Faculté de Médecine
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Definition of quality standards applicable to the purification and the expansion of mesenchymal stem cells for their use in haematopoietic, immunosuppressive and regenerative cellular therapy

Définition des critères de qualité applicables à la purification et à l'expansion de cellules souches mésenchymateuses en vue de leur utilisation en thérapie cellulaire hématopoïétique, immunosuppressive et régénérative

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Résumé

Les cellules souches mésenchymateuses (MSCs) résident dans le compartiment stromal de la moelle osseuse et représentent un type cellulaire versatile dont les propriétés pro-hématopoïétique, immunosuppressive et régénératrice restent mal caractérisées. Une caractérisation des préparations de MSCs a été effectuée à chaque passage dans les milieux d'expansion MSCGM et α-MEM + 20% FBS. Nous avons obtenu trois fois plus des cellules après cinq passages dans le MSCGM que dans l' α-MEM + 20% FBS. Nous avons analysé le phénotype des MSCs après chaque passage par cytométrie en flux. A chaque passage et dans les deux milieux, les cellules sont négatives pour le CD45, fortement positives pour les antigènes CD73 et CD90 et faiblement positives pour les antigènes CD105 et CD106. Nous voulions évaluer la capacité des MSCs à se différencier en adipocytes, ostéoblastes et chondroblastes. A chaque passage testé et dans les deux milieux, les MSCs se sont différenciées dans ces trois tissus lorsqu'elles ont été placées dans des milieux de différenciation adéquats. Nous avons utilisé des RayBio® Human Cytokine Antibody Array pour analyser la présence de plusieurs cytokines dans le milieu conditionné par les MSCs. A chaque passage et dans les deux milieux, IL-6, IL-8 et VCAM-1 sont plus fortement exprimés que les autres cytokines. Nous avons utilisé le CFU-F assay pour évaluer la fréquence et la pureté des MSCs en culture. Nous avons observé que le nombre de CFU-F atteint un maximum au passage 3 et 4 après expansion dans le MSCGM et l'α-MEM + 20% FBS. Nous avons observé une plus grande proportion de CFU-F pour les cellules amplifiées dans le MSCGM comparé aux cellules amplifiées dans l'α-MEM + 20% FBS. Des cultures à long terme ont été effectuées pour analyser la capacité des MSCs à favoriser l'hématopoïèse in vitro. Nous avons observé une population plus importante de colonies hématopoïétiques lorsque les cultures à long terme sont effectuées avec des MSCs de 1er ou 2ème passages que lorsque les cultures sont effectuées avec des MSCs de 3ème, 4ème et 5ème passages. A des souris NOD/SCID, nous avons greffé des cellules CD34+ non cultivées ou le produit d'expansion des cellules CD34+ cultivées pendant une semaine avec des MSCs. Le chimérisme humain était présent dans chaque condition. Nous avons observé un chimérisme plus important quand les cellules CD34+ sont cultivées avec des MSCs du passage 4 par rapport aux passages antérieurs. Dans toutes les conditions, l'expression simultanée des antigènes CD19 ou CD33 sur les cellules humaines CD45+ démontre la présence de cellules repopulatrices avec un potentiel lympho-myéloïde. En conclusion, bien que les MSCs ont le même phénotype, le même potentiel de différenciation et le même profil sécrétoire à chaque passage et dans les deux milieux, les MSCs n'ont ni la même capacité à former des CFU-F, ni la même capacité à soutenir l'hématopoïèse in vitro ou in vivo.

Abstract

Mesenchymal stem cells (MSCs) reside within the stromal compartment of bone marrow and represent a plastic cell population for which pro-haematopoietic, immunosuppressive and regenerative properties are poorly characterized. A characterization of MSC preparations was carried out with each passage in MSCGM or α-MEM + 20% FBS expansion media.

We obtained 3 times more cells after 5 passages in MSCGM than in α-MEM + 20% FBS. We analysed the phenotype of MSCs after each passage by flow cytometry. At all passages and in both media cells were tested negative for CD45, strongly positive for CD73 and CD90 antigens and weakly positive for CD105 and CD106 antigens. We wanted to assess the MSC ability to differentiate into adipocytes, osteoblasts and chondroblasts. At all tested passages and in both media, MSCs differentiated in these three tissues when they were placed in the adequate induction medium. We used RayBio® Human Cytokine Antibody Array to analyse the presence of several cytokines in MSC-conditioned medium. At all passages and in both media, IL-6, IL-8 and VCAM-1 were more strongly expressed than the other cytokines. We used the CFU-F assay to evaluate the frequency and purity of MSCs in culture. We observed that CFU-F number reached a maximum at passages 3 and 4 after expansion in both MSCGM and in α-MEM + 20% FBS. We also observed a greater proportion of CFU-F for the cells expanded in MSCGM compared to the cells expanded in α-MEM + 20% FBS. To assess the haematopoietic supporting ability of MSCs, long term cultures were performed. We observed more colony-forming cells when long-term cultures were done with 1^{st} and 2^{nd} passages MSCs than when cultures were done with 3^{rd}, 4^{th} and 5^{th} passages MSCs. In NOD/SCID mice we transplanted CD34+ uncultured cells or the expansion product of CD34+ cells co-cultured for one week with MSCs. Human chimerism was present in all conditions. We observed a greater human chimerism when CD34+ cells were co-cultured with MSCs from passage 4 than in the other conditions. In all conditions simultaneous expression of CD19 or CD33 antigens on human CD45+ cells demonstrated the presence of repopulating cells with lympho-myeloid potential.

Despite the fact that MSCs had the same phenotype, differentiation potential and cytokine secretion profile at each passage and in both media, MSCs did have neither the same capacity to form CFU-F nor the same capacity to support haematopoiesis in vitro and in vivo.

