenicity, proliferation and differentiation capacity (13), in the present experiments we investigated apoptotic characteristics of MSC throughout their time in culture, as apoptosis is a biological process of any cell type, and we also examined the effect of long-term culture on the main biological features of MSC.

Assessing the effect of time in culture on biological characteristics of MSC, replicative aging of cells, represented by DT increase and CFU-F decrease, was observed during prolonged culture. Clonogenicity declines with

passage progression due to elimination of more primitive cells, depicted by reduction in number of highly proliferating CFU-F colonies, while proliferative capacity also decreases from P4 and thereafter. Additionally, more cells are in non-quiescent state in the more advanced passages, and they need more time to complete the various cell cycle phases with sequential passaging, as shown by increase in cell doubling time, but higher percentage of cycling cells. This shift towards more cycling cells (which is consistent with a population progressively undergoing commitment (21)) is evident following the first three passages (30–45 days in culture). The latter finding differs from those emerging from respective studies that refer to bone marrow MSC from adult donors, in which progressive loss of proliferation is clearly evident after the first passage, and differentiation potential in mesenchymal lineages is only maintained for one additional passage (21).

In the present study, MSC showed no spontaneous apoptosis as indicated by 7-AAD staining. The percentage of apoptotic cells did not increase, even with progression of 10 consecutive passages, which equals approximately 100–150 days of culture. Although MSC highly express CD95, the Fas apoptotic pathway did not seem to trigger apoptosis, since after addition of anti-Fas antibody there was no increase in the apoptotic population.

Moreover, even under conditions that usually cause apoptosis, such as nutrient deprivation, no substantial apoptosis of MSC was noted. The culture was serum free for up to 72 h without any impact on clonogenicity, differentiation potential, proliferative capacity and cell-cycle properties of the cells. This was further confirmed by the following finding: MSC which survived under serum deprivation conditions were sequentially set in standard culture serum-containing medium. They were followed for 2 additional passages and it was observed that they expanded unaffected. Serum-deprived cells were additionally tested for their ability of tri-lineage differentiation and retention of this property was confirmed by histology and molecular analysis. This finding adds more to the investigation of biological features of MSC from the clinical point of view, and is of great importance for cell therapy, which depends on maintenance of viable and functional cells. An ideal MSC transplant would be expanded under conditions devoid of animal products. Addition of serum, an animal derivative, harbours the risk of alloimmunogenicity or transmission of known or unknown animal pathogens to the recipient. Results of this present study could be applied in the transplantation setting, since cells could be temporarily 'washed out' of animal serum for a period of time, a procedure that highly reduces amounts of allogenic proteins in MSCs, before administering them to patients (22).

Serum deprivation has been reported to induce massive human MSC death, when occurring concomitantly with prolonged hypoxia to which they are exposed due to tissue ischaemia, when transplanted *in vivo* (23,24). Results reported in the above studies concern adult bone marrow MSC. In another study by Pochampally *et al.* (25) though, MSC that survived serum deprivation conditions for culture periods of as long as 2–4 weeks, constituted a subpopulation of very early progenitors with enhanced *Oct-4* and other gene expression characteristics of embryonic cells. Enhanced expression of gene characteristic of more primitive cells is currently under investigation. We can speculate that survival after serum withdrawal was prominent in our population, due to the much shorter serum deprivation period and to the fact that MSC derived from bone marrow from children exhibit some qualitative differences compared to their adult counterparts, as has been previously shown (13). Another possible mechanism is that MSC retain some serum proteins in their cytoplasm, explaining maintenance of some effects of serum and their survival (22). Finally, the fact that the culture was FGF-2 supplemented may have resulted in selection of a particular subset of cells with distinct intrinsic features that rendered them resistant to apoptosis under serum-deprived culture conditions comparable to those reported earlier (26) where FGF-2-supplemented bone marrow stromal cell primary cultures initially displayed increase in telomere size followed by gradual decrease.

While further assessing apoptotic behaviour of MSC, high expression of CD95, which is the key molecule for initiation of the Fas-Fas ligand apoptotic pathway, led to addition of anti-Fas antibody to the culture medium, for various time periods, to test functionality of the pathway. Incubation time was longer than has been reported in the literature (27). No substantial apoptotic effect was observed, a result suggesting that this pathway is not effective in mesenchymal stromal cells, at least in standard culture conditions.

As for functionality of MSC derived from bone marrow of children, findings of the present study indicate that the younger the age of the patient, the easier it is for the cells to expand and even be maintained in culture for a longer period.

