Characteristics of Human Bone Marrow Mesenchymal Stem Cells Isolated by Immunomagnetic Selection

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 1, pp. 3-7, January, 2006
Original article submitted November 16, 2005

Immunophenotype of human bone marrow mesenchymal stem cells was studied after several culturing passages and after cryopreservation. Immunocytochemical analysis showed that bone marrow mesenchymal stem cells acquired homogeneity during *in vitro* culturing, but initially contained heterogeneous populations.

Key Words: *mesenchymal stem cells; bone marrow; immunocytochemical analysis*

Bone marrow mesenchymal stem cells (MSC) are usually isolated by the method described in the 1970s by A. Ya. Friedenstein [3,5]. Modern tasks of cell biology and cell technologies require accurate definitions and characteristics of MSC. We analyzed expression of markers of MSC enriched by the immunomagnetic separation method and cultured *in vitro* during several passages and before and after cryopreservation.

MATERIALS AND METHODS

Human bone marrow aspirate for isolation of mononuclear cells was treated with anticoagulant (heparin, 100 U/ml), diluted 2-fold with RPMI 1640, and incubated for 45 min with 0.02% collagenase II and 10 U/ml DNase I. Mononuclear cells were isolated by centrifugation in HISTOPAQUE 1077 (Sigma) according to manufacturer's instruction.

Bone marrow $CD45^{-}Gly^{-}$ fraction was isolated by negative magnetic separation on MACS columns (Miltenyi Biotec) using antibodies with magnetic balls against glycophorine (Gly) and CD45. $CD45^{-}Gly^{-}$ cells were cultured in IMDM with 20% FBS and passaged after attaining confluence. Some cells were cryopreserved after each passage.

Immunocytochemical analysis was carried out by indirect fluorescent labeling using a panel of primary mouse monoclonal antibodies to human antigens (Table 1; Chemicon, Dako, BD Pharmingen) and second goat antibodies to mouse IgG labeled with Alexa 546 or Alexa 488 (Molecular Probes) according to instructions. Cell preparations were examined under fluorescent Leica DMR and optic Nikon TS100 microscopes.

Adipogenesis was induced over 3 weeks in IMDM with 10% FBS, 50 μM indomethacin, 1.6 μM bovine insulin, 0.5 mM 1-methyl-3-isobutylxanthine, 10^{-7} M dexamethasone (Sigma) or over 2 weeks in IMDM with 10% FBS and 5000 U/ml LIF (Chemicon).

Adipocytes were stained with 0.3% Sudan III (Sigma) in 60% isopropanol for 10 min at ambient temperature.

RESULTS

Bone marrow aspirates were received from A. N. Bakulev Research Center of Cardiovascular Surgery, Russian Academy of Medical Sciences. The

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0007-4888/06/1411-0112

cells were isolated in Ficoll density gradient, after which CD45⁻Gly⁻ fraction was selected by negative immunomagnetic selection. The cells passed 8-10 passages and were analyzed visually and immunocytochemicaly during each passage, before and after crypreservation.

The phenotype of MSC during passages 0-2 was not homogeneous, which was previously noted by other authors [6]. Primary culture consisted of cells with 3 main phenotypes: 20-50-μ large flat cells (Fig. 1, *1*) constituting 10-20% population, small elongated cells (~7 μ, about 80%, Fig. 1, *2*), and small round cells (5-10%) observed only up to passage 2 (Fig. 1, *3*) transforming into 7-μ fraction. We examined 2 round cells, which were photographed every 2 h over 12 h in order to follow up gradual alteration of their morphology towards the fibroblast-like type (Fig. 2). The relationship between these three subpopulations is not yet clear. Our observations indicate that phenotypical heterogeneity of MSC disappears by passage 3.

Immunocytochemical analysis showed that starting from passage 2, the cells were homogeneous by more than 20 markers (Table 1). Blood cells markers (CD45, glycophorine, CD3, CD8, CD14) were not detected even during passage 0, though few cells (less than 1%) expressed CD34 and CD38 during early passages. Some authors indicate that these markers can be expressed by MSC [7], others did not detect these surface markers [2]. During all passages the cells expressed mesenchymal cell markers (vimentin, desmin, prolyl-4-hydroxylase, CD105), osteogenic precursor markers (osteonectin, osteopontin, bone sialoprotein, and alkaline phos-

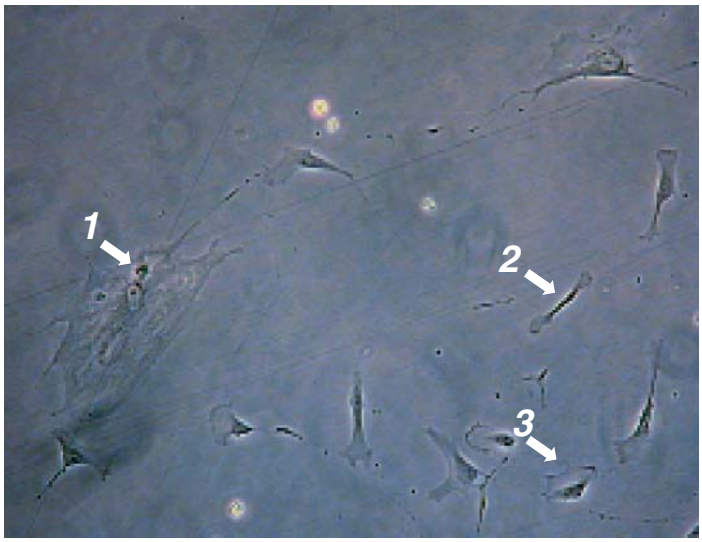

Fig. 1. Phenotypical heterogeneity of mesenchymal stem cell population of passage 0 (5 days in culture); ×10 objective. *1*) subpopulation 1; *2*) subpopulation 2; *3*) subpopulation 3.

