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Expansion of Mesenchymal Stem Cells Isolated From Pediatric and Adult Donor Bone Marrow

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Abstract The enormous plasticity of mesenchymal stem cells (MSCs) suggests an improvement of a standard protocol of isolation and ex vivo expansion for experimental and clinical use. We isolated and expanded MSCs from bone marrow (BM) of pediatric and young adult donors, to analyze the growth kinetic, immunophenotype, telomere length, karyotype during ex vivo expansion. Seventeen BM samples were collected from young adult donors and 8 from pediatric donors. MSCs isolated from two groups showed no morphological differences while their cell growth was strictly related to the donor's age. The MSCs isolated from pediatric donors reached a cumulative PD almost twice as high as MSCs isolated from young adult donors after 112 days (10.2 ± 1.9 versus 5.5 ± 3.7). Furthermore, we analyzed the modulation of antigen expression in the MSCs isolated from two groups until 10th passage (77 days) and there was no significant difference between the modulation of antigen expression. In particular, at the first passage, MSCs showed a low contamination of hemopoietic cells which became insignificant in the following passages. There was a high expression of CD90, CD29, CD44 and CD105 and variable and moderate expression of CD166 and CD106 at the start of MSC culture and at each passage during expansion. No chromosomal alteration or evidence of cellular senescence were observed in all analyzed samples. All these data suggest that MSCs can be isolated and expanded from most healthy donors, providing for an autologous source of stem cells. J. Cell. Biochem. 97: 744–754, 2006.

Key words: mesenchymal stem cells; expansion; pediatric donor; adult donor

Bone marrow (BM) contains two different stem cell populations: those of the hemopoietic lineage, hemopoietic stem cells (HSCs), which reconstitute the hemopoietic system with all blood cell lineages, and mesenchymal stem cells (MSCs), mesoderm precursors which normally differentiate into multiple mesodermal tissue, including: bone [Haynesworth et al., 1996]; cartilage [Yoo et al., 1998]; adipose [Park et al., 1999]; muscle [Wakitani et al., 1995]; tendon [Awad et al., 1999]; stroma [Majumdar et al., 1998]; and neuron cells overcoming their germinal commitment [Kopen et al., 1999; Brazelton et al., 2000; Black and Woodbury, 2001; Kim et al., 2002; Woodbury et al., 2002].

Although present in very low numbers in BM, MSCs are easily isolated from BM as they can adhere to plastic and are capable of substantial proliferation and expansion in culture [Pittenger et al., 1999]. Undifferentiated MSCs exhibit a fibroblastic like morphology and a characteristic pattern of cell-surface antigens maintaining the ability to differentiate into multiple cell types, establishing their stem cell nature [Mareschi et al., 2001]. They can be numerically expanded in vitro and cryopreserved with no loss of phenotype or differentiation potential [Lazarus et al., 1995; Bruder et al., 2000]. Moreover, MSCs produce several cytokines, growth factors, and cell adhesion molecules; important factors which influence the hemopoietic microenvironment [Eaves et al., 1991; Haynesworth et al., 1992; Majumdar et al., 1998]. They constitute an important component of the myelosupportive stroma which is damaged by the conditioning regimen for stem cell transplantation and preliminary results suggest that co-transplantation with expanded MSCs resulted

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in faster engraftment and a reduction in the incidence and severity of graft-versus-host in some patients [Lazarus et al., 2005].

Ex vivo expanded cells undergo several divisions which might induce excessive cellular senescence, therefore determination of the aging is relevant before an extensive clinical application of ex vivo manipulated MSCs [Hodes, 1999]. Progressive shortening of telomere length has been suggested as acting as a mitotic clock which may contribute to cellular senescence and as a good predictor of the remaining replicative capacity of the somatic cells [Vaziri et al., 1994].

Previous studies have shown that human MSCs expanded in vitro tend to lose their proliferative potential, homing capacity, and in vivo bone forming efficiency aging [Mets and Verdonk, 1981; Digirolamo et al., 1999; D'Ippolito et al., 1999; Banfi et al., 2000; Rombouts and Ploemacher, 2003; Stenderup et al., 2003].

From these observations it is clear that different variables and parameters must be considered in the isolation and expansion of MSCs for experimental and clinical use.

Several studies were performed on the age-related effect on the number, cellular growth, and biological characteristics of osteoprogenitor cells identified as MSC or stromal cells. Some studies reported an age-related decrease in the number of osteoprogenitor cells, while other studies showed no effects or an age-related increase [Brockbank et al., 1983; Xu et al., 1983; Tsuji et al., 1990; Egrise et al., 1992; Quarto et al., 1995; Bergman et al., 1996; Oreffo et al., 1998; D'Ippolito et al., 1999; Nishida et al., 1999; Stenderup et al., 2001]. Stenderup et al. [2003] demonstrated that aging is associated with a decreased proliferate capacity of MSCs but not with a function capacity. Recently, Baxter et al. [2004] reported the effect of in vitro expansion on the replicative capacity of MSCs isolated from pediatric and adult (59–75 years aged) donors on the basis of the telomere loss during the in vitro expansion. These studies were all performed on MSCs isolated from adult donors, which were divided into young and old (over 50 years of age) adult donors.