Introduction

Mesenchymal stem cells (MSCs) reside within the stromal compartment of bone marrow and have the capacity to differentiate into cells of the haematopoietic microenvironment: fibroblast, endothelial cells, adipocytes and osteoblasts. In vitro, after specific induction, MSCs have also the potential to differentiate in mesodermal tissue (skeletal myocytes and chondrocytes), ectodermal tissue (neurons and astrocytes) and endodermal tissue (hepatocytes) (Jiang et al, 2002; Prockop, 1997; Pittenger et al., 1999; Sanchez-Ramos et al., 2000). MSCs represent a very small fraction (0,001-0,01%) of the total population of nucleated cells in bone marrow (Pittenger et al., 1999). These stem cells secrete a number of cytokines and regulatory molecules that play important roles in the proliferation and maturation of haematopoietic stem cells. MSCs in culture secrete notably IL-6, SCF, TPO and Flt3-ligand (Ueda et al., 2000). Phenotypically MSCs are identified by the absence of the CD45 haematopoietic cell marker and are positive for Thy-1 (CD90), endoglin (CD105), vascular cell adhesion molecule-1 (VCAM-1/CD106) and ecto-5'-nucleotidase (CD73) (for a review see Deans and Moseley, 2000; Minguell et al., 2001; Pittenger et al., 1999). Clinical applications of MSC transplantation are potentially large. Recent studies show that co-infusion of haematopoietic stem cells (HSCs) and MSCs is associated with rapid haematopoietic recovery (Koç et al., 2000). MSCs have also a potent immunosuppressive effect in vivo; studies show that transplantation of haploidentical MSCs treats severe acute graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (LeBlanc et al., 2004). Other studies suggest that MSCs distribute widely to a variety of non-haematopoietic tissues following systemic infusion and may possess the capacity to proliferate within these tissues (Devine et al., 2003). Currently neither purification nor expansion of MSCs are standardized. According to preparation techniques of MSCs different cellular populations with different biological properties may be obtained. We wish to specify the essential parameters to be measured on MSC preparations to predict their biological activities of potential clinical use. In the present study, we analysed the influence of purification and expansion procedures on the biological activity of MSCs. A phenotypical and functional characterization of MSC preparations was performed in order to define the criteria which most accurately reflect their biological activity. Bone marrow mononuclear cells were isolated by centrifugation over Ficoll-Paque^{TM} Plus and seeded in alpha-minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum (FBS) or in mesenchymal stem cell growth medium (MSCGM). MSCs were selected by plastic adherence and elimination of nonadherent cells. The adherent cells were expanded until the fifth passage. A characterization of MSC preparations was carried out with each passage in both expansion media.

Materials and methods

Human bone marrow mesenchymal stem cell preparation

A human bone marrow sample was obtained from a normal adult volunteer. Mononuclear cells were isolated by centrifugation over Ficoll-Paque^{TM} Plus (Amersham Biosciences, Uppsala, Sweden) and washed in DPBS (Cambrex, Verviers, Belgium). We seeded 5 x 10^4 cells/cm² in alpha-minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin or in mesenchymal stem cell growth medium (MSCGM) (all from Cambrex). MSCs were selected by plastic adherence and elimination of nonadherent cells by replacing the medium 24 hours after cell seeding. When cultures reached 90% confluence, cells were detached with trypsin-EDTA solution (Cambrex), and sub-cultured at 5 x 10^3 cells/cm² during five passages.

Human haematopoietic cell isolation

Human umbilical cord blood samples were obtained following full-term vaginal delivery. All samples were processed within 24 hours after delivery. In experiments requiring large cell numbers, samples were pooled. All material was acquired with informed consent and used according to the guidelines established by the Ethical Committee of the University of Liège. Mononuclear cells were isolated by centrifugation over Ficoll-Paque^{TM} Plus and washed in DPBS. CD34+ cells were purified using magnetic activated cell separation (MACS) CD34 isolation kits (Miltenyi Biotech, Gladbach, Germany). Approximately 95% of the separated cells were found to be CD34+ cells by flow cytometric analysis.

Flow cytometric analysis of MSC preparations

The following antibodies were used to phenotypic characterization of MSCs: fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (Becton Dickinson, Erembodegem, Belgium), phycoerythrin (PE)-conjugated anti-CD73 (Becton Dickinson), PE-conjugated anti-CD90 (Becton Dickinson), PE-conjugated anti-CD105 (Serotec) and PE-conjugated anti-CD106 (Becton Dickinson). Cells were incubated with antibodies or isotype-matched control IgG for 30 minutes at 4℃ in the dark. Cells were washed in DPBS 1% calf serum (Cambrex) and fixed in DPBS 1% formaldehyde (Vel, Leuven, Belgium). Data were acquired on a FACScalibur flow cytometer (Becton Dickinson) and analysed by using CellQuestPro software (Becton Dickinson).

MSC differentiation assays

For osteogenesis cells were plated at 3 x 10^3/cm² and incubated until confluence. The cultures were then incubated in MSCGM or α-MEM + 20% FBS that was supplemented with 60 µM ascorbic acid (Sigma, Bornem, Belgium), 10 mM β-glycerophosphate (Sigma), 0,1 µM dexamethasone (Sigma) (Tondreau et al., 2004). The medium was changed 2 times a week for 3 weeks. The cells were fixed with 10% formalin for 20 minutes at room temperature and stained with Alizarin Red, pH 4,1 (Sigma) for 20 minutes at room temperature.

For adipogenesis cells were plated at 2 x 10^4/cm² and incubated in MSCGM or α-MEM + 20% FBS that was supplemented with 1 µM dexamethasone, 60 µM indomethacin (Sigma), 5 µg/ml insulin (Sigma) (Tondreau et al., 2004). The medium was changed 2 times a week for 3 weeks. The cells were fixed with 10% formalin for 20 minutes at room temperature and stained with Oil Red O (Sigma) for 20 minutes at room temperature.
For chondrocyte differentiation a pellet culture system was used. A total of 5 x 10^5 cells were placed in a 10 ml polystyrene tube and centrifuged to pellet. The pellet was cultured in 500 µl MSCGM or α-MEM + 20% FBS that was supplemented with 10 ng/ml transforming growth factor β 3, 50 µM ascorbic acid, 0,5 µg/ml insulin (Tondreau et al., 2004). Half of the medium was changed 2 times a week for 3 weeks. For microscopy the pellets were embedded in paraffin, cut into 4 µm sections and stained with Toluidine blue Sodium Borate.

RayBio® Human Cytokine Antibody Array

At each passage, when MSC cultures reached 90% confluence, the medium was changed and incubated for 72 hours. The conditioned medium was removed and freezed at -80°C for further analyses. To analyse the presence of BMP-4, BMP-7, Flt-3L, G-CSF, GM-CSF, IL-10, IL-15, IL1-α, IL-3, IL-2, IL-6, IL-7, IL-8, LIF, MMP-9, SCF, SDF-1, TGF-β, Thrombopoietin and VCAM-1 in conditioned medium, RayBio® Human Cytokine Antibody Array (RayBiotech, Inc, Boechout, Belgium) was used. We selected these cytokines form previously published data (for a review see Deans and Moseley, 2000; Minguell et al., 2001). The membrane was incubated at room temperature for 30 minutes in blocking buffer. Blocking buffer was decanted. The membrane was incubated with 1 ml of conditioned medium at room temperature for 2 hours. The medium was decanted. The membrane was washed 3 times with 2 ml of wash buffer I at room temperature with shaking (5 min per wash). Then the membrane was washed 2 times with 2 ml of wash buffer II at room temperature with shaking (5 min per wash). The membrane was incubated with 1 ml of biotin-conjugated antibodies at room temperature for 2 hours. Then the membrane was washed as described above. The membrane was incubated with 2 ml of HRP-conjugated streptavidin at room temperature for 2 hours. The membrane was washed as described above. The membrane was incubated with mixed detection buffer at room temperature for 2 minutes (all from RayBiotech, Inc). X-ray film (Kodak, Aarschot, Belgium) was exposed to the membrane for 1 to 5 seconds and signals were detected using film developer.

