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Identification of Cultured Progenitor Cells From Human Marrow Stroma

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Abstract The marrow stromal cells (MSC) are essential for regulation of bone remodeling and hematopoiesis. It is of prime importance to isolate MSC and to expand the proliferating cells ex vivo. In this study, we analyzed cultured MSC for various cellular parameters, including cell morphology, cell cycle, and expression of cell surface antigens by flow cytometry. MSC were divided based on cell size to small (S-cells) and large (L-cells) and were visualized by light and electron microscope. The S-cells were proliferating cells correlated with G_0/G_1 phase of cell cycle, and expressed cFOS. The expression of surface markers CD-34, -44, -51, -61, -62E, -62P, -62L was quantified using flow cytometry. CD-44 was ubiquitously expressed by S and L cells, CD-51 and -61 were expressed by 30%–38% of S-cells. CD-34 and -62 expressed 20% positive of the analyzed cells that were of the proliferating progenitors (S-cells). This study enables the identification of subpopulations from MSC with special attention paid to the proliferating cells from ex vivo cultures of marrow stroma. J. Cell. Biochem. 87: 51–57, 2002. © 2002 Wiley-Liss, Inc.

Key words: marrow stromal cells; surface markers; cell cycle

The marrow stromal cells (MSC) are of mesenchymal origin capable to differentiate into distinctive cell types. These cells support the growth of hemopoietic cells, and play a role in the maintenance of the skeleton throughout life. MSC are represented by heterogeneous populations of cells at different stages of maturation, including progenitor cells with high proliferating capacity that can differentiate into various lineages, including fibroblasts, adipocytes, cartilage, myogenic, and osteogenic cells [Friedenstein et al., 1978; Owen and Friedenstein, 1988; Benayahu et al., 1989; Haynesworth et al., 1992; Triffitt et al., 1998; Benayahu, 2000; Aubin, 2001]. Cell differentiation through distinct maturational stages into specific characterized cells involves coordination and activation of different sets of genes. Distinct transcription factors and master genes have been reported to control discrete differentiation steps for various cell lineages. Peroxisome proliferator-activated receptor-gamma 2 (PPAR-γ2) is expressed by adipocytes [Tontonoz et al., 1994; Rosen and Spiegelman, 2000]; Myf5, myogenin, and MRF4 are expressed by muscle cells [Buckingham, 1994; Black and Olson, 1998; Beauchamp et al., 2000] and cbfa1 by osteoprogenitors [Nakashima et al., 2002]. Osteoprogenitors express alkaline phosphatase, CD10/NEP, and cell surface markers that are variable along the cells' differentiation stages from progenitors to mature osteoblasts [Benayahu et al., 1989, 1991, 1995; Indig et al., 1990; Fried et al., 1993; Fried and Benayahu, 1996; Pittenger et al., 1999; Benayahu, 2000; Aubin, 2001]. The progenitors of MSC are suggested as a source for cell-based therapies and tissue engineering, and mimic the physiology process of the cells in tissues, such as bone, cartilage, and muscle [Bruder et al., 1994; Bruder and Fox, 1999; Weissman, 2000]. Thus, the

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ultimate goal is to identify the progenitor cells in culture and to expand these proliferating cells before their differentiation. This requires the detailed characterization of the proliferating progenitor MSC.

Present study describes the morphology, cell cycle, surface, and nuclear antigens expression by ex vivo cultured MSC with high self-renewal capacity. Studied parameters enable the identification of proliferating subpopulation of MSC adds insight into progenitor cells isolation from human donors and the cultured cells maintenance in vitro.

MATERIALS AND METHODS

In Vitro Culture

Human bone MSCs were collected from surgical aspirates of bone marrow to prepare ex vivo culture plated at low-density (1.5×10^4 cells/cm^2). Cells were derived from normal 2–13 years old donors ($n = 8$), and cultured in growth medium containing Dulbecco's modified essential Medium (DMEM) with the addition of 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Bet-Haemek, Israel). To generate large number of cells from the primary cultures, the cells were trypsinized and single cell suspensions were re-cultured for 7 days and grew up to 80% confluence (Fig. 1).