In this study, we isolated and analyzed MSCs from healthy donor bone marrow (BM) to analyze the modulation of the immunophenotypic characteristics, telomere length, and karyotype modifications during their cellular expansion and to verify whether MSCs isolated from pediatric and young adult donors showed any differences.

MATERIALS AND METHODS

Harvest and Preparation of MSCs

BM cells were harvested from the iliac crest of adult or pediatric Caucasian donors who underwent BM collection for a related patient after informed consent. For our experiments, we used an unfiltered BM collection bag (Baxter Healthcare Corporation, IL) which was normally discarded before the BM infusion. The bag was washed three times with phosphate buffer saline (PBS) 1× (Cambrex Bioscience, Versviers, Belgium) and the cells were collected at 900*g* for 10 min. The cells were then layered on a Percoll (Sigma Aldrich, St. Louis, MO) gradient (density: 1.073 g/ml) and centrifuged at 1,100*g* for 30 min, according to a previously reported method (17). The cells in the interphase were recuperated, washed twice with PBS 1× (200*g* for 10 min) and seeded at a density of 800.000/cm^2 in alfa-MEM (Cambrex Bioscience) at 10% of fetal bovine serum (FBS) in 75 cm^2 T-flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and maintained at 37°C with an atmosphere of 5% CO_2. After 3 days, the nonadherent cells were removed and re-feed every 3–4 days.

In order to expand the isolated cells, the adhered monolayer was detached with trypsin/EDTA (Cambrex Bioscience) for 5 min at 37°C, after 15 days for the first passage and every 7 days for successive passages. During in vitro passaging, the cells were seeded at a density of 8.000/cm^2 and expanded for several passages until they no longer reached confluence.

Analysis of MSCs

At each passage the cells were counted and analyzed for cellular growth, viability, and immunophenotype analysis by cytofluorimetric analysis. The evaluation of telomere length and the karyotype analysis was also performed.

Cellular Expansion

Cellular expansion growth rate was evaluated by cell count in a Burker Chamber at each passage and expressed in terms of population doubling (PD) as performed in the studies reported by Stenderup et al. [2003].

Cytofluorimetric Analysis of MSCs

The identification of adherent cells was performed by flow cytometry analysis. At each passage 200–500 cells were stained for 20 min with anti-CD45 fluoroisothyocyanate (FITC), CD14 phycoerytrin (PE), CD90FITC, CD106-PE, CD29FITC, CD44PE, CD105PE, CD166F-ITC, and with 7-aminoactinomycin D (AAD) (Becton Dickinson, San Jose, CA) for the viability. Labeled cells were thoroughly washed with PBS 1× and were analyzed on a Epics XL cytometer (Beckman Coulter, CA) with the XL2 software program. The percentage of positive cells was calculated using the cells stained with Ig FITC/PE as a negative control.

Evaluation of Telomere Length

In order to determine senescence during culture, telomere length was analyzed at each passage by flow fluorescence in situ hybridization (Flow-FISH) [Rufer et al., 1998]. Two hundred thousand expanded MSCs were labeled with a telomere-specific conjugated $(C_3TA_2)_3$ peptide nucleotide acid (PNA) probe (Perseptive Biosystems, Framingham, MA) and the same number were analyzed as a control without a probe. The same number of hemopoietic stem cells (HSCs) was isolated with an immunomagnetic system using CD34 microbeads conjugated antibody from BM at the moment of collection and used as examples of long telomeres. Stained cells were analyzed on a FACS Calibur cytometer (Beckon Dickinson). At the beginning of each experiment, the fluorescence signals from four different populations of FITC-labeled microbeads (Quantum-TM-24 FITC Premix, Flow Cytometry Corporation, IN) were acquired. The voltage and amplification of the FL1 parameter were set in such a way that blank, 6,288, 12,825, and 26,904 molecular equivalents of soluble fluorochrome (MESF) units per bead corresponded to channel numbers ranging from 10 to 15, 150 to 160, 450 to 460, 800 to 820, respectively, in the FL1 channel on a linear scale. The resulting calibration curve ($y = 0.029x$) was used to convert telomere fluorescence into MESF units, to compare the experiment results.

Karyotype Analysis

MSCs were cultivated overnight with fibroblast growth factor (FGF) (Sigma Aldrich), 10 ng/ml and Colcemid (Invitrogen Corporation, Grand Island, NY) 100 ng/ml for 24 h before harvesting with trypsin-EDTA. The cells were then lysed with hypotonic KCL and fixed in a solution of methanol/acetic acid (3:1). Fifty metaphases were analyzed after GTG banding using MackType software (Nikon Corporation, Japan).

Statistical Analysis

Statistical analysis was performed using Statistica StatSoft, version 6.0, software. Data were assessed using Student's *t*-test, used to compare the means of the two groups of adults and pediatric donors.

RESULTS

Study Subject

Seventeen BM samples were collected from donors (6 male and 11 female) over 18 years of age (range of age: 20–50 years) and 8 (4 male and 4 female) with ages younger than 18 years (range of age: 2–13 years). All donors had no evidence of any concurrent illness and were not receiving any medications that could affect bone. The study was conducted according to the Helsinki Declaration.

