

An efficient method for isolation of murine bone marrow mesenchymal stem cells

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ABSTRACT Mesenchymal stem cells (MSCs) have been isolated based on the ability of adherence to plastic surfaces. The potential of these cells to differentiate along multiple lineages is the key to identifying stem cell populations in the absence of molecular markers. Here we describe a homogenous population of MSCs from mouse bone marrow isolated using a relatively straightforward and novel approach. This method is based on the combination of frequent medium change (FMC) and treatment of the primary cultures with trypsin. Cells isolated using this method demonstrated the MSCs characteristics including their ability to differentiate into mesenchymal lineages. MSCs retained the differentiation potentials in expanded cultures up to 10 passages. Isolated MSCs were reactive to the CD44, Sca-1, and CD90 cell surface markers. MSCs were negative for the hematopoietic surface markers such as CD34, CD11b, CD45, CD31, CD106, CD117 and CD135. The data presented in this report indicated that this method can result in efficient isolation of homogenous populations of MSCs from mouse bone marrow.

KEY WORDS: murine, bone marrow, mesenchymal stem cell, isolation

Introduction

Stem cells are undifferentiated cells with the ability to proliferate and produce a large number of differentiated progeny (Fuchs and Segre, 2000). Bone marrow is the main source of hematopoietic stem cells (HSCs) and MSCs. MSCs isolated from bone marrow display multilineage differentiation potentials (Pittenger *et al.*, 1999). MSCs have also been named colony-forming fibroblastic cells (Friedenstein *et al.*, 1976), marrow stromal stem cells (Bianco *et al.*, 2001, Prockop, 1997) and mesenchymal progenitor cells (Sun *et al.*, 2003). MSCs have been considered as an appropriate source for cell and gene therapy tools for treatment in a number of congenital and degenerative diseases (Baksh *et al.*, 2004). Promising evidences have been reported with the use of cells in a number of animal models for human diseases, including models for osteogenesis imperfecta (Horwitz *et al.*, 2002), spinal cord injury (Sasaki *et al.*, 2001), stroke (Chen *et al.*, 2003), and Parkinsonism (Schwarz *et al.*, 1999).

Bone marrow derived MSCs have been isolated and characterized from many species including rat (Schwarz *et al.*, 1999), cat (Martin *et al.*, 2002), dog (Kadiyala *et al.*, 1997), baboon

and rhesus monkeys (Devine *et al.*, 2001, Izadpanah *et al.*, 2005), rabbit (Wakitani *et al.*, 1994), pig (Awad *et al.*, 1999), goat (Mosca *et al.*, 2000) and sheep (Jessop *et al.*, 1994). In contrast, the isolation and purification of murine MSCs (mMSCs) from mouse bone marrow has been more difficult than that in human and other species due to the unwanted growth of non-MSCs for long a time in cultures (Meirelles Lda and Nardi, 2003, Peister *et al.*, 2004, Phinney *et al.*, 1999).

To date, several techniques have been developed to obtain pure cultures of MSCs by the reduction or elimination of non-MSCs from bone marrow cultures (Baddoo *et al.*, 2003, Eslaminejad *et al.*, 2006, Modderman *et al.*, 1994, Peister *et al.*, 2004, Sun *et al.*, 2003). Nevertheless, isolation of pure MSCs from murine bone marrow remains an unsolved problem. The present study describes a novel method for isolation of mMSCs. Based on this technique, cell culture media was changed several

Abbreviations used in this paper: CFU-F, colony-forming unit-fibroblast; FACS, fluorescence-activated cell sorting; FMC, frequent medium changes; HSCs, hematopoietic stem cells; MSC, mesenchymal stem cell; mMSC, murine mesenchymal stem cell; WFMC, without frequent medium changes.

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times upon processing and plating the harvested bone marrow in a timely manner. Further analysis of MSCs confirmed the purity of the culture.