Transmission Electron Microscopy

Cultured cells were washed in phosphate-buffered saline (PBS) and EDTA released, cells in pellet were fixed in 4% paraformaldehyde in PBS, pH, 7.4 for 1 h at room temperature (RT).

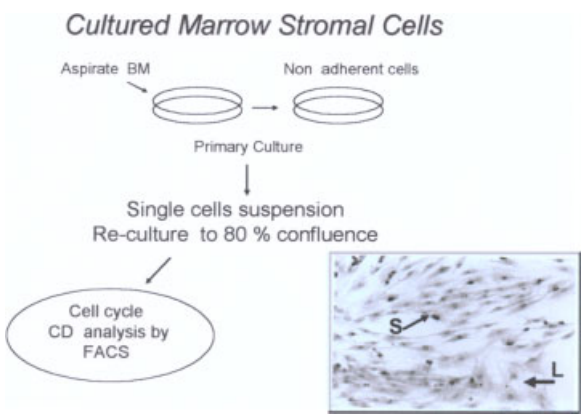

Fig. 1. Schematic diagram of cells cultured from bone marrow to form adherent layer of MSC. Different cells (size and morphology) were visualized by phase contrast microscope.

After washing, the cells were post-fixed with 1% osmium tetra-oxide in PBS, pH, 7.4 for 2 h dehydrated in ethanol and embedded in araldite. Ultra thin sections were cut with a diamond knife in LKB ultra microtome and sections were mounted onto formavar coated grids. The grids were stained with uranyl acetate and lead citrate, and the sections were examined with a transmission electron microscope (Jeol, JEM 100CII).

Immunofluorescence Staining

Cells were stained with antibodies (Ab) to focal adhesion, cytoskeleton and to phosphotyrosine. The focal adhesion was detected with mouse anti-Vinculin. Cytoskeleton was stained with mouse anti Tubulin (Sigma, Israel). Phosphotyrosine was stained with mouse antibody (PY-20, Pharmingen). The cells were incubated with specific Ab for 1 h in RT. The cells were then washed and incubated with goat anti-mouse biotinylated-Ab (Dako, Denmark), for 30 min in RT. Finally, incubation with Extravidine FITC-conjugated (Sigma, St. Louis, USA) for 30 minutes in the dark in RT. Nuclear staining was detected using 4,6 diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma) on cells fixed in 200-mM KCl. The staining with 0.25 μg/ml DAPI dissolved in 100-mM NaCl, 10-mM EDTA, and 10-mM Tris buffer. Cells were visualized by Zeiss fluorescence microscope, and pictures were taken using Zeiss Axiophot photomicroscope and Kodak TMAX 3200 film.

Flow Cytometry Analysis (FACS)

Surface and intracellular antigens were analyzed according to Pharmingen technical protocols (http://www.pharmingen.com). We used single cells suspension that were EDTA released and stained with various Ab–biotin conjugated to CD-44, -34, -51, -61, -62 E, P, L (Pharmingen). For each antibody, 10^6 cells were re-suspended in PBS containing 1% FCS, (blocking buffer), and were incubated for 30 min on ice in 50 μl of each antibody solution. After washing, cells were stained with secondary Extravidine-FITC-labeled antibody (Sigma). For cell cycle analysis, intracellular staining was performed with anti-cFOS (Oncogene Science, Inc.) and anti-Cyclin B1 (Pharmingen). Negative control for the intracellular staining was isotype-matched IgG of irrelevant specificity used at the same concentration as the

antibody of interest. In addition, propidium iodide (PI, 50 μg/ml; Sigma) staining for DNA of single cell suspensions, cells were treated with 0.2% Triton-X-100 (Sigma) with PI. Cells (1×10^4) were collected for each sample, and statistical analysis was performed using software from Becton Dickinson.