CFU-F assay

We used the colony forming unit-fibroblast (CFU-F) assay to evaluate the frequency of MSCs in mononuclear cells and after each passage. At each passage MSCs were plated at 5 to 2000 cells/cm² in Mesencult™ complete medium (Stem Cell Technologies, Myelan, France). The medium was changed every 3 days during 21 days. Cells were fixed in methanol and stained with Giemsa solution at room temperature for 5 minutes. All visible colonies were counted.

LTC-IC assays

A total of 1250 cord blood CD34+ cells were overlayed with confluent MSCs of each passage, in 1,5 ml Myelocult (Stem Cell Technologies). Cultures were maintained at 33℃ in 5% CO_2 with weekly half medium changes. After 5 weeks cultures were trypsinized and the cell suspension was transferred in duplicate progenitor assays in a semi-solid medium consisting in 0,9% methylcellulose (Stem Cell Technologies), 30% FBS, 10% 5637 bladder carcinomacell line conditioned medium, 3 U erythropoietin (Janssen-Cilag), 50 mmol/L 2-mercaptoethanol, 200 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in Iscove's modified Dulbecco's medium (IMDM). After an additional 2-week incubation at 37℃ secondary colony-forming cells were scored.

Transplantation of human cells into NOD/SCID mice

Two-month old NOD/SCID mice were sublethally irradiated with 200 cGy from a X-ray source (Stabilivolt, Siemens) or 300 cGy from a ^{137}Cs source (GammaCell 40, Nordion, Ontario, Canada). Mice received 1 x 10^5 CD34+ uncultured cells or the expansion product of 1 x 10^5 CD34+ cells co-cultured for one week with 1 x 10^5 MSCs from passages 2, 3 and 4. Cells were transplanted by intravenous tail injection. After 6 weeks, mice were killed by cervical dislocation and bone marrow cells were harvested from femurs and tibias by flushing the bones with IMDM + 10% FBS. Mononuclear low-density cells were isolated by centrifugation over Ficoll-Paque™ Plus (Amersham Biosciences), washed and resuspended in IMDM with 10% FBS.

Flow cytometric analysis of engraftment

Between 1 x 10^5 and 6 x 10^5 NOD/SCID bone marrow cells were pelleted and incubated with various mouse antihuman monoclonals for 30 minutes at 4℃ in the dark. Allophycocyanin (APC)-conjugated anti-CD45 (Becton Dickinson), FITC-conjugated anti-CD33 (Becton Dickinson) and PE-conjugated anti-CD19 (Immunotech, Marseille, France) were used. Cells were washed in DPBS 1% calf serum (Cambrex) and fixed in DPBS 1% formaldehyde (Vel). Data were acquired on a FACScalibur flow cytometer (Becton Dickinson) and analysed using CellQuestPro software (Becton Dickinson). Positive cells were identified by comparison with isotypic controls.

Results

Amplification and characterization of MSCs

We seeded 5 x 10^4 primary cells/cm² in α-MEM supplemented with 20% FBS or MSCGM. After removal of non adherent cells, adherent cells were sub-cultured at 5 x 10^3 cells/cm² during five passages. At each passage we had on average 43 x 10^3 cells/cm² in MSCGM and 37 x 10^3 cells/cm² in α-MEM supplemented with 20% FBS. Each passage lasted on average 10 days in MSCGM and 14 days in α-MEM supplemented with 20% FBS. We obtained 3 times more cells after 5 passages in MSCGM than in α-MEM supplemented with 20% FBS (Figure 1).

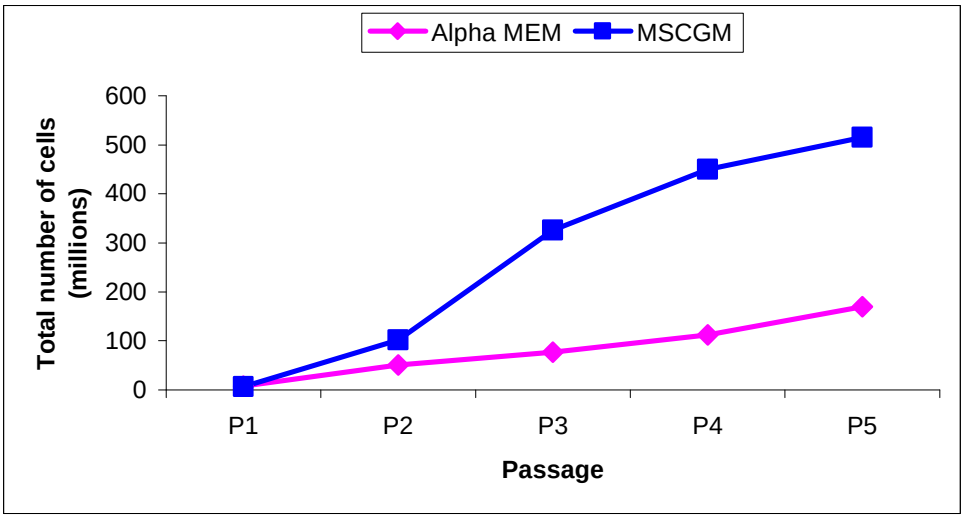


Figure 1: Growth curve of MSCs expanded in α-MEM + 20% FBS (in pink) and in MSCGM (in blue). Initial cell dose was 90 x 10^6 mononuclear cells in both media.

We analysed the phenotype of bone marrow mononuclear cells and MSCs after each passage by flow cytometry. Bone marrow mononuclear cells were negative for the CD73, CD90, CD105 and CD106 antigens and were positive for the CD45 antigen (Figure 2 A). After the first passage cells were tested negative for CD45, strongly positive for CD73 and CD90 antigens and weakly positive for CD105 and CD106 antigens (Figure 2 B-C). This phenotype was maintained during five passages and was similar for cells expanded in both media.