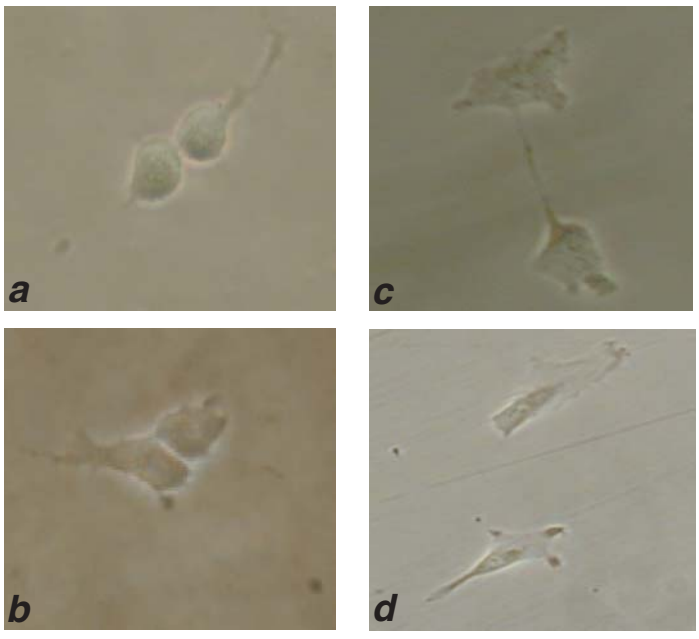

Fig. 2. Mesenchymal stem cells, passage 0, subpopulation 1. Phenotype alteration; ×40 objective. *a*) 0 h; *b*) 4 h; *c*) 8 h; *d*) 12 h.

TABLE 1. Results of Immunocytochemical Analysis of CD45$^-$Gly$^-$ Mesenchymal Stem Cell Cultures at Different Passages

Antibody	MSC (passage 0)	MSC (passage 2)	MSC (passage 8)	MSC (passage 1)	MSC (passage 3) after cryopreservation
CD105	+	+	+	+	+
Prolyl-4-hydroxylase	+	+	+	+	+
Vimentin	+	+	+	+	+
Desmin	+	+	+	N/d	+
CD133	Some positive	-	-	N/d	-
CD34	Some positive	-	-	Some positive	-
CD31	-	-	-	-	-
CD38	Some positive	-	-	Some positive	-
SSEA-4	Some positive	-	-	N/d	-
CD11b	-	-	-	-	-
CD142	-	-	-	-	-
Nestin	+	+	-	+	+/-
CD45	-	-	-	-	-
wWF	-	-	-	-	-
Cytokeratin 19	-	-	-	-	-
Oct-4	-	-	-	-	-
CD41a	-	N/d	N/d	-	-
CD10	Some positive	-	-		+
CD90	Some positive	+	+	+	+/-
CD13	Some positive	+	+	+	N/d
CD117 (c-kit)	Some positive	-	-	N/d	-
Collagen IV	+	+	+	+	+
Osteopontin	+	+	+	+	+
Osteonectin	+	+	+	N/d	+
Bone sialoprotein II	+	+	N/d	N/d	N/d
Endogenous alkaline phosphatase	+	+	+	+	+

Note. "+": expression; "-": no expression; "+/-": weak expression, N/d: no data.

TABLE 2. Antigens Differently Expressed by MSC during Early and Late Passages

Antibody	CD45$^-$Gly$^-$ MSC, passage 0, 10 days in culture	MSC, passage 1	MSC, passage 8
Nestin	+	+ and -	-
CD90	+ and -	+	+
CD10	+ and -	+ and -	-
CD13	+ and -	+ and -	+
CD117 (c-kit)	+ and -	-	-

Note. "+": all cells positive; "+ and -": positive and negative cells; "-": no expression.

phatase), but expression of these antigens and the enzyme varied from one donor to another and in cell cultures (Fig. 4). Few MSC expressed SSEA-4 antigen during passage 0. This antigen is expressed by human fetal carcinoma and fetal stem cells. No expression of SSEA by human MSC was detected previously. We detected several antigens expressed differently by cells of early and late passages (Table 2). Expression of nestin (Fig. 4, *b*), a marker of immature cells, decreased with increasing the number of passages. In addition, expression of CD90, CD117, CD10, and CD13 markers differed in cells of passages 0-1. Subfraction of cells less than 7 μ in size expressed CD117, but not CD10. In order to clear out whether MSC retain the capacity to differentiation after isolation by immunomagnetic