Experimental Protocols

Isolation and expansion of MSCs

Balb/c Mice, 6-8 weeks old, were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from adherent soft tissue. The tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with Dulbecco's Modified Eagle's Medium (DMEM; Gibco). The bone marrow cells were filtered through a 70- μ m nylon mesh filter (BD,

Falcon, USA). Cells were plated into 6-well plastic cell culture plate at a density of 25×10^6 cells per well in DMEM containing 15% fetal bovine serum (FBS; Sigma), 2mM L-glutamine (Gibco, USA), 100 u/ml penicillin (Sigma) and 100 u/ml streptomycin (Sigma). Cultures were kept at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. When primary cultures became nearly confluent, the culture was treated with 0.5ml of 0.025% Trypsin containing 0.02% EDTA for 2 minutes at room temperature. The cells which were lifted within 2 minutes were harvested and cultured in a 25 cm² flask. Once the culture reached 70-80% confluence, the cells were harvested for further experiments. Furthermore, the cells not lifted after two minutes of trypsin treatment were kept in culture media for two weeks, at which point these cells were collected for further analysis.

In addition, some bone marrow cells were cultivated without frequent medium changes (referred to as WFMC cells) as a cell culture control, WFMCs were cultured in 6-well culture plates at a density of 25×10^6 cells per well in DMEM and the culture medium was changed after 72 hours for the first time. Cell cultivation was continued for two weeks at which time the cells were analyzed.

Tumorigenic assay

Immunodeficient mice (SCID mice; n=3), 4 weeks old, were subcutaneously injected with 5×10^5 cells in 50 μ l of DMEM containing FBS, L-glutamine and antibiotic. Three SCID mice were inoculated with GL 26 glioma cells as controls. Injected mice were monitored for tumor formation for 60 days.

Colony forming unit-Fibroblast (CFU-F) assay

The clonogenic potential of the isolated cells (first passage) was tested for the colony formation potentials. For this assay, 100 cells were plated on a 60 mm² cell culture dish and incubated for 7 days. Subsequently, the plates were stained with 3% crystal violet in methanol for 10 minutes. All visible colonies were counted. This assay was repeated on 15 donors.

Differentiation assays

The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was examined. For osteogenesis, the cultured cells were incubated in osteogenic conditioned medium described by Eslaminejad *et al.* (Eslaminejad *et al.*, 2006). Briefly, DMEM was supplemented with 10 mM β -glycerol phosphate (Sigma), 50 μ g/ml ascorbate-2-phosphate (Sigma) and 10^{-7} M dexamethasone (Sigma) (Eslami nejad *et al.*, 2006). The culture medium was changed two times per week for up to three weeks. The cells were fixed with methanol for 10 minutes at room temperature and stained with alizarin red with pH=4 for 5 minutes at room temperature. Treated cells were subjected to RNA extraction and RT-PCR analysis.

For adipogenesis, the cultured cells were incubated in adipogenic medium DMEM supplemented with 50 μ g/ml indomethacin (Sigma), 10^{-7} M dexamethasone (Sigma), and 50 μ g/ml ascorbate-2-phosphate (Sigma) (Eslaminejad *et al.*, 2006). The culture medium was changed two times per week for up to three weeks. The cells then were fixed in methanol for 45 minutes and stained with Oil Red (Sigma). The cells were also used for RNA extraction and RT-PCR analysis of adipogenic gene expression.