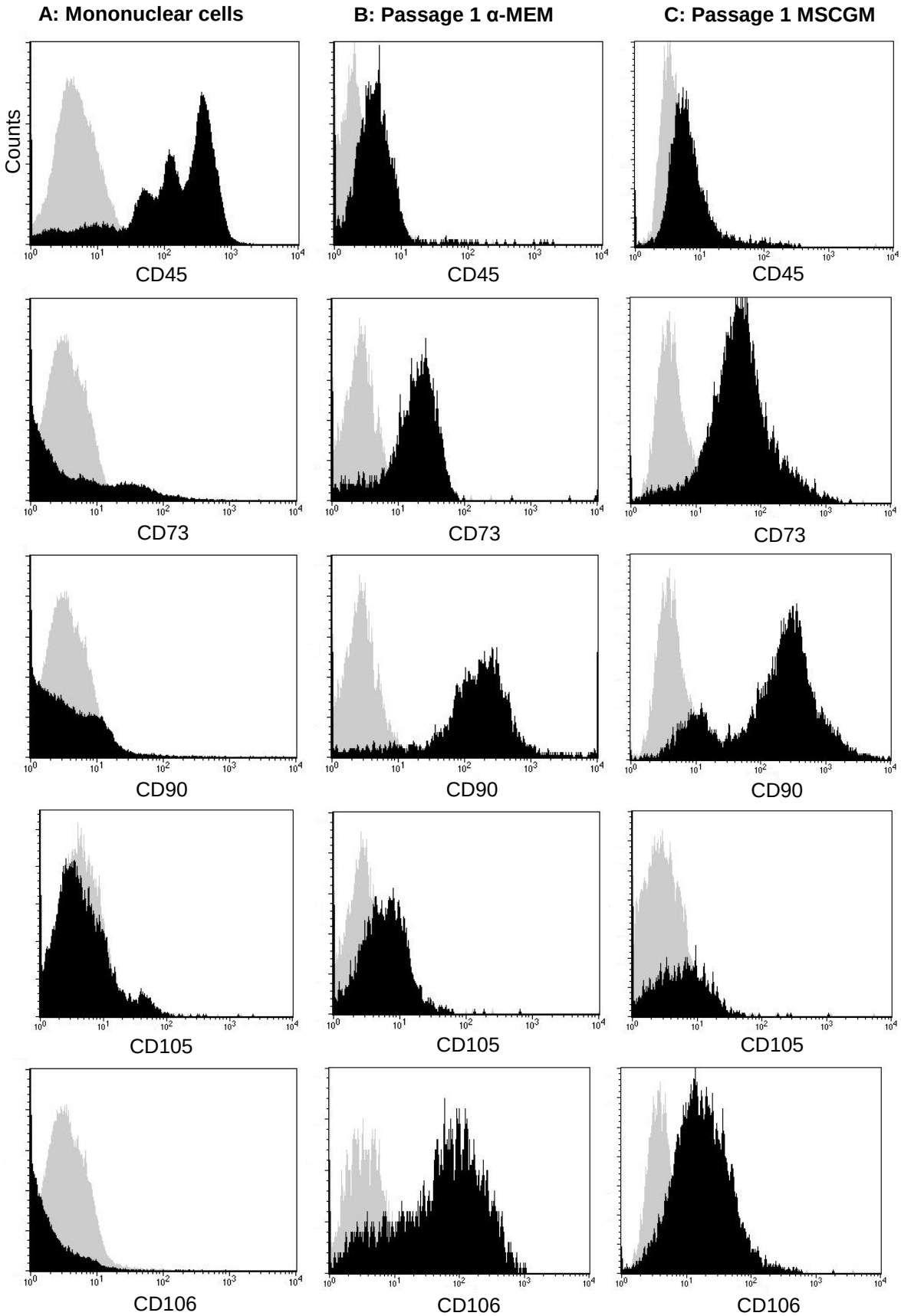


Figure 2: Flow cytometric analysis of the expression of CD45, CD73, CD90, CD105 and CD106 antigens; Column A: bone marrow mononuclear cells; Column B: passage 1 cells expanded in α-MEM + 20% FBS; Column C: passage 1 cells expanded in MSCGM; in each panel staining with isotype-matched mouse IgG control is indicated in grey.

MSC differentiation

We wanted to assess the MSC ability to differentiate into three tissues from mesodermal origin. MSCs from passages 1, 3 and 5 in both media differentiated into fat cells when they were placed in the adequate induction medium (Figure 3 A-B). MSCs from passages 1, 3 and 5 in MSCGM and from passage 5 in α-MEM + 20% FBS differentiated into mineral cells (Figure 3 C-D). MSCs from passages 3 and 5 expanded in both media differentiated into chondrocyte cells (Figure 3 E-F). Lipid vacuoles, calcium deposits and chondrogenic differentiation were revealed respectively with Oil Red O, Alizarin Red, and Toluidine blue (Figure 3).