Isolation of MSCs

Adherent cells were observed in all samples after 3 days' culture and in the following 15 days an adherent monolayer was achieved. BM cells rapidly generated a confluent layer of cells possessing an elongated, fibroblastic shape. No morphological differences were observed on the MSCs isolated from adult and pediatric donors, but when early passage cells were compared with late passage cells, MSCs showed a different morphology. The cells increased in size and showed a polygonal morphology with evident filaments in the cytoplasm especially when isolated from the adult donors.

Analysis of MSCs

MSCs isolated from pediatric donors were expanded for a median of 20 passages (range: 20–27 passages), while MSCs from adult donors were expanded for a median of 18 passages (range: 6–26 passages) before the growth stopped. The cell growth of expanded in vitro MSCs was strictly related to the donor's age. In fact, in vitro expanded MSCs isolated from pediatric donors showed a median fold increase of 3.1 (range: 1.6–4.6), 1.5 (range: 1.0–2.4), 1.3

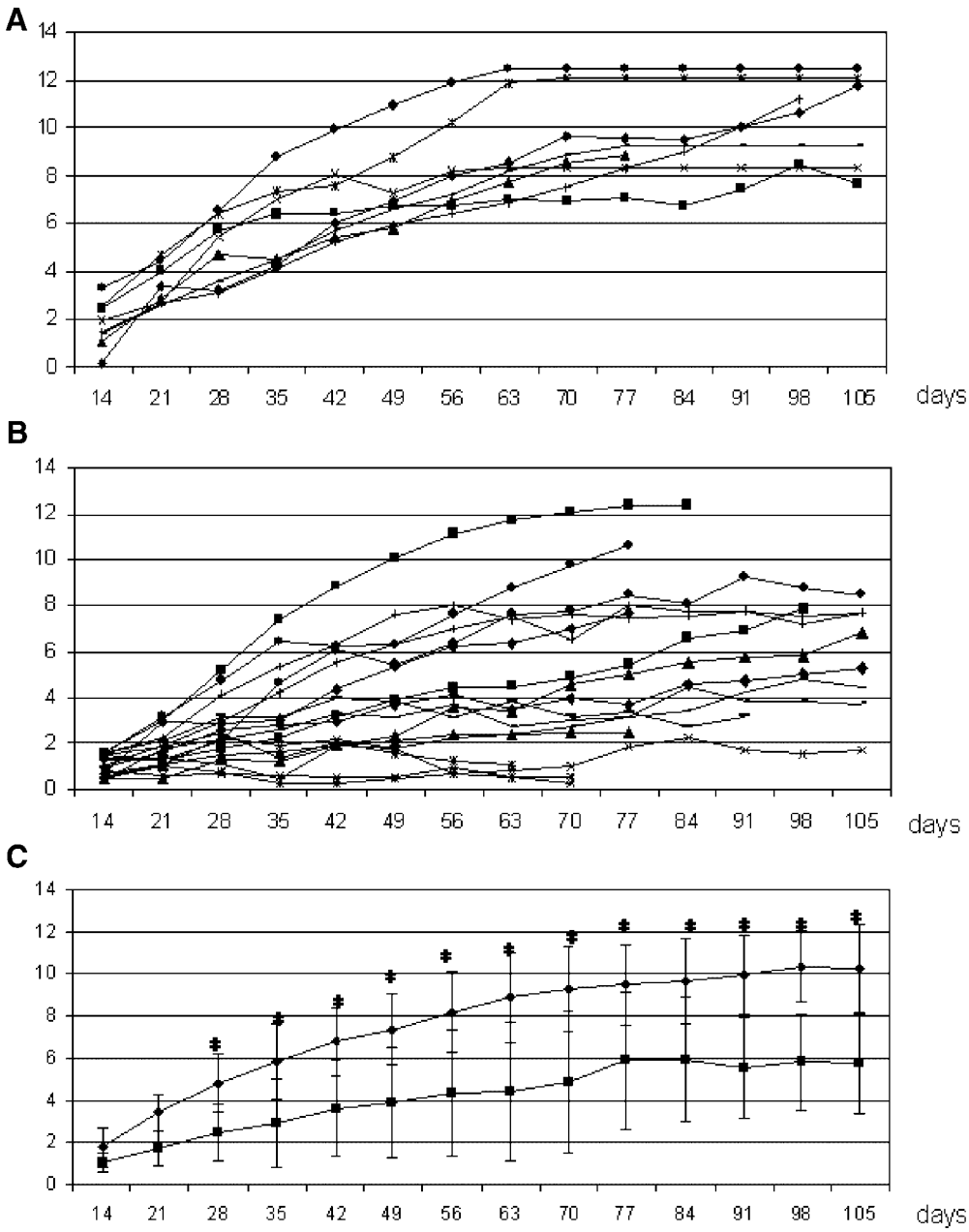

Fig. 1. Growth kinetics of human MSCs culture showing cumulative PD as a function of time in culture. **A**: Long-term growth curves each obtained from an individual pediatric donor (n = 8). **B**: Long-term growth curves each obtained from an individual adult young donor (n = 17). **C**: Mean values of pediatric (●) and adult donors (■). Each point represents mean ± SD. $^*P < 0.05$. PD, population doubling; bp, base pair.