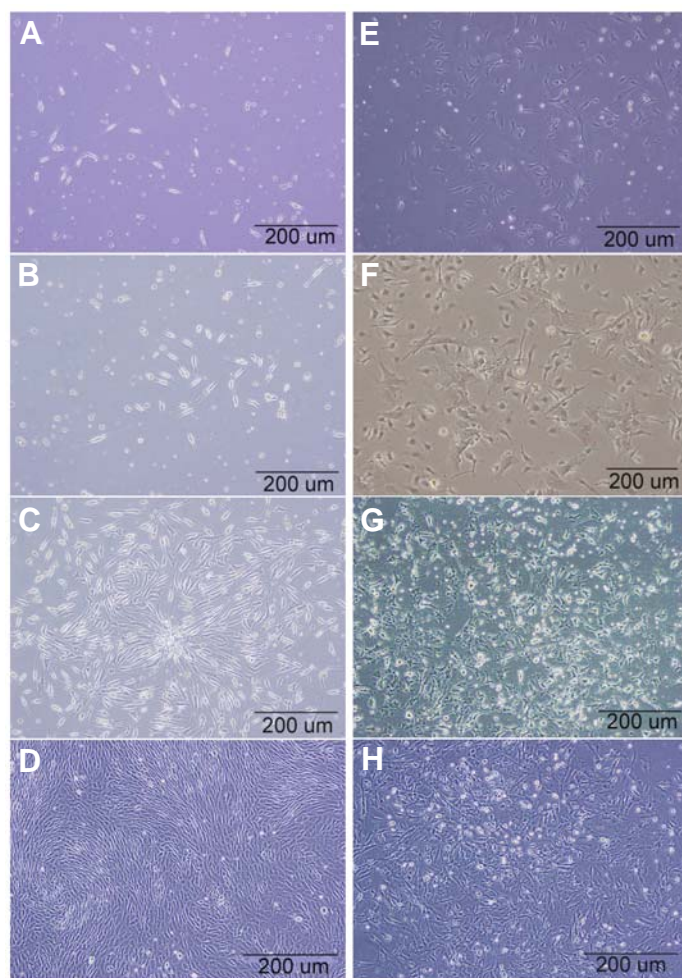


Fig. 1. Mouse bone marrow cells culture. (A-D) Cultures exposed to frequent medium changes (FMC). (E-H) Cultures without frequent medium changes (WFMC). (A) Morphological features of in vitro expanded spindle-shaped FMC cells which appeared on day 3. (B) More confluent FMC culture at 8 days and (C) 15 days after isolation (D). Purified populations of spindle-shaped cells appeared on the first passage. (E) Three days after culture initiation: some WFMC cells demonstrated fibroblastic morphology. (F) Eight days after culture initiation, an admixture of WFMC cells was observed. (G) Small round cells appeared on adherent cells after 15 days in culture. (H) A heterogeneous population of WFMC cells appeared on the first passage. Original magnification, $\times 10$

RNA extraction and RT-PCR analysis of gene expression

Total RNA was isolated from cells by using the Nucleospin RNAII kit (Macherey-Nagel, Germany). Prior to reverse transcription (RT), RNA samples were digested with DNase I (EN0521; Fermentas) to remove contaminating genomic DNA. DNase I was dissolved in 10X reaction buffer with $MgCl_2$, and 1 μ l of DNase I was added per 1 μ g of RNA and incubated for 30 min at 37°C. DNase I activity was arrested following addition of 1 μ l of 25 mM EDTA and incubated at 65°C for 10 minutes. Standard RT was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) and 2 μ g total RNA, 0.5 μ g oligo (dt_{18}) per reaction, according to the manufacturer's instructions. Reaction mixtures for PCR, included 2.5 μ l cDNA, 1x PCR buffer (AMS™, Sinagen, Iran), 200 μ M dNTPs, 0.5 μ M of each of Forward and Reverse primers and 1U Taq DNA polymerase (Fermentas, MD, USA). The primers are listed in Table 1. Polymerase chain reactions were performed at 94°C for 1 min, 25-30 cycles 94°C for 30 s, 55-63°C for 30 s, and 72°C for 30 s and 72°C for 10 minutes. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidium bromide (10 μ g/ml) and photographed on a UV transilluminator (uvidoc, UK).

Flow cytometry analysis

The cells were detached from culture dish with Trypsin/EDTA and counted. About 2×10^5 cells were divided into aliquots in amber-tinted 5 ml centrifuge tubes and 3% rat serum was added. The cells were incubated on ice for 30 minutes, resuspended in 400 μ l PBS and pelleted by centrifugation for 10 minutes at 400Xg. Then the cells were stained with Fluorescent isothiocyanate (FITC)-conjugated rat anti-mouse Sca-1, CD34, CD11b, CD45, CD90.2 (Thy1.2), CD31, CD106 (Vcam-1), CD117 (c-Kit), and also Phycoerythrin (PE)-conjugated rat anti-mouse CD44, CD135 (eBioscience, San Diego, CA) at a concentration of 2 μ g/ml at 4°C for 30 minutes. The cells stained with FITC-or PE-labeled rat anti-mouse IgG served as controls. The cells were pelleted, washed twice with PBS and fixed with 1% paraformaldehyde in PBS. Cells were examined by FACS Calibur cytometry (Becton Dickinson, San Jose, CA) and analyzed using cell quest software. WinMDI 2.8 software (Scripps Institute, CA) was used to create the histograms. The SPSS software package (Version 12.0; SPSS, Chicago, IL, USA) was used for the statistical tests. Data were presented as mean \pm standard deviation. The Student t-test was used to analyze the surface markers of frequent medium change (FMC), WFMC, mMSCs (first passage cells) and non-lifted cells.