RESULTS

Bone marrow cells were cultured under conditions promoting the hematopoietic cells death in culture and formation of an adherent layer of MSC. The cultured cells appeared heterogeneous in shape and size; they posses a phenotype of small spindle-shaped fibroblastic or large body size cells (Fig. 1). We used electron microscope to analyze the ultra structure of cells of different size: large (L-cells, Fig. 2A) or small cells (S-cells, Fig. 2B). The cells contained a pale nucleus and their plasma membrane was ruffled and posses many cytoplasm processes. The cells have a respective characteristic pattern of rough endoplasmic reticulum (RER) with dilated cisternae with or without dense content and vesicles of different size and shape. Stromal cells are active in matrix production, an extracellular matrix (M) is visible adjacent to the cell (Fig. 2B,C).

Cells Size

Small (S) and large (L), cell size and cell cycle stage were analyzed by FACS (Fig. 3). Approximately, 80% of the cells were of small size (S-cells) (less then 30% of maximum cell size, Fig. 3A). The cell cycle stage of these cells was analyzed by the expression of cFOS and cyclin B1 (Fig. 3B). The expression of c-FOS (measured cells at G1 to S phase) and Cyclin-B1 (measured cells at S to G2/M phase) was $63 \pm 7\%$ and $28 \pm 3\%$, respectively (Fig. 3B). In addition, PI staining for DNA revealed that 75% of cells were at $G0/G_1$ phase (Fig. 3C). FACS was used to analyze the surface markers expression for CD-34 receptor, CD-44-hyaluronic acid receptor, CD-61 and -51-subset of integrin family $\alpha_v\beta_3$ and of selectin family CD-62 E, P, L (Table I, Figs. 4–6). The expression of CD-44 was $85 \pm 8\%$ of the cultured cells (Fig. 4), with 60% of cells defined as S-cells (Figs. 5 and 6). The expression for CD-51^+ and CD-61^+ cells was $30.2 \pm 12.6\%$ and $38.25 \pm 12\%$, respectively (Fig. 4), and most were found to be of S-cells population (Figs. 5 and 6). Approximately, 20% of the cells were of subtypes from CD-62^+, and most belong to the S-cells (Figs. 4 and 5). A similar pattern was detected for MSC cells analyzed for CD-34 expression ($22.4 \pm 8\%$, Figs. 5 and 6) most cells, 97% were found to be S-cells (Fig. 6). In summary, CD44 was ubiquitously expressed, CD-51 and -61 stained ~35% of cells, CD-62 and -34 stained 20% of the cells. These surface markers identified mainly the S-cells proliferating progenitors cells.

The adherent cells interact with the substrate through focal adhesion located at the edges of cell processes. We used antibody to vinculin (V) to stain an anchor protein that is specifically associated with cell–ECM and cell–cell interactions (arrow Fig. 7B–C). The cells were speared out and posses a well-developed cytoskeleton demonstrated with antibody to tubulin (T) (Fig. 7A–C). When cells were attached to the substrate, they expressed high level of phosphotyrosine (PY-20) (Fig. 7D).

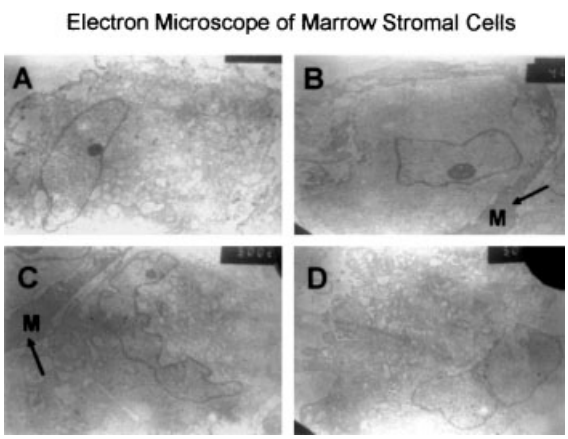

Fig. 2. MSC ultrastructure study by TEM. The cells have different size; large cells were visualized at magnification 5,400× (**A**), and smaller cells are at 8,000× (**B**), 10,000× (**C** and **D**). The cells are active in matrix (M) production (B, C). They have a pale nucleus and a well-developed RER with dilated cisternae and many cytoplasm processes (D).