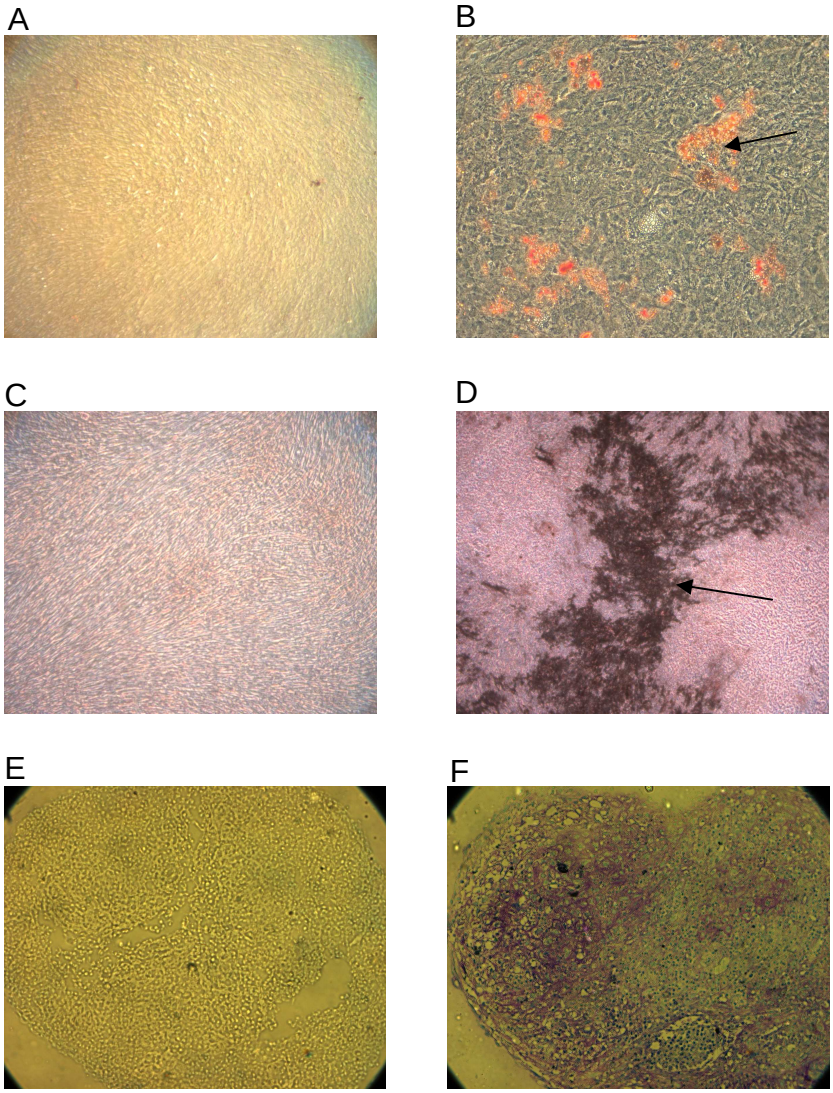


Figure 3: MSC differentiation of passage 1 (A-D) and passage 5 (E-F). A: Control culture incubated in MSCGM and stained with Oil Red O; B: Induction of fat cell differentiation and staining with Oil Red O (magnification 20x); arrow indicates lipid vacuoles; C: Control culture incubated in MSCGM and stained with Alizarin Red; D: Induction of osteoblast differentiation and staining with Alizarin Red (magnification 20x); arrow shows calcium deposits; E: Control culture incubated in MSCGM and stained with Toluidine blue; F: Induction of chondrogenic differentiation and staining with Toluidine blue (magnification 20x).

Cytokine array

We used RayBio® Human Cytokine Antibody Array to analyse the presence of BMP-4, BMP-7, Flt-3L, G-CSF, GM-CSF, IL-10, IL-15, IL1-α, IL-3, IL-2, IL-6, IL-7, IL-8, LIF, MMP-9, SCF, SDF-1, TGF-β, Thrombopoietin and VCAM-1 in MSC-conditioned medium at each passage. This panel was selected according to previously published data and included the main soluble factors identified in regulation of haematopoiesis. All cytokines were present in both unconditioned media (Table 1). At first passage, IL-6, IL-8 and VCAM-1 were more strongly expressed than the other cytokines. This expression was maintained during four passages and was similar for cells expanded in both media. The other cytokines were present in all conditions at a weak level.

	(a) MSCGM				
	control	P1	P2	P3	P4
BMP-4	+	+	+	+	+
BMP-7	+	+	+	+	+
Flt-3L	+	+	+	+	+
G-CSF	+	+	+	+	+
GM-CSF	+	+	+	+	+
IL-10	+	+	+	+	+
IL-15	+	+	+	+	+
IL1-α	+	+	+	+	+
IL-3	+	+	+	+	+
IL-2	+	+	+	+	+
IL-6	+	+++	+++	+++	+++
IL-7	+	+	+	+	+
IL-8	+	++	++	++	++
LIF	+	+	+	+	+
MMP-9	+	+	+	+	+
SCF	+	+	+	+	+
SDF-1	+	+	+	+	+
TGF-β	+	+	+	+	+
Thromb.	+	+	+	+	+
VCAM-1	+	++	++	++	++

	(b) α-MEM				
	control	P1	P2	P3	P4
BMP-4	+	+	+	+	+
BMP-7	+	+	+	+	+
Flt-3L	+	+	+	+	+
G-CSF	+	+	+	+	+
GM-CSF	+	+	+	+	+
IL-10	+	+	+	+	+
IL-15	+	+	+	+	+
IL1-α	+	+	+	+	+
IL-3	+	+	+	+	+
IL-2	+	+	+	+	+
IL-6	+	+++	+++	+++	+++
IL-7	+	+	+	+	+
IL-8	+	++	++	++	++
LIF	+	+	+	+	+
MMP-9	+	+	+	+	+
SCF	+	+	+	+	+
SDF-1	+	+	+	+	+
TGF-β	+	+	+	+	+
Thromb.	+	+	+	+	+
VCAM-1	+	++	++	++	++

Table 1: Presence of different cytokines in unconditioned medium and conditioned medium MSCGM (a) or α-MEM + 20% FBS (b) at each passage. Intensity of signal: strong (+++), weak (+), middle (++); Thromb.: Thrombopoietin; P: passage; control: unconditioned medium.

CFU-F assay

We used the CFU-F assay to evaluate the frequency and purity of MSCs in culture. We observed that CFU-F number reached a maximum at passages 3 and 4 after expansion in both MSCGM and in α-MEM supplemented with 20% FBS (Figure 4). We also observed a greater proportion of CFU-F for the cells expanded in MSCGM compared to the cells expanded in α-MEM supplemented with 20% FBS. Indeed, at passage 3, while 10% of cells expanded in MSCGM were CFU-F, only 1% of cells expanded in α-MEM + 20% FBS had maintained colony-forming activity (Figure 4).