Results

Cell culture

Bone marrow was harvested from Balb/c ($n=5$) and plated into 35 mm culture dish at the density of 25×10^6

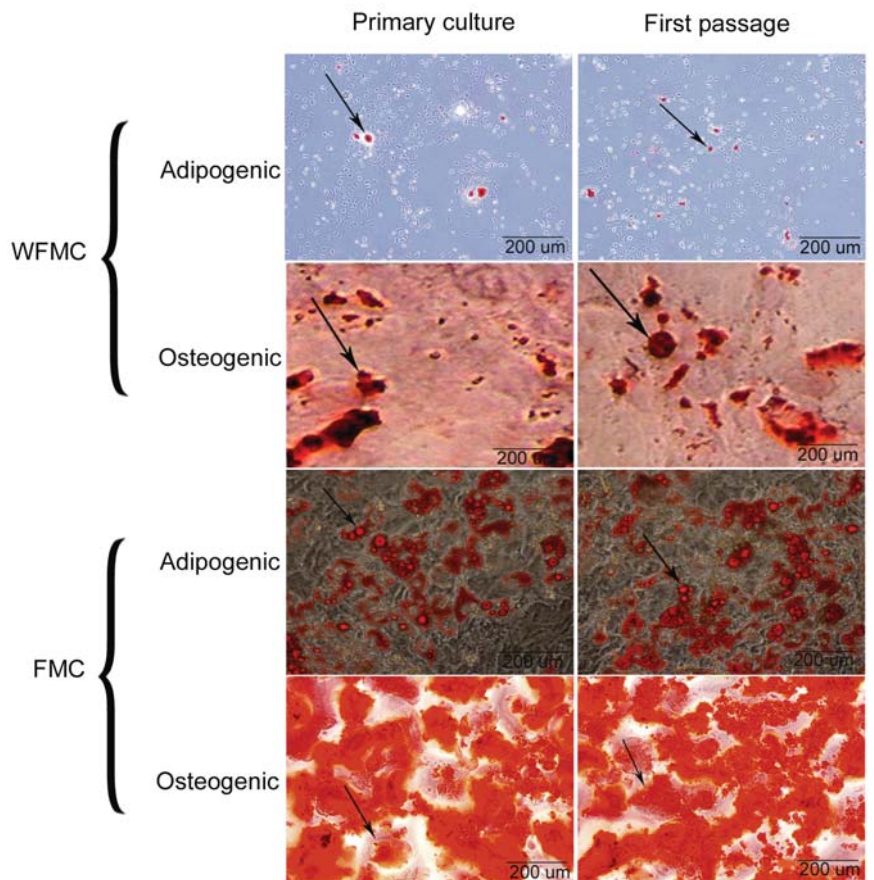


Fig. 2. *In vitro* differentiation of the cells. Osteogenic and adipogenic differentiation of WFMC and FMC cells were examined after first passage. Arrowheads indicate the nodule-like structures stained with Alizarin red and adipose droplet stained with Oil red. Original magnification, X10

cells. Non-adherent cells were carefully removed after 3-4 hours and 1.5 ml fresh medium was replaced. Thereafter, this step was repeated every 8 hours for up to 72 hours of initial culture. Then, the adherent cells (passage 0) were washed with phosphate-buffer saline (PBS), and fresh medium was added every 3-4 days. The initial adherent spindle-shaped cells appeared as individual cells on the third day (Fig. 1A). In 4-8 days culture became more confluent (Fig. 1B), and reached 65-70% of confluence within two weeks (Fig. 1C).

