A New Approach to Evaluation of Osteogenic Potential of Mesenchymal Stromal Cells

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We developed a new approach to evaluation of the intensity of osteogenic differentiation of mesenchymal stromal stem cells based on measurement of optical density of mesenchymal stromal cell cultures in a concentration range 625-10,000 cells per well after their culturing in osteogenic induction medium and staining of calcium deposits by the method of von Kossa. The proposed method allows comparative semiquantitative evaluation of osteogenic properties of mesenchymal stem cells depending on tissue sources (bone marrow, adipose tissue, placenta), *in vitro* cell density, number of passages, duration of culturing, and concentration of serum growth factors in the microenvironment. The developed approach makes it possible to compare functional activity of mesenchymal stromal cells in various pathologies. The proposed method can be used in traumatology and orthopedics for improving the efficiency of transplantation of mesenchymal stromal cells for stimulation of reparative osteogenesis.

Key Words: *mesenchymal stromal cells; bone marrow; adipose tissue; placenta; differentiation*

Mesenchymal stromal cells (MSC) belong to the class of somatic polypotent precursor cells characterized by capacities to self-maintenance and differentiation into various cell types (osteoblasts, chondrocytes, adopoicytes, endotheliocytes, neuronal cells, *etc.*) [20]. MSC were first detected in the bone marrow [12], but later these cells were isolated from other sources (adipose tissue, umbilical blood, placenta, and amniotic fluid) [2,4,5,11,13,18,22, 24]. Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC: MSC must be plastic-adherent when maintained in standard culture conditions; MSC must express CD73, CD90, and CD105, and lack expression of CD45, CD34, and HLA-DR surface molecules, and must differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* [10].

New approaches are now intensively developed for the use of MSC for stimulation of reparative osteogenesis in patients with various local and systemic osteogenesis disturbances [1,3,16]. The use of autologous cell material is the most safe mode, because it allows to avoid the problems of histocompatibility and ethical and legal problems and excludes the risk iatrogenic infection associated with hetero- and allotransplantations.

However, the use of MSC for autotransplantation dictates the need for preliminary evaluation of their differentiation potential. The capacity of MSC to targeted differentiation *in vitro* is a key parameter reflecting their functional activity. However, the efficiency of cell therapy with MSC is largely determined by maintenance of their diffe-

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rentiation potencies, which in turn directly depend on various factors (age and genetic peculiarities of the patient, character and spreading of the pathological process, previous drug therapy, source of MSC, methods of their isolation and expansion *in vitro, etc.*).

According to modern protocols, osteogenic differentiation of MSC is induced by their culturing for 18-21 days in an induction medium containing dexamethasone or 1,25-dihydroxy vitamin D_3, β-glycerophosphate, and ascorbic acid-2-phosphate [8,17, 18]. Several approaches are applied for evaluation of osteogenic differentiation of MSC [17,19,14]. Histochemical analysis of cell monolayer after staining of calcium deposits in the extracellular matrix with special dyes (alizarin red S or silver nitrate by the method of van Kossa) is most often applied. The presence of mineralization as a result of osteogenic differentiation of MSC is evaluated visually by light microscopy. Sometimes flow cytofluorometry or real-time PCR are used for evaluation of the expression of surface markers or mRNA of genes specific for osteoblasts [9,15]. These approaches are qualitative methods detecting osteogenic differentiation of MSC (yes/no) without evaluation of its intensity.

For quantitative evaluation of osteogenic potential of MSC, calcium content (Ca^{2+} in mmol/μg protein) and/or alkaline phosphatase activity (in nmol/min/μg protein) in supernatants from lysates of monolayer cells and extracellular matrix are measured [19,23]. However, these methods require preparation of cell lysate and special reagents, construction of calibration curves, and standardization of the result for protein content. These drawbacks make the analysis technically complex and considerably increase its time and costs.

The aim of the present study is the development of a new approach to evaluation of osteogenic potential of MSC, which not only simplifies the procedure of the analysis, but also allows comparative semiquantitative evaluation of osteogenic properties of MSC depending on the type of tissue source, cell density *in vitro,* number of passages and time of culturing, and concentration of serum growth factors in the microenvironment.