(range: 0.2–1.9) at each passage after 5, 10, 15 passages, respectively, and reached a mean number of cumulative PDs at the 10th passage of 9.5 ± 1.9 and at the 15th passage of 10.3 ± 2.0. In the MSCs isolated from adult donors, the median fold increase at each passage was of 1.8 (range: 0.9–3.9), 1.5 (range: 0.9–2.4), 1.1 (range: 0.2–1.5) after 5, 10, and 15 passages, respectively, while the cumulative PD after 10 passages was 5.9 ± 3.2 and after 15 passages, 6.2 ± 2.7. The growth curves expressed in cumulative PD established for each donor in pediatric (A) and in adult (B) group are given in Figure 1. The means with standard deviations (Fig. 1C) of the two groups showed a showed a significant statistic difference ($P < 0.05$).

TABLE IA. Median Values of the Antigens Expression (With Ranger Expressed in Percentage) of MSCs Isolated From Pediatric Donors and Analyzed at Each Passage

	CD45+	CD14+	CD90+	CD106+	CD166+	CD29+	CD44+	CD105+
Passage 1	3.66% (0.88–16.44)	2.20% (1.45–2.94)	70.75% (43.80–97.70)	8.37% (4.53–12.20)	10.67% (6.02–15.32)	71.75% (45.50–98.00)	97.85% (97.80–98.20)	97.95% (97.60–98.30)
Passage 2	6.65% (0.52–15.90)	8.42% (0.97–17.40)	95.05% (68.20–96.60)	24.40% (1.93–70.00)	8.21% (1.87–72.40)	82.75% (57.10–98.20)	98.75% (72.90–99.90)	96.25% (89.90–99.50)
Passage 3	1.41% (0.09–9.67)	2.56% (0.43–6.66)	96.95% (51.20–99.70)	63.40% (0.27–75.50)	17.70% (0.49–71.00)	87.30% (81.40–99.30)	91.35% (41.96–99.80)	96.20% (84.10–100.00)
Passage 4	4.42% (0.02–16.10)	4.08% (0.27–23.40)	84.65% (72.36–92.30)	36.90% (0.69–66.20)	36.30% (0.39–92.50)	85.20% (79.96–96.20)	90.85% (39.30–98.30)	83.75% (74.10–95.70)
Passage 5	4.75% (0.14–15.39)	1.70% (0.18–19.52)	99.20% (82.70–99.70)	27.30% (0.45–98.30)	29.00% (2.32–73.50)	98.65% (82.50–99.40)	97.50% (84.60–99.70)	92.65% (83.90–99.60)
Passage 6	1.01% (0.05–22.70)	8.47% (0.71–20.90)	89.30% (73.20–99.70)	20.72% (0.90–98.30)	13.80% (1.55–56.90)	84.80% (54.70–89.80)	93.75% (84.60–99.70)	81.40% (64.80–99.40)
Passage 7	3.50% (0.76–10.36)	3.40% (0.32–18.70)	98.60% (89.20–98.60)	32.50% (0.80–91.90)	31.30% (4.67–60.30)	89.80% (43.80–95.80)	96.60% (96.00–99.20)	98.00% (70.20–99.30)
Passage 8	3.17% (0.35–10.99)	2.54% (1.10–13.97)	83.85% (77.90–89.80)	27.81% (2.92–52.70)	23.86% (3.42–44.30)	92.70% (89.00–93.60)	91.95% (89.30–94.60)	92.80% (89.70–95.90)
Passage 9	4.23% (0.29–13.10)	4.22% (2.07–7.99)	95.80% (48.80–99.30)	15.23% (2.78–54.30)	9.72% (2.00–82.70)	84.20% (33.90–95.00)	98.85% (97.30–99.20)	97.30% (96.80–97.40)
Passage 10	4.32% (0.06–11.25)	4.50% (2.00–9.12)	99.00% (47.90–100.00)	20.60% (2.90–52.60)	32.30% (3.25–89.60)	80.50% (35.20–98.30)	99.20% (93.56–99.80)	98.70% (97.10–99.20)

TABLE IB. Median Values of the Antigens Expression (With Ranger Expressed in Percentage) of MSCs Isolated From Young Adult Donors and Analyzed at Each Passage