TABLE 1

SPECIFIC PRIMERS USED FOR PCR AMPLIFICATION

Annealing temperature (C°)	Size (bp)	Primer sequences	Genes
62	376	F: 5' GAG GAC ACT TGT CAT CTC ATT C3' R: 5' CCT TCT TAT TGGTCA GAC TTC C 3'	Lipoprotein lipase (LP)
57	309	F: 5' ATG GTA TGA TGT GCA GAG TGT AG 3' R: 5' CAC ACA TCA TGT TAA TGG TGA C3'	Adipsin
60	276	F: 5' GAC CAT CTT TCT GCT CAC TCT G 3' R: 5' GTG ATA CCA TAG ATG CGT TTG TAG 3'	Osteocalcin (OC)
67	377	F: 5' CAG TGA TTT GCT TTT GCC TGT TTG 3' R: 5' GGT CTC ATC AGA CTC ATC CGA ATG 3'	Osteopontin (OP)
67	448	F: 5' GAC AAG CTG CTC AAG GAA GTT CTG 3' R: 5' GGA ATA TCC CAC GGT GTA GAT CAT G 3'	PTH receptor
63	317	F: 5' GGAACATAGCCG TAAACTGC 3' R: 5' TCACTGTGCCTGAACCTACC 3'	β -Tubulin

Many cells with spindle-shape morphology were lifted and cultured in another flask (25 cm²). These cells (lifted cells along first passage of FMC cultures) were cultured for one week until confluence was achieved. Cell population appeared to be more homogeneous of the spindle-shaped cells (Fig. 1D).

WFMC cultures demonstrated heterogeneous cell populations with diverse morphologies including flat, spindle-shaped, and polygonal cells (Fig. 1E-H).

To ensure that the purified cell population did not contain cells with abnormal proliferative characteristics, we tested tumorigenic potentialities in immunodeficient mice. Total number of 5×10^5 spindle-shape cells (passage 2) were injected into the flank area of SCID mice ($n=3$). No sign of formation of tumor was observed after four weeks (data not shown). The GL26 glioma cells (5×10^5 cells) were also injected into SCID mice ($n=3$) as controls. All GL26 glioma injected animals developed tumor within 2 weeks. In summary, these data showed that our cell population was free of transformed or tumorigenic cells.

Colony forming unit - fibroblast (CFU-F) assay

CFU-F assay provides a convenient means of assessing the proliferation and clonogenic capacity of the cells (first passage) expanded in culture. The results showed that 70 ± 3.4 and 20 ± 4.2 (Mean \pm SD) colonies were formed from FMC and WFMC cultures, respectively.

Differentiation assay

The first passage cells (bipolar fibroblast-like cells) were readily differentiated into osteocyte cells and adipocytes by culturing in appropriate induction media. None of the lifted cells in primary culture differentiated into mesenchymal lineages (data not shown). A lower level of osteogenic and adipogenic differentiation was

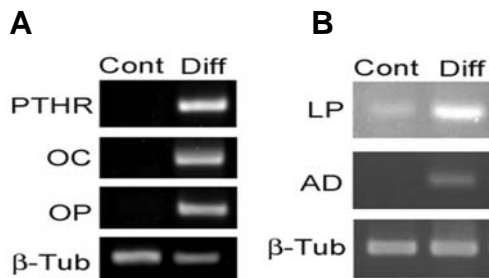


Fig. 3. RT-PCR analysis of differentiation. (A) The osteoblast markers, osteocalcin (OC), osteopontin (OP) and parathyroid hormone receptor (PTHr) were expressed in differentiated cultures (day 21), whereas control undifferentiated cultures were negative for these genes. (B) The adipocytic markers, lipoprotein lipase (LP) and adipisin (AD) were expressed in differentiated cells (day 21). β -Tubulin is shown as a loading control.