MATERIALS AND METHODS

Human bone marrow, adipose tissue, and placenta were used as the sources of MSC.

Bone marrow was obtained from donors (n=19) and patients with various pathologies (n=11); informed consent was signed by all participants. In brief, trepanobiopsy was performed by a hematologist under surgical conditions, bone marrow (5-20 ml) was aspirated from the iliac crest and placed into a sterile flask containing heparin (50 U/ml aspirate). Then the aspirate was incubated at 37°C for 40 min. After gravitation separation, the upper fraction presented by leukocytic suspension in autoplasma was centrifuged (250g, 15 min). The sediment was suspended in PBS containing 10 U/ml heparin and centrifuged in Ficoll-verografin density gradient (1.078 g/liter) for 20 min at 1000g. The isolated bone marrow mononuclear cells were 3 times washed in PBS, resuspended in α-MEM (BioloT), and the total number of isolated cells and the percent of viable cells after staining with trypan blue were determined.

We also used placenta tissue samples (n=10) obtained after timely physiological delivery (informed consent was obtained in all cases). The adipose tissue samples (n=13) were obtained during lipoaspiration procedures (informed consent was also obtained in all cases). The placental and adipose tissues were subjected to mechanical and enzymatic dissociation with 0.25% trypsin or 0.1% type I collagenase (Sigma-Aldrich) and EDTA for 30-40 min at 37°C. After inactivation of the enzymes with PBS containing 2% albumin, the cell suspension was centrifuged at 250g for 10 min, the cells were resuspended in α-MEM and passed through filters (100 μ pore size) for removal of cell debris. The purified cells were suspended in α-MEM, nucleated cells (NC) isolated from the adipose and placental tissues were counted and their viability was determined.

For isolation of enriched MSC population, the bone marrow MNC and NC and NC isolated from the adipose and placental tissues were cultured in 25- and 75-cm^2 plastic flasks (Nunc) at a seeding density of 10^6 cell/cm^2 in α-MEM containing 20% FCS (BioloT) at 37°C and 5% CO_2. Nonadherent cells were removed after 24 h, while adherent cells were cultured until 80-90% confluence. MSC were harvested with 0.25% trypsin and 0.02% EDTA (8-10 min at 37°C), counted, and their viability was determined.

The morphology of isolated cells was visually evaluated under a phase-contrast microscope. The initial content of MSC in the examined tissues was determined by the number of fibroblast CFU (CFU-F). To this end, bone marrow MNC and NC or NC isolated from the adipose and placental tissues (1×10^6) were cultured in Petri dishes in α-MEM supplemented with 20% FCS for 14 days, then stained by the method of Romanowsky—Giemsa, and the number of colonies containing ≥50 cells were counted.

The intensity of MSC division was evaluated by the time of attaining confluence (passage 1) and

by the number of daughter cells per one CFU-F. The number of cell population doublings was evaluated by the formula logN/log2, where N is the ratio of the number of cells after passage 1 to the initial number of CFU-F.

The morphometry of MSC was performed using a computer-assisted video system with build-in interface (MicroMed Images 1.0) followed by evaluation of cell diameter distribution in a range from <10 to >60 μ (with 10-μ intervals).

The phenotypic analysis of MSC was performed by the method of laser flow cytofluorometry (FACSCalibur, Becton Dickinson) using FITC- or propidium iodide-labeled monoclonal CD3, CD14, CD16, CD20, CD34, CD73, CD90, CD105, HLA-DR antibodies (Becton Dickinson or Sorbent).