DISCUSSION

MSC are important in vivo for the maintenance of the hematopoietic microenvironment and the skeleton. The MSC are of mesenchymal origin and contain progenitor cells that differentiate through multiple maturation pathways [Friedenstein et al., 1978; Owen and Friedenstein, 1988; Benayahu et al., 1989; Triffitt et al., 1998; Benayahu, 2000; Aubin, 2001]. In this study, we identified the proliferating MSC by morphological means, markers for cell cycle,

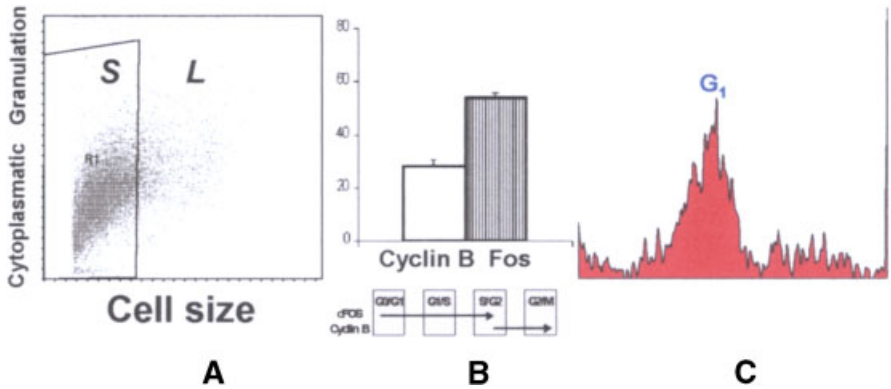

Fig. 3. FACS analysis of MSC distribution based on (**A**) cells size (S, small; L, large). **B**: Cell cycle distribution based on the cfos and Cyclin B1 expression. **C**: The cell cycle stage was determined using PI for DNA staining.

and surface protein expression. The MSC morphology was demonstrated using light and electron microscope (cell size and shape). It was shown that early progenitors are small spindle-shaped (S-cells) with high proliferating capacity. The S-cells are different from the L-cells that have many cellular processes and to represent advanced stages of differentiation [Fried et al., 1993]. In the present study, we analyzed MSC cultured from young donors (up to age 13) and identified cells with high proliferating capacity presented by the small (S) cells that composed 80% of total cultured MSC, while the large (L) cells composed 20%. Cell cycle regulatory proteins govern cell proliferation. It was demonstrated that MSC express low levels of cyclin B1 (~25%) and high levels of cFOS (~70%). The findings are consistent with multiple lines of evidence linking activity of these early response genes to regulation of cell growth. The levels of cell cycle regulatory proteins (cyclins A, B, and D1) were studied and indicated a marked increase of cyclin B in the late differentiation stage. An elevation of cyclin B is observed in osteoblasts maintained under culture conditions that support differentiation [Smith et al., 1995]. cFOS is expressed in osteoprogenitor cells and not in mature osteoblasts in vitro and in vivo. Proliferating cells exhibit a prolonged induction of Fos/Jun family members in response to serum. The reduction of proliferation capacity is accompanied by the cells progress towards differentiation into specific lineage [Machwate et al., 1995]. The goal to identify and maintain in vitro proliferating MSCs requires better characterization of these cells. We further demonstrated that the diversity of cells based on their size is correlated with differences in cell surface antigens expression. MSC were analyzed by flow cytometry for the expression of surface antigens in association with cell size. Hence, we elaborated the differential expression of surface markers in relation to cell proliferation used to discrete the population of S or L cells. The CD44, hyaluronic acid receptor that mediates cell attachment to hyaluronate was quantified in both small and large cells. Other studied demonstrated the ubiquitous expression of CD-44 by MSC [Benayahu et al., 2000; Deschaseaux and Charbord, 2000]. CD-61 and -51, subsets of integrin family receptors were expressed mostly by

TABLE I. Classes of Cell Surface Receptors Analyzed for the Expression by MSC Cells