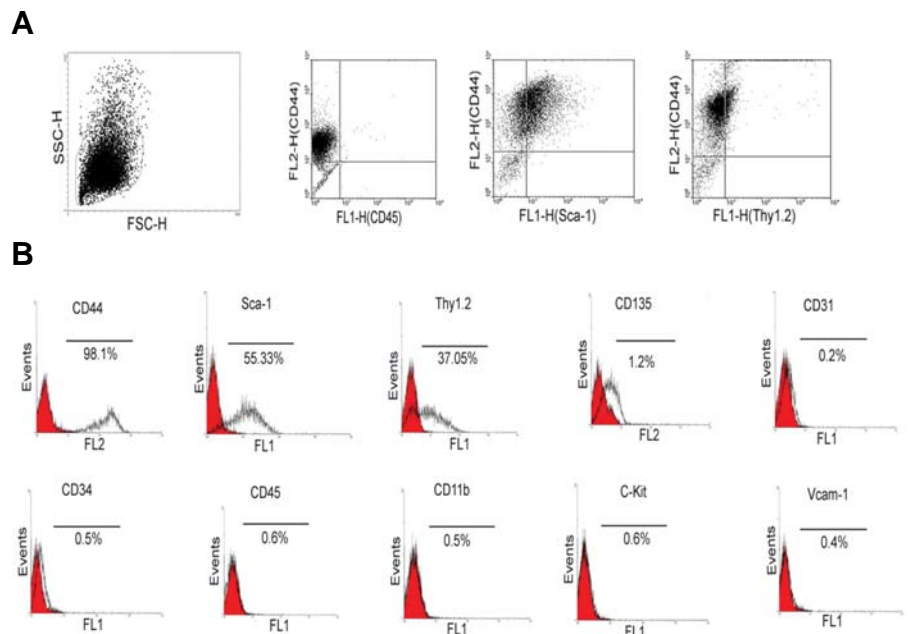


Fig. 4. Assay of cell-surface antigens on murine mesenchymal stem cells (mMSCs). (A) Characteristic homogeneous FSC-HxSSC-H and double-stained dot plot exhibited by Fibroblast-like cells (first passage). (B) Histogram analyses of cell surface markers of mMSCs. The respective isotype control is shown as red.

observed in WFMCs (Fig. 2).

In osteogenic cultures nodule-like structures were observed which stained with alizarin red (Fig. 2). Osteocyte specific genes such as parathyroid hormone receptor (PTHr), osteocalcin (OC) and osteopontin (OP) were expressed in differentiated cells after three weeks of induction (Fig. 3A).

In adipogenic cultures, intercellular lipid vacuoles were stained with Oil red (Fig. 2). Expression level of adipocyte genes such as lipo protein lipase (LP) and adipisin (AD) genes were elevated in differentiated cells, confirming the adipogenic potentials of MSCs (Fig. 3B). The mMSCs retained the osteoblastic and adipocytic differentiation potential up to passage 10.

Analysis of cell surface markers

The cells (WFMC and FMC culture, first passage and non lifted cells in first passage of FMC culture) were analyzed for cell surface antigens. Results showed that mMSCs from BALB/c mice were negative for CD31, CD34, CD11b, CD45, c-Kit, Vcam-1, and CD135 antigens. mMSC were positively stained with monoclonal antibodies against CD44, Sca-1, and Thy1.2 (CD90) (Fig. 4A,B). Our results indicated some significant differences among bone marrow cells cultivated with FMC and WFMC (Fig. 5A). Furthermore, non-lifted cells in the first passage showed significantly less expression of CD45, CD11b antigens compared with mMSCs (first passage) (Fig. 5B).

Discussion

Isolation and purification of murine MSCs is far more challenging than that of other species due to the low frequencies of MSCs in bone marrow and also unwanted growth of non-mesenchymal

cells during the cultures (Eslaminejad *et al.*, 2006). In present study, spindle-shaped fibroblast-like cells from Balb/c mice bone marrow were purified by repeated medium change at initial hours of culture and diminishing the trypsinization time.

Bone marrow-derived adherent cells have been found to contain different cell types including fibroblasts, hematopoietic progenitor cells, macrophages, endothelial cells and adipocytes (Phinney *et al.*, 1999; Tavassoli and Takahashi, 1982; Dexter *et al.*, 1984; Zuckerman and Wicha, 1983). Previous studies have demonstrated that these cells remain in the culture, contaminating fibroblastic cells (Meirelles and Nardi, 2003; Phinney *et al.*, 1999; Xu *et al.*, 1983).