	CD45+	CD14+	CD90+	CD44+	CD105+	CD106+	CD166+	CD29+
Passage 1	15.45% (1.55–29.50)	12.67% (1.40–2.90)	95.85% (46.10–98.00)	95.40% (92.40–99.30)	96.10% (92.2–98.50)	23.20% (1.90–75.00)	67.95% (3.67–78.50)	93.00% (63.20–98.90)
Passage 2	3.05% (0.10–11.70)	9.08% (0.13–29.8)	94.60% (74.30–99.10)	97.70% (54.50–99.10)	96.70% (8.16–99.30)	36.80% (3.88–96.70)	21.30% (1.62–61.60)	88.30% (8.72–97.40)
Passage 3	3.59% (0.06–6.61)	5.69% (0.32–6.9)	93.70% (74.5–99.3)	98.90% (80.64–99.60)	93.90% (68.80–99.70)	22.05% (2.55–63.10)	19.00% (1.58–57.00)	91.20% (1.92–97.30)
Passage 4	2.24% (0.05–5.63)	2.73% (0.16–4.57)	95.80% (74.1–98.60)	98.30% (74.20–99.60)	96.15% (73.40–99.30)	17.20% (9.54–31.40)	11.70% (2.72–33.30)	77.50% (69.6–96.8)
Passage 5	2.40% (0.00–4.89)	2.50% (0.23–5.06)	85.30% (42.40–96.10)	93.85% (81.20–93.20)	89.90% (83.20–94.40)	20.70% (9.3–41.50)	11.94% (2.53–18.56)	75.80% (35.00–97.70)
Passage 6	2.42% (0.05–6.80)	2.53% (0.14–6.43)	95.60% (55.60–99.70)	97.5% (40.00–99.10)	95.10% (68.80–99.30)	17.60% (8.18–60.40)	25.33% (1.30–64.20)	77.35% (32.30–98.40)
Passage 7	3.97% (0.02–6.75)	4.12% (4.12–6.06)	96.50% (95.70–99.50)	98.30% (98.30–99.70)	94.70% (94.70–98.40)	8.48% (7.13–52.12)	20.43% (2.23–51.32)	86.00% (33.20–97.80)
Passage 8	0.04% (0.00–7.02)	0.40% (0.06–6.93)	84.40% (77.20–95.50)	96.00% (94.53–98.20)	93.7% (79.70–95.60)	4.99% (9.30–48.23)	29.80% (1.89–45.60)	78.30% (34.32–98.21)
Passage 9	0.64% (0.00–1.23)	0.81% (0.20–2.32)	86.70% (83.20–85.96)	97.60% (95.32–98.69)	93.30% (81.40–98.20)	4.28% (4.55–15.52)	6.26% (2.03–11.30)	76.90% (29.53–89.23)
Passage 10	0.50% (0.05–1.30)	0.42% (0.03–1.020)	86.90% (83.26–86.96)	94.50% (92.35–97.32)	92.00% (93.53–99.6)	10.80% (4.32–19.23)	7.27% (3.05–12.36)	78.63% (32.69–92.36)

Viability Evaluation

Trypan blue staining analysis showed a viability of between 98% and 100% in all samples analyzed with no differences between the two groups. The same results were confirmed after 7AAD staining in cytofluorimetric analysis.

Immunophenotype Analysis by Flow Cytometry

During the first 10 passages, the cells were analyzed at each passage for the expression of: CD45 and CD14, hemopoietic surface antigens; CD90 (a membrane glycoprotein, also called Thy-1), used as a stem cell marker; CD29, the β subunit of the fibronectin receptor; CD44, receptor-III of extracellular matrix; CD105 or endoglin; CD166 and CD106, cell adhesion molecules.

Tables IA and IB show the median values of the antigen expression (with ranges) of MSCs isolated from pediatric and adult donors, respectively, and analyzed at each passage.

At the first passage, MSCs isolated from pediatric donors were CD45, CD14 negative because antigen expression was less than 5% (the median was 3.66% with a range of 0.88%–6.44%, and 2.20% with a range of 1.45%–2.94%, respectively), while they showed a high expression of CD90 (median of 70.75%, range: 43.80%–97.70%), CD29 (median of 71.75%, range: 45.50%–98.00%), CD44 (median of 97.85%, range: 97.80%–98.20%), and CD105 (median of 97.95%, range: 97.60%–98.30%) and a low expression of CD106, CD166 adhesion molecules (median of 8.37% with a range of 4.53%–12.20% and 10.67% with a range of 6.02%–15.32%, respectively). During the expansion time, the MSCs were negative for the hemopoietic antigen while at each passage they expressed high percentages of CD90, CD29, CD44, and CD105 positive cells with the median antigen expression being over 80% (Fig. 2A).