Interestingly, although no statistically significant correlation was evident between total number of CFU-F and age of the donor, a tendency was observed towards a higher number of CFU-F in younger patients in all passages and additionally MSC of younger patients gave more large-sized CFU-F, which are considered to be derived from more primitive cells. In addition, when the number of CFU-F was adjusted for age, effect of passage on CFU-F number was highly significant. From the present study, it was also evident that slowly progressing samples (high DT) produce less CFU-F.

The above results provide evidence that MSC from younger patients and from earlier passages (preferably P2) can be considered better candidates for clinical use in transplantation compared to MSC from adult donors, as they can be maintained in an earlier progenitor state, longer.

In conclusion, it seems that MSC from children retained their functional characteristics throughout serial passages and were very stable under conditions that can usually cause apoptosis, although progression in passages resulted in lower number of cells maintaining quiescence. This could be very useful in the setting of transplantation, together with the finding that elimination of serum from culture medium for extended periods of time did not affect the functional characteristics of *in vitro* expanded MSC. These features make bone marrow-derived MSC from children, especially those of early passages, optimal candidates for use in transplantation and cell therapy.

Acknowledgements

This work was supported in part by the GENOSTEM (EU-FP6 Integrated Project). We thank Helen Koutala for technical advice and support in flow cytometry.

References

- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
- Gerson SL (1999) Mesenchymal stem cells: no longer second class marrow citizens. *Nat. Med.* **5**, 262–264.
- Herzog EL, Chai L, Krause DS (2003) Plasticity of bone marrow derived stem cells. *Blood* **102**, 3483–3493.
- Kassem M (2004) Mesenchymal stem cells: biological characteristics and potential clinical applications. *Cloning Stem Cells* **6**, 369–374.
- Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S (1998) Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *J. Orthop. Res.* **16**, 155–162.
- Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY *et al.* (2002) Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc. Natl. Acad. Sci. USA* **99**, 8932–8937.
- Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM *et al.* (2007) Cotransplantation of *ex vivo* expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* **110**, 2764–2767.
- Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J *et al.* (2007) Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* **8**, 1733–1738.
- Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M *et al.* (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* **363**, 1439–1441.
- Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H *et al.* (2006) Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**, 1390–1397.
- Rubio D, Garcia-Castro J, Martín MC, de la Fuente R, Cigudosa JC, Lloyd AC *et al.* (2005) Spontaneous human adult stem cell transformation. *Cancer Res.* **65**, 3035–3039.
- Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A *et al.* (2007) Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res.* **67**, 9142–9149.
- Dimitriou H, Linardakis E, Martimianaki G, Stiakaki E, Perdiko-gianni Ch, Charbord P *et al.* (2008) Properties and potential of bone marrow mesenchymal stromal cells from children with hematologic diseases. *Cytotherapy* **10**, 125–133.
- Locatelli F, Maccario R, Frasson F (2007) Mesenchymal stromal cells, from indifferent spectators to principal actors. Are we going to witness a revolution in the scenario of allograft and immune-mediated disorders? *Haematologica* **92**, 872–877.
- Lecoeur H, Ledru E, Prevost M-C, Gougeon M-L (1997) Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin V and 7-AAD cytofluorometric staining methods. *J. Immunol.* **209**, 111–123.
- Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD *et al.* (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* **92**, 4857–4861.
- Liu J, Farmer SR (2004) Regulating the balance between peroxisome proliferator-activated receptor γ and β -catenin signaling during adipogenesis. A glycogen synthase kinase 3 β phosphorylation-defective mutant of β -catenin inhibits expression of a subset of adipogenic genes. *J. Biol. Chem.* **279**, 45020–45027.
- Li F, O'Keefe RJ, Chen D (2005) TGF-beta signaling in chondrocytes. *Front. Biosci.* **10**, 681–688.
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271–279.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63.
- Banfi A, Muraglia A, Dozin B, Mastrogiacomio M, Cancedda R, Quarto R (2000) Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Exp. Hematol.* **28**, 707–715.
- Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ *et al.* (2004) Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol. Ther.* **9**, 747–756.
- Zhu W, Chen J, Cong X, Hu S, Chen X (2006) Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Stem Cells* **24**, 416–425.
- Potier E, Ferreira E, Meunier A, Sedel L, Logeart-Avramoglou D, Petite H (2007) Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng.* **13**, 1325–1331.
- Pochampally RR, Smith JR, Ylostalo J, Prockop DJ (2004) Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* **103**, 1647–1652.
- Bianchi G, Banfi A, Mastrogiacomio M, Notaro R, Luzzatto L, Cancedda R *et al.* (2003) *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp. Cell Res.* **287**, 98–105.
- Maurillo L, Del Poeta G, Venditti A, Buccisano F, Battaglia A, Santinelli S *et al.* (2001) Quantitative analysis of Fas and bcl-2 expression in hematopoietic precursors. *Haematologica* **86**, 237–243.