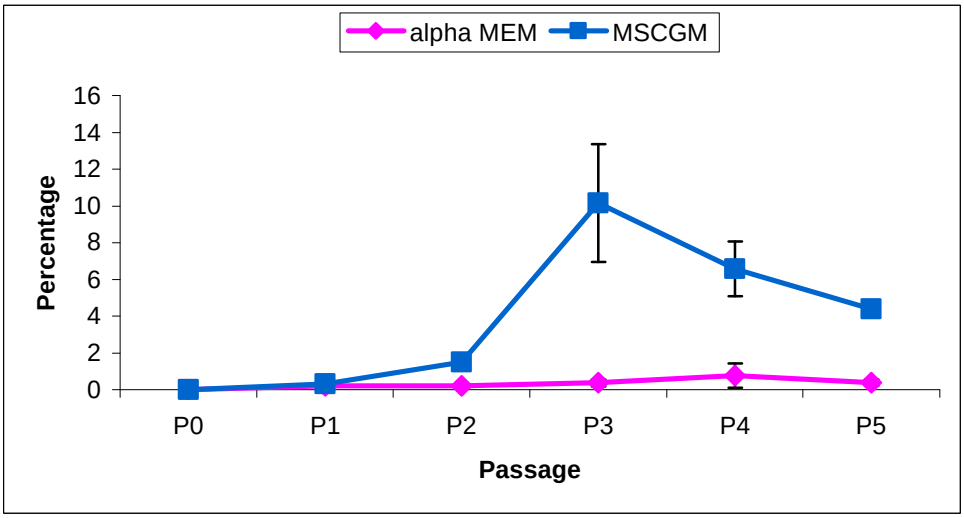
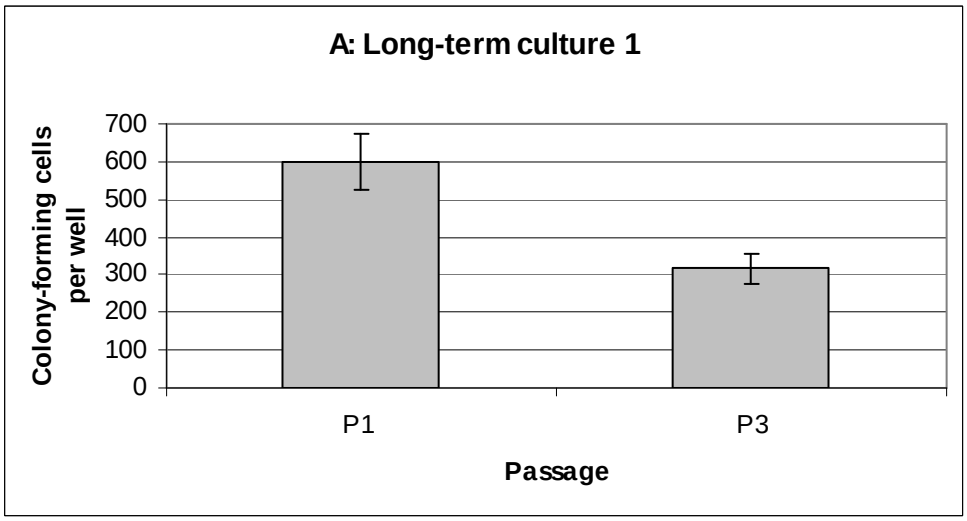


Figure 4: Percentage of CFU-F related to the total cell number after expansion in α-MEM + 20% FBS (in pink) and in MSCGM (in blue).

LTC-IC assays

A total of 1250 CD34+ cells were overlayed with MSCs expanded in MSCGM and harvested at distinct passage at confluence. After 5 weeks of long-term culture secondary colony-forming cells were scored.
We did a first experiment with MSCs from passages 1 and 3. We observed more colony-forming cells when long-term cultures were done with 1st passage MSCs than when cultures were done with 3rd passage MSCs (Figure 5 A). We repeated the same experiment with MSCs from passages 2, 4 and 5. Colony-forming cell number decreased when cultures were done with 4th and 5th passages MSCs compared to passage 2 MSCs (Figure 5 B).



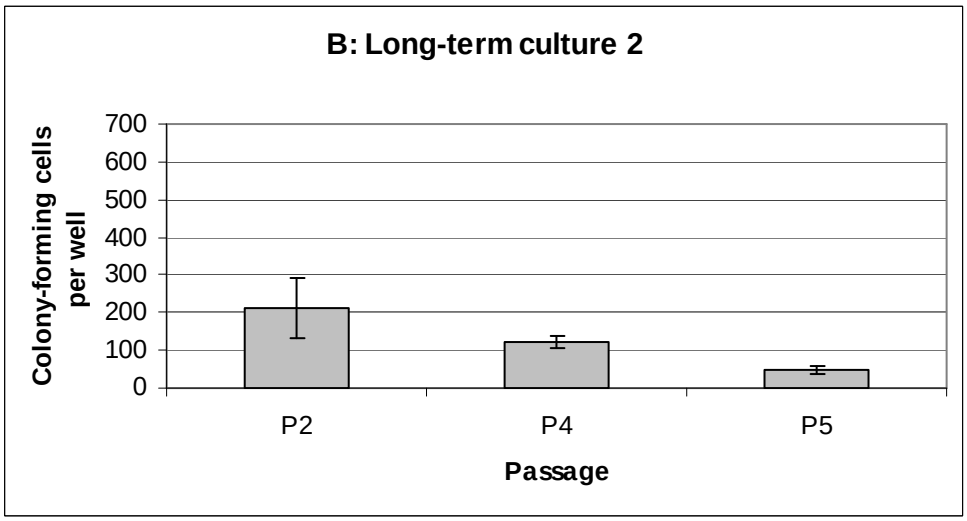


Figure 5: Colony-forming cells per well after 5 weeks of long-term culture with MSCs harvested at indicated passage (P).

Transplantation of human cells into NOD/SCID mice

Two-month old NOD/SCID mice were sublethally irradiated with 200 cGy or 300 cGy. Mice received 1 x 10^5 CD34+ uncultured cells or the expansion product of 1 x 10^5 CD34+ cells co-cultured for one week with 1 x 10^5 MSCs from passages 2, 3 and 4. Cells were transplanted by intravenous tail injection. After 6 weeks mice were killed and bone marrow cells were harvested from femurs and tibias. Between 1 x 10^5 and 6 x 10^5 NOD/SCID BM cells were incubated with various mouse antihuman monoclonals: APC-conjugated anti-CD45, FITC-conjugated anti-CD33 and PE-conjugated anti-CD19. Human chimerism was present in all conditions (Table 2). We observed a greater human chimerism when mice were irradiated with 300 cGy than 200 cGy (Figure 6). We also observed a greater human chimerism when CD34+ cells were co-cultured with MSCs from passage 4 than in the other conditions (Figure 6). In all conditions simultaneous expression of CD19 or CD33 antigens on human CD45+ cells demonstrated the presence of repopulating cells with lympho-myeloid potential (Figure 7).

	Irradiation 200 cGy					Irradiation 300 cGy			
Graft	Mouse	% human chimerism	Mean	SEM	Graft	Mouse	% human chimerism	Mean	SEM
CD34+ alone	1	0,015			CD34+ alone	1	0,19		
	2	0,079				2	0,82		
	3	dead				3	dead		
	4	dead	0,047	0,032		4	dead	0,505	0,315
Passage 2	1	0,055			Passage 2	1	0,22		
	2	0,023				2	0,38		
	3	0,022				3	0,15		
	4	dead	0,033	0,011		4	1,75	0,625	0,378
Passage 3	1	0,093			Passage 3	1	0,6		
	2	0,032				2	0,18		
	3	0,023				3	0,23		
	4	dead	0,049	0,022		4	0,18	0,2975	0,102
Passage 4	1	0,885			Passage 4	1	1,24		
	2	0,021				2	2,23		
	3	0,282				3	dead		
	4	dead	0,396	0,256		4	dead	1,735	0,495

Table 2: Percentage of human chimerism of each mouse, mean and standard error of the mean (SEM).