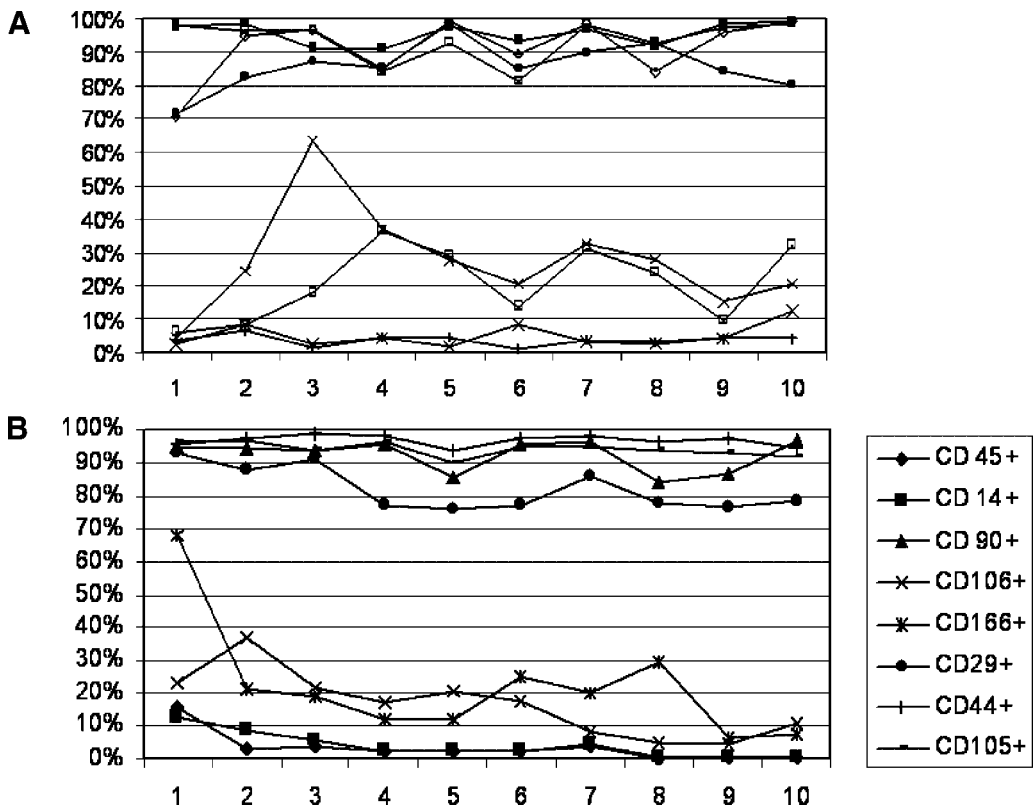

Fig. 2. Modulation of median values of antigen expression analyzed at each culture passage during expansion. Median values of immunophenotypic antigens in MSCs isolated from pediatric (**A**) and young adults (**B**) during expansion from the 1st to the 10th passages are indicated. The percentage of positive cells was calculated using the Ig FITC/PE stained cells as a negative control.

At first passage, the MSCs isolated from young adult donors showed a low contamination of hemopoietic cells (a median of 15.45% with a range of 1.55%–29.50% of CD45 and 12.67% with a range of 1.40%–32.90% of CD14 positive cells) which decreased in the following passages. A high level of CD90, CD44 and CD105, and CD29 was expressed from these cells with a median of 95.85% (range: 46.10%–98.00%), 95.40% (range: 92.40%–99.30%), and 96.10% (range: 92.20%–98.50%) at the first passage and remained high throughout the whole period of expansion as shown in Figure 2B. The expression of CD106 and CD166 antigens was variable during the expansion in both groups. There was no significant difference between the modulation of antigen expression in the MSCs isolated from the two groups.

In one only sample (MSC culture from an adult donor) was the hemopoietic contamination persistent until the 10th passage and this sample was excluded from the analysis of the modulation of the antigen expression.

Evaluation of Cellular Senescence

Ten MSC samples (7 from pediatric and 3 from adult donors) were analyzed for cellular senescence after 10 cumulative PDs in the expansion. The mean telomere length at the first PD showed no significant differences between MSCs isolated from pediatric and adult donors with a mean of 10.11 ± 0.5 kb or of 10.30 ± 0.70 kb. After 10 PDs, the mean telomere length was 8.36 ± 0.75 kb and 9.2 ± 1.4 kb with a mean shortening value of 1.8 ± 0.6 and of 1.1 ± 0.8 ($P = 0.3$) in pediatric and adult donors, respectively.

In four samples (three pediatric donors and one adult donor), the telomere length at the first passage obtained by Flow-FISH was compared with the telomere length of CD34 positive cells sorted at the moment of BM collection and the telomere length of CD34 positive cells were comparable with or slightly lower than the MSCs at the start of the culture. In these samples, the median value of telomere length at the 1st passage was 10.22 ± 0.43 kb against a median value of CD34+ cells of 9.16 ± 1.18 kb. During MSC expansion, the telomere shortening was 1.8 ± 0.17 kb (mean value) after 10 cumulative PDs. There was a significant difference between CD34+ cells and MSCs after 10 PDs in a group of three pediatric donors ($P = 0.024$). The shortening trend was not always linear and in one sample during the expansion, the telomere length increased by 2.3 kb. The increase of the telomere length was noted in one sample where the MSCs were isolated and expanded from a 2-year-old child and the karyotypic analysis of this was normal at the 10th passage. Figure 3 shows the modulation of telomere shorting, expressed in bp, in relation with cumulative PD during the expansion in pediatric (A) and adult (B) donors.

Karyotype Analysis

Five samples were analyzed at the 2nd, 5th, and 10th passage and no chromosomal alteration was noted. Two samples analyzed at 10th expansion passage did not show a sufficient number of metaphases to perform the analysis.