Using the method described in this study frequent medium change may prevent adherence of many of the non-MSC and hematopoietic populations to the culture dish. Our result indicated that CD31, Vcam-1, CD34, c-Kit and CD135 antigens have not been present in FMC cells. These antigens were considered as endothelial, myeloid and hematopoietic cell specific antigens (Sun *et al.*, 2003; Baldwin *et al.*, 1994; Kinashi *et al.*, 1995; Ikuta and Weissman, 1992; Simmons and Torok – Storb, 1991). In WFMC cultures, heterogeneous cell populations with diverse flat, spindle-shaped and polygonal cell morphology were observed. These heterogeneous cell populations were partially differentiated along mesenchymal lineages and expressed high levels of hematopoietic antigens. Above mentioned antigens were expressed by a very limited number of control cultures which is in agreement with the previous data indicating the existence of hematopoietic cells in primary culture of the murine bone marrow cells (Phinney *et al.*, 1999; Xu *et al.*, 1983; Wang *et al.*, 1990).

MSCs also were shown to be more responsive to trypsin (Digirolamo *et al.*, 1999) while monocytes and macrophages firmly adhered to cell culture dishes (Sun *et al.*, 2003). Non-lifted cells (along first passage) expressed high levels of both CD11b and CD 45. Previous studies showed that CD11b antigen was expressed in monocytes, granulocytes and natural killer cells, this data also indicated that CD45 was detectable in all cells of hematopoietic lineage (Springer *et al.*, 1978; Ledbetter and Herzenberg, 1979). The differentiation potentials of non-lifted cells into mesenchymal lineages was also examined. The results showed that these cells do not have differentiation potential into osteocytes and adipocytes. The properties of the non-lifted cells allowed us to conclude that they do not show mesenchymal characteristics. Also, these cells had low growth capability, which was possibly due to the absence of MSC populations since MSCs may support the growth of hematopoietic cells (Oostendorp and Dormer, 1997; Janowska-Wieczorek *et al.*, 2001). In the first passage, monocytes and macrophages have been depleted in fibroblast-like cells in comparison with non-lifted cells. The results showed that two minutes is optimum for trypsin treatment which was detected by assaying over a range of trypsinization times (data not shown).

Using this method, purified population of fibroblast-like cells with high proliferation and differentiation potential into mesenchymal lineages was obtained in the first passage (three weeks after the initiation of culture). These cells maintained both potentials up to passage 10. Osteoblastic differentiation was demonstrated by the expression of PTHR, OC and OP and the accumulation of a bone-like mineralized matrix. Adipocytic differentiation was shown by the expression of AD, LP and cytoplasmic lipid accumulation.

This piece of evidence together with fibroblastic morphology and clonogenic capacity of the cells allowed us to conclude that these cells (fibroblast-like cells) were MSCs.

To date, several protocols have been developed for isolation of MSCs from murine bone marrow; among them, Baddo *et al.* (2003) and Tropel *et al.*, (2004) utilized immunodepletion techniques for the isolation of MSCs. mMSCs isolation by