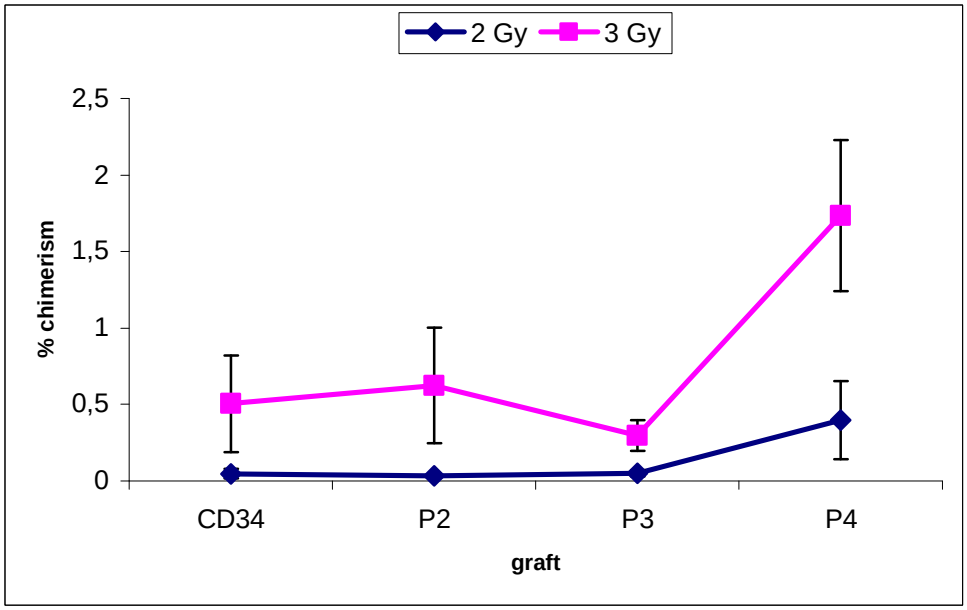


Figure 6: Average percentage of chimeric human CD45+ cells in the bone marrow of mice transplanted 6 weeks previously with uncultured CD34+ (CD34) cells or CD34+ cells co-cultured with MSCs harvested at indicated passage (P); MSCs were previously expanded in MSCGM; mice were sublethally irradiated with 2 Gy (in pink) or 3 Gy (in blue).

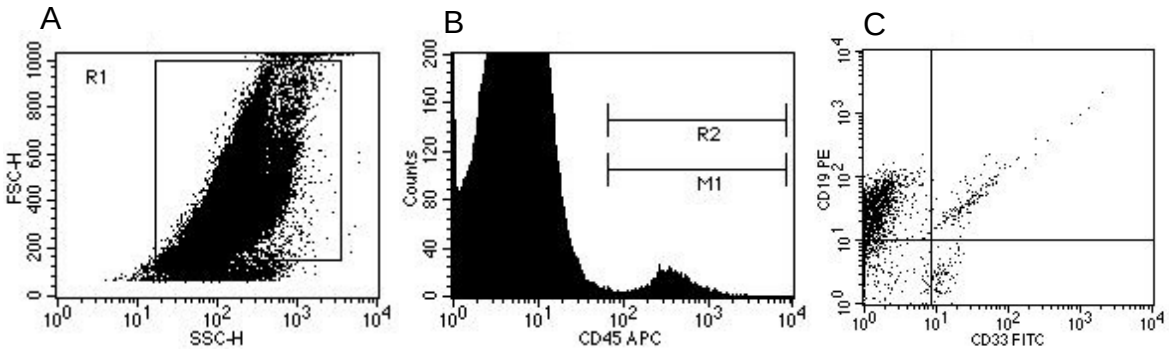


Figure 7: Evaluation of multilineage repopulation in mouse 2 irradiated with 3 Gy which received P4 MSCs. (A) Scatter plot (B) CD45 expression in R1 population (C) Dual-color flow cytometric analysis of CD19 and CD33 in R2 population.

Discussion

Mesenchymal stem cells reside within the stromal compartment of bone marrow and represent a plastic cell population for which pro-haematopoietic, immunosuppressive and regenerative properties are poorly characterized. A characterization of MSC preparations was carried out with each passage in MSCGM or α-MEM + 20% FBS expansion media.

As for phenotype analysis, we showed that after the first passage cells were tested negative for CD45, strongly positive for CD73 and CD90 antigens and weakly positive for CD105 and CD106 antigens. This phenotype was maintained during five passages and was similar for cells expanded in both media. We thus obtained a mesenchymal phenotype as early as passage 1 in both media (for a review see Deans and Moseley, 2000; Minguell et al., 2001; Pittenger et al., 1999).

Then we wanted to assess the MSC ability to differentiate into adipocytes, osteoblasts and chondroblasts. At all tested passages and in both media, MSCs differentiated in these three tissues when they were placed in the adequate induction medium. The differentiation potential in vitro demonstrated the presence of MSCs in our cultures (Pittenger et al, 1999).

Next we analysed the presence of 20 cytokines in MSC-conditioned medium at each passage. At all passages and in both media, IL-6, IL-8 and VCAM-1 were strongly expressed by MSCs. These three cytokines play a role in haematopoiesis or in engraftment of HSCs. It was shown that inactivation of the IL-8 pathways inhibits CD34+ cell proliferation and colony formation (Hermouet et al., 2000). Some studies indicate that IL-6 synergistically acts with other cytokines to support the proliferation of haematopoietic progenitors (Ikebuchi et al., 1987; Ueda et al., 2000). Haematopoietic cell lodgement in the bone marrow can be influenced by VLA4/VCAM-1 adhesion pathway (Papayannopoulou et al., 1995; Simmons et al., 1992).

We used the CFU-F assay to evaluate the frequency and purity of MSCs in culture. We observed that CFU-F number reached a maximum at passages 3 and 4 after expansion in both MSCGM and in α-MEM + 20% FBS. We also observed a greater proportion of CFU-F for the cells expanded in MSCGM compared to the cells expanded in α-MEM + 20% FBS. Despite the fact that MSCs had the same phenotype, differentiation potential and cytokine secretion profile at each passage and in both media, MSCs did have neither the same capacity to maintain CFU-F activity nor the same capacity of expansion. Indeed we obtained 3 times more cells after 5 passages in MSCGM than in α-MEM + 20% FBS.