Family	Example	CD no.	Ligands
Integrin	$\alpha_v\beta_3$	CD51/CD61	Matrix proteins
Selectin	Selectin L	CD-62L	Glycan-1, CD-34, CAM-1, Lectin
	Selectin P	CD-62P	Lectin
	Selectin E	CD-62E	
CD-44	PgP	CD-44	Hyaluronic acid

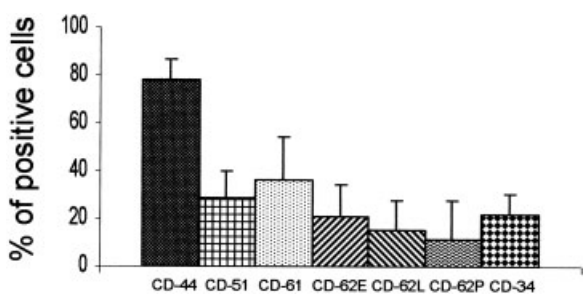

Fig. 4. Histogram summarizes FACS analysis of positive stained MSC for surface antigen expression (CD-44, -51, -61, -62E, L, P, and CD-34). Histogram presents average measurement for various donors ± SD.

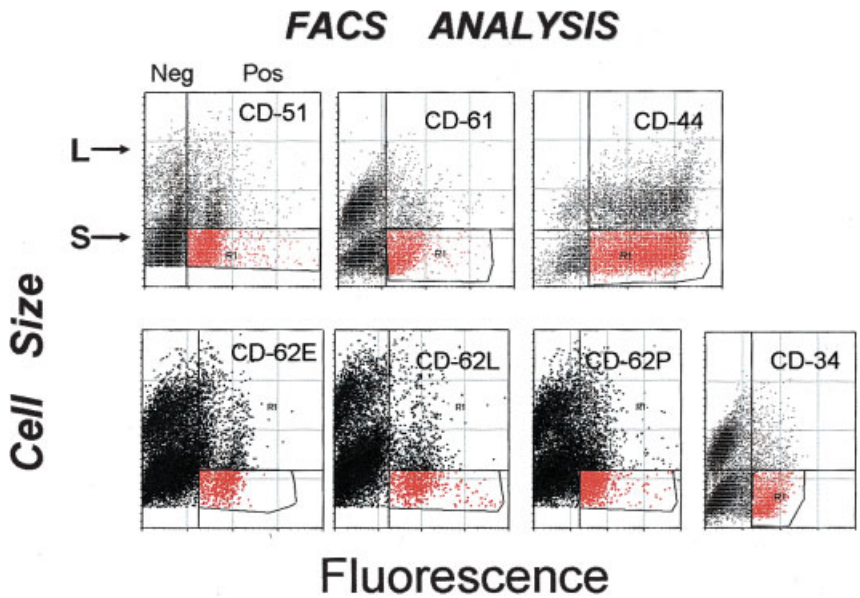

Fig. 5. FACS analysis for each surface antigen expression CD-44, -51, -61, -62E, L, P, and CD-34 measured by fluorescence intensity of the MSC was correlated to cells size (small and large population).

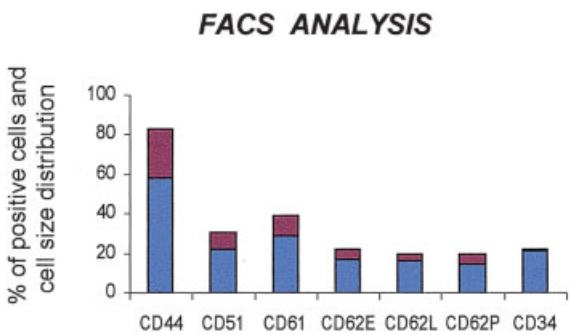

Fig. 6. Histogram summarizes analysis for each surface antigen expression CD-44, -51, -61, -62E, L, P, and CD-34. The results are represented as percentage of positive cells subdivided to small cells (S-cells, blue) and large cells (L-cells, red).