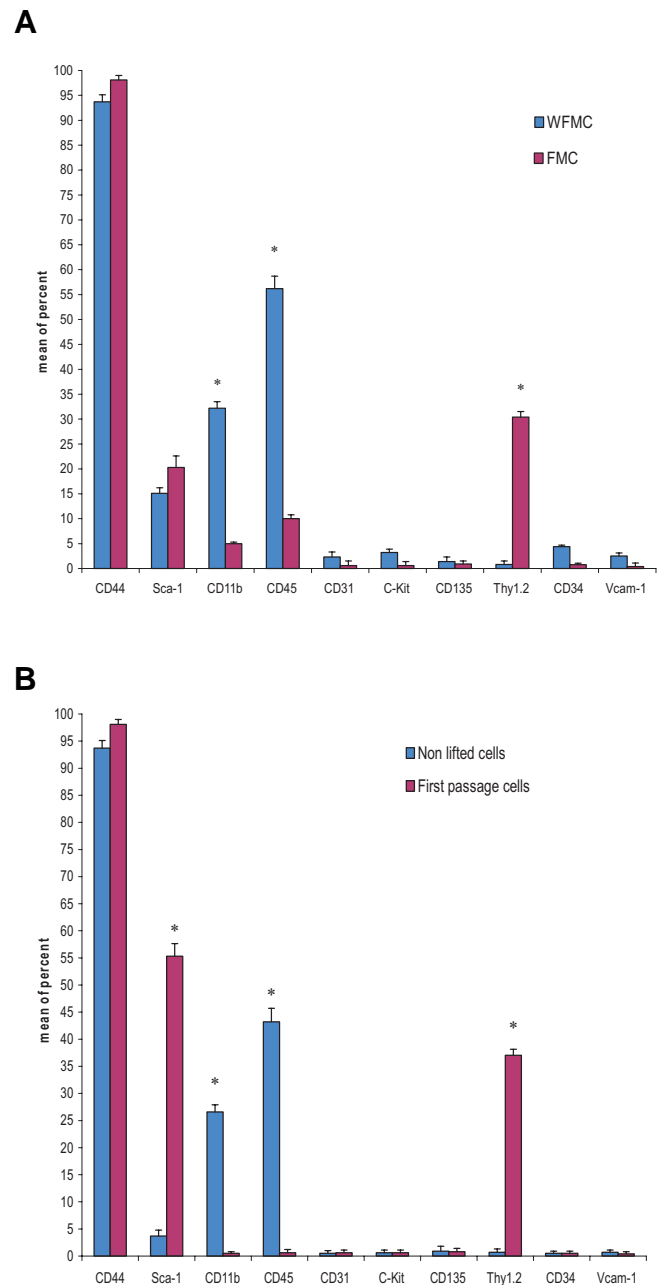


Fig. 5. Flow cytometry analysis of expanded cell cultures. (A) The expression of CD11b, CD45 and Thy1.2 surface antigens in WFMC cultures was significantly different from that of FMC cultures. **(B)** The expression of some cell surface markers on non-lifted cells was significantly different from mMSCs in first passage. Data are expressed as the mean \pm SD, $n=10$ (*, $p \leq 0.001$; Student *t*-test); the standard deviation of the data points is illustrated by bars.

immunodepletion method is relatively effective and the cells prepared with immunodepletion are capable of multilineage differentiation. However, down regulation of some of the genes involved in cell proliferation and cell cycle progression has been reported. Furthermore, Tropel et al., (2004) found that even after eliminating granulomonocytic cells using CD11b antibody, such cells continued to exist in the culture one week after immunodepletion. Results obtained from CFU-F assay and long term expansion of MSCs indicated high proliferation potential of isolated cells. The expression of CD11b was negative throughout the cultivation period. Van Vassler et al., (1999) described the cell sorting technique for the isolation of mMSCs. This approach yielded a cell population exhibiting reduced clonogenicity and osteogenic potential as compared to unsorted cells which was possibly due to the shear forces generated during the sorting procedures. The approach adopted in the present study was simple and exerted minimal stress on fibroblastic cells.

An unknown aspect of mMSCs which can be used to separate them from bone marrow is recognition of surface antigens. There is a dispute between researchers in using a unique surface antigen profile which describes mMSCs (Sun et al., 2003; Peister et al., 2004; Baddoo et al., 2003). In this study, the expression of CD44, Thy1.2 and Sca-1 has been widely observed in mMSCs. CD44 marker was expressed by leukocyte, erythrocyte and non-hematopoietic cells and mediates cell attachment to extracellular matrix (Trowbridge IS, 1982). This marker was expressed on both cell types in a high level. This was not beyond conception related to this project's results. Thy1.2 is an antigen for endothelial and lymphocyte T cells (Baldwin HS, 1994). Its expression at high levels in MSCs, and its lack of expression in non-lifted cells suggested that this marker could be used as an mMSC marker. Sca-1 is considered as a hematopoietic cell marker (Peister et al., 2003). The expression of Sca-1 in mMSCs has been reported (Peister et al., 2003; Baddoo et al., 2003; Sun S et al., 2003). In present study, the percentage of sca-1 positive cells was about 55%. However, it is very likely that some known antigenic determinant for hematopoietic and endothelial cell lineage may be expressed on mMSCs as well.

Collectively, results of our study indicated that we have developed a new protocol for isolation of mMSCs from the heterogeneous mixture of bone marrow cells, mainly based on frequent medium change in primary culture and diminishing the trypsinization time.

Acknowledgments

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