Then we studied the capacity of MSCs to maintain primitive haematopoietic progenitors in long term culture. We observed an increased production of haematopoietic colonies when long-term cultures were done with 1^{st} or 2^{nd} passages MSCs than when cultures were done with 3^{rd}, 4^{th} and 5^{th} passages MSCs.

Finally NOD/SCID mice were sublethally irradiated and received 1 x 10^5 CD34+ uncultured cells or the expansion product of 1 x 10^5 CD34+ cells co-cultured for one week with 1 x 10^5 MSCs from passages 2, 3 and 4. Human chimerism was present in all conditions. We observed a greater human chimerism when CD34+ cells were co-cultured with MSCs from passage 4 than in the other conditions. In all conditions simultaneous expression of CD19 or CD33 antigens on human CD45+ cells demonstrated the presence of repopulating cells with lympho-myeloid potential. We showed an expansion of SRC after one week of co-culture with 4^{th} passage MSCs but not with prior passages. Interestingly, our data indicate that distinct populations of

MSCs support haematopoietic cells in vitro (LTC-IC) or in vivo (SCID repopulating cells, SRC). It has been demonstrated by Gan et al (1997) that LTC-IC and SRC represent different populations of haematopoietic cells. Differences may result from the state of differentiation, SRC being more immature, or from their homing capacity, LTC-IC being unable to reach bone marrow niches in vivo. Our results may contribute to identify specific signals which differentially support LTC-IC or SRC expansion by MSCs. So far, it appears that the haematopoietic supporting capacity of MSCs is not dependent on MSC phenotype, differentiation capacity into mesodermal lineages or soluble cytokine secretion repertoire. It is likely that MSCs support haematopoiesis through membrane-bound factors.

Among these, it would be interesting to identify the signals which lead to the amplification of SRC and which appear at passage 4. It was shown that the notch-ligand Jagged-1 induces the survival and expansion of HSCs capable of pluripotent repopulating capacity (Karanu et al., 2000). An other study shows that Shh functions as a regulator of primitive haematopoietic cells via mechanisms that are dependent on downstream BMP signals (Bhardwaj et al., 2001). Also, critical adhesion molecules mediating binding of HSCs in haematopoietic niches may be differentially expressed in MSC populations. Among these, N-cadherin (Zhang et al., 2003) and osteopontin (Nilsson et al., 2005) are strong candidates. Further studies will be needed to analyse the role of these factors in the haematopoietic supporting MSC ability.

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References

Bhardwaj,G., Murdoch,B., Wu,D., Baker,D.P., Williams,K.P., Chadwick,K., Ling,L.E., Karanu,F.N., & Bhatia,M. (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol*, **2**, 172-180.

Deans,R.J. & Moseley,A.B. (2000) Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol.*, **28**, 875-884.

Devine,S.M., Cobbs,C., Jennings,M., Bartholomew,A., & Hoffman,R. (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood*, **101**, 2999-3001.

Gan,O.I., Murdoch,B., Larochelle,A., & Dick,J.E. (1997) Differential maintenance of primitive human SCID-repopulating cells, clonogenic progenitors, and long-term culture-initiating cells after incubation on human bone marrow stromal cells. *Blood*, **90**, 641-650.

Hermouet,S., Corre,I., & Lippert,E. (2000) Interleukin-8 and other agonists of Gi2 proteins: autocrine paracrine growth factors for human hematopoietic progenitors acting in synergy with colony stimulating factors. *Leuk.Lymphoma*, **38**, 39-48.

Ikebuchi,K., Wong,G.G., Clark,S.C., Ihle,J.N., Hirai,Y., & Ogawa,M. (1987) Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proc Natl Acad Sci U S A*, **84**, 9035-9039.

Jiang,Y., Jahagirdar,B.N., Reinhardt,R.L., Schwartz,R.E., Keene,C.D., Ortiz-Gonzalez,X.R., Reyes,M., Lenvik,T., Lund,T., Blackstad,M., Du,J., Aldrich,S., Lisberg,A., Low,W.C., Largaespada,D.A., & Verfaillie,C.M. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*, **418**, 41-49.

Karanu,F.N., Murdoch,B., Gallacher,L., Wu,D.M., Koremoto,M., Sakano,S., & Bhatia,M. (2000) The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med*, **192**, 1365-1372.

Koc,O.N., Gerson,S.L., Cooper,B.W., Dyhouse,S.M., Haynesworth,S.E., Caplan,A.I., & Lazarus,H.M. (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol.*, **18**, 307-316.

Le Blanc,K., Rasmusson,I., Sundberg,B., Gotherstrom,C., Hassan,M., Uzunel,M., & Ringden,O. (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*, **363**, 1439-1441.

Minguell,J.J., Erices,A., & Conget,P. (2001) Mesenchymal stem cells. *Exp Biol.Med (Maywood.)*, **226**, 507-520.

Nilsson,S.K., Johnston,H.M., Whitty,G.A., Williams,B., Webb,R.J., Denhardt,D.T., Bertoncello,I., Bendall,L.J., Simmons,P.J., & Haylock,D.N. (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*, **106**, 1232-1239.

Papayannopoulou,T., Craddock,C., Nakamoto,B., Priestley,G.V., & Wolf,N.S. (1995) The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proc Natl Acad Sci U S A*, **92**, 9647-9651.

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., & Marshak,D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science*, **284**, 143-147.

Prockop,D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*, **276**, 71-74.

Sanchez-Ramos,J., Song,S., Cardozo-Pelaez,F., Hazzi,C., Stedeford,T., Willing,A., Freeman,T.B., Saporta,S., Janssen,W., Patel,N., Cooper,D.R., & Sanberg,P.R. (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol.*, **164**, 247-256.

Simmons,P.J., Masinovsky,B., Longenecker,B.M., Berenson,R., Torok-Storb,B., & Gallatin,W.M. (1992) Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood*, **80**, 388-395.

Tondreau,T., Lagneaux,L., Dejeneffe,M., Massy,M., Mortier,C., Delforge,A., & Bron,D. (2004) Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. *Differentiation*, **72**, 319-326.

Ueda,T., Tsuji,K., Yoshino,H., Ebihara,Y., Yagasaki,H., Hisakawa,H., Mitsui,T., Manabe,A., Tanaka,R., Kobayashi,K., Ito,M., Yasukawa,K., & Nakahata,T. (2000) Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest*, **105**, 1013-1021.

Zhang,J., Niu,C., Ye,L., Huang,H., He,X., Tong,W.G., Ross,J., Haug,J., Johnson,T., Feng,J.Q., Harris,S., Wiedemann,L.M., Mishina,Y., & Li,L. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, **425**, 836-841.