DISCUSSION

On the basis of their capacity to adhere to plastic, we isolated MSCs from BM of pediatric and young donors using a Percoll gradient and expanded them in alfa-MEM + 10% FBS without adding growth factor. As we previously reported [Lazarus et al., 2005], these multipotent cells had substantial proliferation and expansion in culture and did not differentiate spontaneously during culture expansion, but did differentiate in adipocytes, chondrocytes, and osteoblasts when they grew in lineage-specific culture conditions.

Our results showed that BM MSCs generated an adherent layer initially formed by individual cells or colonies composed of a few fibroblast-like cells, which rapidly reached confluence. We observed an exponential growth of these cells with 98% of viability at each passage and noted that cell growth of expanded in vitro MSCs was strictly related to the donor's age. Indeed, the MSCs isolated from pediatric donors reached a cumulative PD almost twice as high as MSCs isolated from young adult donors after 112 days (10.2 ± 1.9 vs. 5.5 ± 3.7). In the successive passages, MSCs isolated from adult donors increased in size and showed a polygonal morphology and the different morphology was related to a decreasing proliferation capacity (data not shown).

Our data are in accordance with a series of studies reporting a negative correlation between donor age and the number and the proliferative capacity of MSCs isolated from

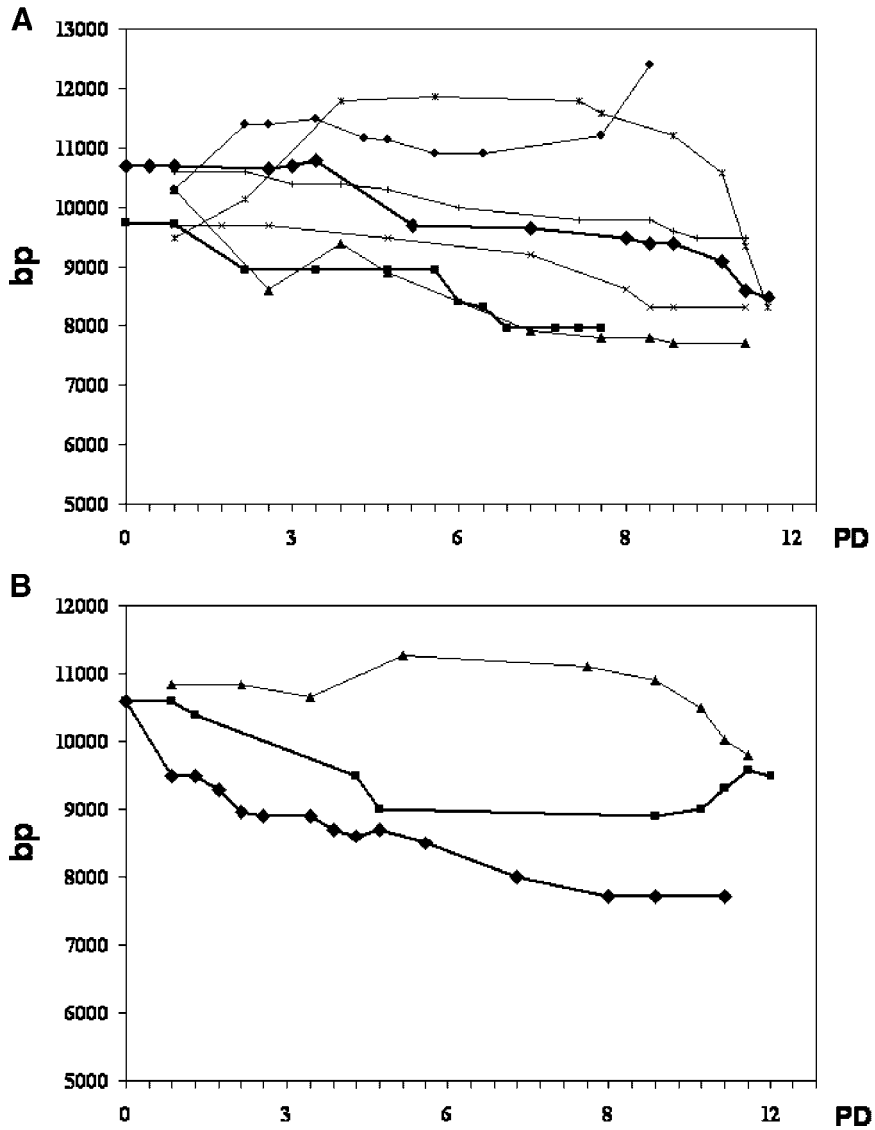

Fig. 3. Telomere length of human MSCs during in vitro expansion. Each point represents telomere length at each passage in relation to cumulative PD. **A**: Telomere length expressed by bp in pediatric MSCs (n = 7). **B**: Telomere length expressed by bp in adult MSCs (n = 3). PD, population doubling; bp, base pair.

young and old donors in short-term early passage cultures [Martin et al., 1970; Schneider and Mitsui, 1976; Majors et al., 1997; D'Ippolito et al., 1999; Nishida et al., 1999; Stenderup et al., 2003]. However, there are no studies on the modulation of MSCs isolated from pediatric and young adult donors.