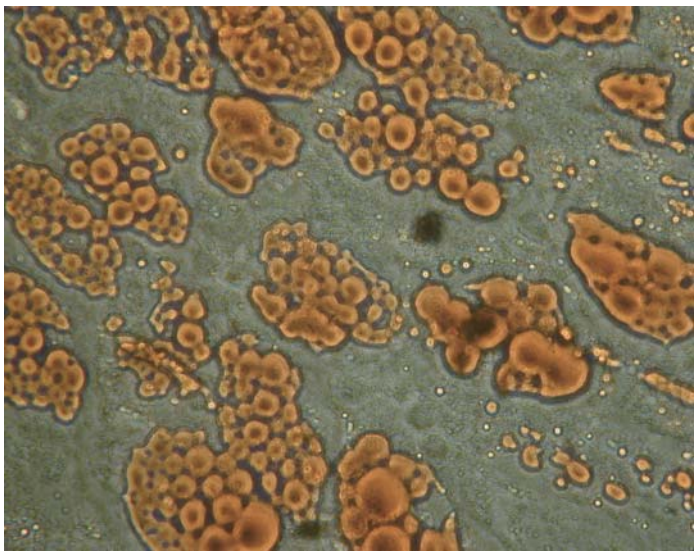

Fig. 3. Adipocytes from mesenchymal stem cells. Sudan III staining; ×20 objective.

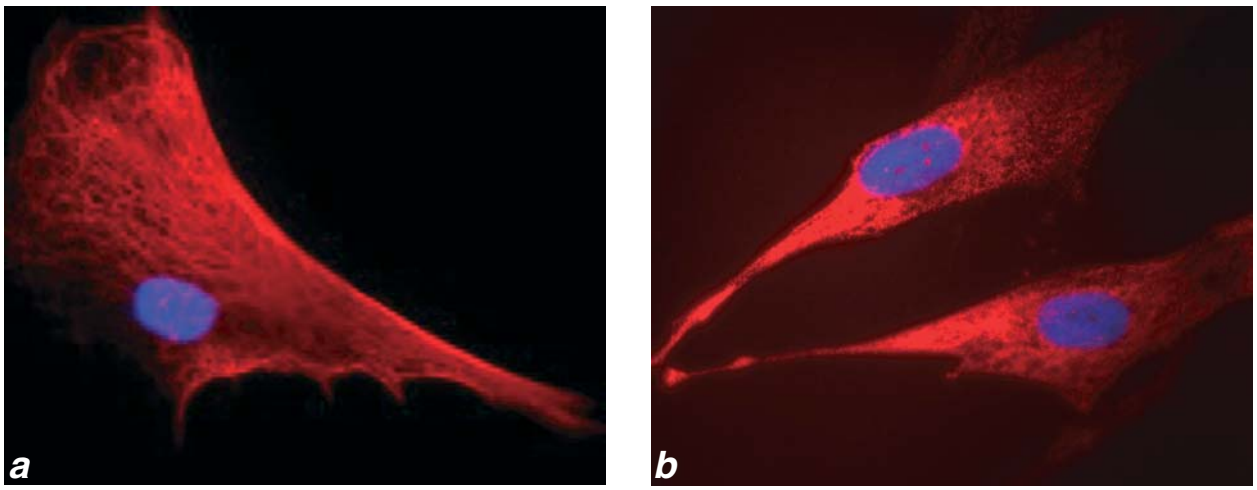

Fig. 4. Immunohistochemical analysis of human CD45$^-$Gly$^-$ mesenchymal stem cells, passage 2. *a*) staining with antibodies to vimentin; *b*) staining with antibodies to human nestin. Nuclei stained by DAPI; ×100 objective.

separation and culturing for several weeks, the cells of different passages were subjected to directed differentiation into adipocytes and osteoblasts. MSC differentiation was evaluated by Alisarine Red staining of mineralized matrix (data not presented). Adipogenesis in cell cultures was determined morphologically (by the presence of lipid droplet incorporations) and by Sudan III staining (Fig. 3).

Cells of earlier passages (0-3) slower differentiated into osteoblasts and adipocytes than populations of later passages, but the percentage of differentiated cells (80-90%) during earlier passages was higher than in cells of passages 6-8 (40-50%). Our observations showed that cells of later passages partially loose bipotency and exhibit a trend to predominant differentiation into osteoblasts or adipocytes. These results confirm the data indicating that bone marrow MSC gradually loose multipotency during culturing [1,4]. Hence, it is not yet possible to produce a great number of MSC *in vitro* without loosing their differentiation potential. Presumably, this task can be solved thorough selection of growth factors in concentrations maintaining the balance between self-support and differentiation.

We failed to differentiate MSC into neuron-like cells by any of the methods (retinoid acid treatment, incubation in suspension in neurogenic medium with bFGF and EGF) (data not presented).

Hence, human MSC express markers of mesenchymal cells and osteogenic precursors, do not express neurogenic markers, acquire homogeneity during *in vitro* culturing, but initially contain phenotypically and immunophenotypically heterogeneous populations.

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