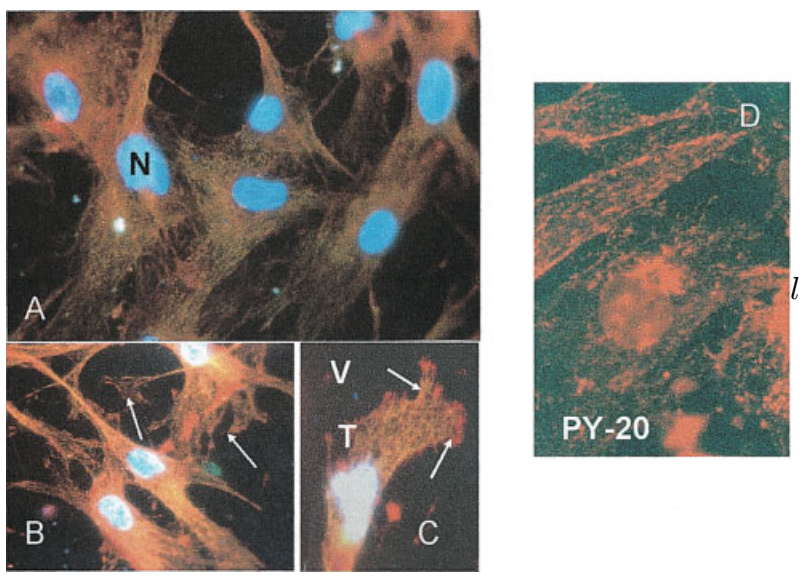

Fig. 7. (**A–C**) MSC visualized by immuno-fluorescence staining for cytoskeleton by antibody to tubulin (T), and for focal adhesion using antibody to vinculin (V) (white arrow). (**D**) The well spread cells were then stained for tyrosine phosphorylation with PY-20 antibody. Cell nucleus (N) was stained with DAPI.

the S-cells of cultured MSCs. The receptors of the integrin family bind to many matrix proteins via an RGD sequence. The attachment of the cell is important for its activation in a specific microenvironment. ECM composition and physical properties affects the activity of adherent cells through integrin receptor that mediates the cytoskeleton assembly and intracellular signaling. Integrins are associated with two distinct types of adhesions."Classical" type displays a high level of tyrosine phosphorylation, is enriched with paxillin, vinculin, actinin, and FAK, localizes at the termini of actin stress fibers. The integrins are localized on cell membrane, form fibrillar adhesions of cytoskeletal components, and posses low levels of tyrosine phosphorylation [Damsky, 1999; Katz et al., 2000]. Herein, we demonstrate that cultured MSC are spread out and interact with the substrate, forming focal adhesion at the edges' of their processes. The attached cells also posses well-developed cytoskeleton, and express high levels of phosphotyrosine.

Another set of surface proteins, selectins, were reported to provide interaction between hematopoietic cells and stromal cells [Sackstein, 1997]. The regulation of hematopoietic progenitors is dependent upon bone marrow microenvironment created by discrete cell–cell and cell–matrix based on receptor–ligand interactions. The selectin proteins are part of the variety of adhesion proteins that were detected by progenitor cells, the S-cell subset of the stromal cells. Interestingly, we identified the $CD\text{-}34^{+}$ expressed by the small percentage of MSC mostly S-cells, while in contrast, L-cells did not exhibit this marker. The CD-34 is a glycoprotein attributed to early hematopoietic progenitors, and is important in maintaining interaction between stromal elements (cells and/or matrix) to hemopoietic. We identified also the CD-34 expression by stromal precursors from human adult bone marrow. Our data is in agreement with the earlier report that demonstrated correlation of CD-34 and STRO-1 expression identified on multipotential MSC [Simmons and Torok-Storb, 1991a,b; Gronthos et al., 1999].

In conclusion, our results describe series of cellular markers expressed by progenitors of primary cultured human stromal cells. The markers are useful for providing the necessary control for the identification and maintenance of proliferating cells from the ex vivo cultured MSC. These cells are the basis for further studies in the field of tissue engineering, and the ability to manipulate such progenitor cells will provide a powerful tool for the development of new techniques for generating models of cell therapy strategies.

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