Furthermore, we analyzed the modulation of antigen expression in the MSCs isolated from two groups until 10th passage (77 days). At the first passage, MSCs showed a low contamination of hemopoietic cells which became insignificant (<5%) in the following passages during expansion. There was a high expression of CD90, CD29, CD44, and CD105 at the start of MSC culture and at each passage because the antigen expression median was always more than 75%. The antigens CD166 and CD106, and adhesion molecules, showed a variable and moderate expression during the expansion. As far as antigens are concerned there was no significant difference between the modulation of antigen expression in the MSCs isolated from the two groups. It was also noted that hemopoietic contamination was more frequent in adult donor MSCs. Pediatric donor MSCs

probably have a higher proliferative advantage than adult donor MSCs.

Extensive proliferation, however, can induce cells senescence, which was analyzed by telomere length measurement by Flow-Fish. This cytofluorimetric method was validated and compared with the molecular method by Rufer et al. [1998] and has the additional advantage that the analysis can also be made on a small quantity of cells. Telomere length between the two groups was similar at the start and its shortening during expansion was not significantly different, data which differed from our expectations and from Baxter et al. [2004]. They, in fact found that mean telomere restriction fragment (mTRF) in pediatric MSCs was significantly longer than adult MSCs. Adult donor age, Baxter's study, was between 59 and 75 years, with an age range much higher than our donors. We analyzed telomere shortening from cells at growth arrest after about 10 cumulative PDs, which was the minimal expansion of MSCs used for transplantation in Osteogenesis Imperfecta [Horwitz et al., 1999] and there was significant difference between the two groups (1.8 ± 0.6 and 1.1 ± 0.8 in pediatric and adult donors). As shown by Baxter et al. [2004], an equal number of PDs in vitro causes equivalent telomere erosion regardless of donor age. In one sample of the pediatric group, during expansion, the telomere length increased by 2.3 kb. One limit of our study is the low number of analyzed samples. However, comparing the telomere shorting with cumulative PD, we found that the average total loss at each cellular division was less than 200 bp in each group and these data can be correlated with a physiological phenomenon, as reported by different groups [Harley et al., 1990; Allsopp et al., 1992] and, more recently, by Gammaitoni et al. [2004]. The increase of the telomere length (but not karyotypic alteration) observed in only one sample, where the MSCs were isolated and expanded from a 2-year-old child, might be due to the immature state of these cells.

Recent studies [Serakinci et al., 2004; Rubio et al., 2005] have show the human adult mesenchymal stem cells (MSCs) can become neoplastic cells after long-term in vitro culture. Serakinci et al. [2004] analyzed the expression of the transformed phenotype during long-term culture of three different cell lines derived from MSCs transduced with the telomerase hTER gene at various PD levels. The authors showed that the MSCs acquired gene aberration and formed tumors when implanted subcutaneously in immune-deficient mice, only after reaching elevated PD levels (256 PD). On the other hand, Rubio's more alarming study reported a spontaneous transformation of MSCs which, after spontaneously bypassing the senescence phase, accelerated the cell cycle rate compared with pre-senescence MSCs. The senescence phase was evident after about 2 months from the isolation and the senescence duration varied from 1 to 8 weeks. Moreover, the authors also morphologically distinguished the cells into a senescence phase and an in cell crisis. These data suggest a particular caution in using long-term ex vivo expanded cells in clinical protocols.

However, our data excluded chromosomic alterations, early cellular senescence, no modification of the immunophenotype during the expansion of MSCs for 112 days and after 10 PD level. All these data suggest that MSCs can be isolated and expanded from most healthy donors irrespective of age, since the donor's age is only correlated to a defect of cellular growth kinetics, maintaining the other peculiar MSC characteristics.

The variability of results depends on the reduced number of analyzed samples and, above all, on the heterogeneous nature of the MSC cultures. Therefore, the diverse MSC characteristics might depend on the donor to donor variability as Phinney et al. [1999] also suggest. They observed that growth properties and functional capabilities of the MSCs might be different, not only on the basis of the donor nature, but also on samples obtained at different times from the same donor.

There might be numerous clinical applications of MSCs. In particular, promising results have been obtained using human MSCs in clinical trials for Osteogenis Imperfecta [Horwitz et al., 1999, 2001, 2002], metachromatic leukodystrophy, and Hurley syndrome [Koc et al., 2002]. In addition, for their immunosuppressive proprieties and capacity to support hemopoiesis, MSCs have been used to reduce acute and chronic graft versus disease and facilitate the hemopoietic engraftment after BM transplantation [Koc et al., 2000; Lazarus et al., 2005]. Moreover, MSCs are being tested in clinical trials for tissue regeneration and engineering [Horwitz et al., 2002; Mangi et al., 2003].

It is now clear that MSCs might be a useful tool for new therapeutic strategies for the cure of multiple diseases. Our data regarding healthy donor BM showed that MSCs isolated and expanded from pediatric and adult young donors did not present any fundamental differences regarding cellular characterization, except that MSCs isolated from pediatric donors have a faster growth rate than MSCs isolated from adult donors. These results might reflect the higher proliferative potential of pediatric MSCs than adult MSCs and suggest an improvement of a standard protocol of isolation and ex vivo expansion for experimental and clinical use, both in adult and pediatric patients.

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