For evaluation of the osteogenic potential of MSC, the cells obtained after passage 1 were transferred into 96-well flat-bottom plates in serial 2-fold dilutions from 625 to 10,000 in α-MEM supplemented with 20% FCS. The medium was replaced after 72 h; inductors of osteogenic differentiation of MSC 10 mmol/liter β-glycerophosphate, 100 nmol/liter dexamethasone, and 0.2 mmol/liter ascorbate 2-phosphate (Sigma-Aldrich) were added to experimental wells, while MSC in control wells were incubated under standard conditions. The medium (standard and induction) was replaced twice a week. After 18 h, the efficiency of osteogenic differentiation of MSC was evaluated by the method of von Kossa. To this end, the experimental and control MSC cultures were fixed with 10% formaldehyde for 10-20 min, treated with 1% silver nitrate for 20 min under UV light, washed twice with distilled water, and maintained in 5% sodium thiosulfate for removal of nonspecific silver excess. The presence of calcium deposits in the extracellular matrix (stained black with sodium nitrate) attested to osteogenic differentiation of MSC. The intensity of color reaction was evaluated on a Multiskan Ascent multichannel photometer (Thermo Electron Corp.) at λ=492 nm. Optical density (extinction units) in the control and experimental samples was measured and index of osteogenic differentiation (IOD) was calculated by the formula: IOD=E/C, where E and C are optical density in experimental and control samples, respectively.

The data were processed statistically using Statistica 5.0 software.

RESULTS

Adherent fibroblast-like cells were isolated from all tissues. The initial content of MSC precursors in the analyzed tissues was determined by the number of CFU-F (Table 1). In the bone marrow from healthy donors, the number of CFU-F varied from 16 to 70 (mean 33.6±6.8) colonies of 10^6 MNC. The mean content of CFU-F in the fraction of NC from the adipose tissue was 2-fold higher (75.0±37.9), but this parameter was more variable. The initial content of clonogenic precursors in the placental tissue was minimum (6.0±1.5, $p<0.05$). All tested cells irrespective of tissue source were capable of intensive proliferation. In cultures of bone marrow and adipose tissue cells, the confluent growth of MSC was observed after 13-15 days. The cell population doubled 10 times during this interval and each CFU-F gave rise to 1-2 thousands daughter cells. At the same time, placental cell cultures attained confluence after 25 days ($p<0.05$), which was probably determined by low initial content of CFU-F. The number of cell population doublings (13.0±0.5) and the yield of MSC per one CFU-F (8770±2133) by the day of subculturing were significantly higher than in bone marrow cell cultures, which attested to principal possibility of quantitative expansion of placental MSC *in vitro*.

It was noted that the cells from different sources were characterized by peculiar type of growth. For instance, bone marrow MSC were characterized by homogenous growth and formed a uniform monolayer. MSC from the placenta formed large foci with high cell density, which then fused. MSC from the adipose tissue in most cases were characterized by a unique type of growth: the cell formed honeycomb structures, which considerably complicated the formation of the dense monolayer.

MSC are morphologically heterogeneous; subpopulation of cells with a diameter about 10 μ is characterized by the highest proliferation/self-maintenance capacity [21]. Comparative morphometry of MSC from different cell sources showed that medium-size cells (diameter 20-40 μ) predominated in the studied cultures, while small cells (<20 μ) constituted about $^1/_3$ of total population. At the same time, the content of relatively large cells (>40 μ) was minimum in placental MSC cultures (1.6±1.0 *vs.* 7.0-12.9% in bone marrow and adipose tissue MSC, $p<0.05$).

Phenotype analysis of cells obtained during passage 1 showed that the cells irrespective of their tissue origin express markers specific for MSC (CD73, CD90, CD105) [10]. Minor admixtures of cells expressing lineage markers (*e.g.* $CD14^+$, $CD20^+$, $HLA\text{-}DR^+$) were present in primary MSC cultures. However, the relative content of $HLA\text{-}DR^+$ cells in the population of MSC from the bone marrow, placenta, and adipose tissue sharply decreased (practically to zero) during their further culturing and subculturing.

TABLE 1. Comparative Characteristics of MSC Isolated from Human Bone Marrow, Adipose Tissue, and Placenta (*M*±*SE*)

Parameter	MSC obtained during passage 1		
	bone marrow (*n*=7)	adipose tissue (*n*=8)	placenta (*n*=8)
CFU-F, $\times 10^6$ initial cells	33.6±6.8	75±37.9	6.0±1.5*
Time of attaining subconfluence, day	15.5±0.9	13.3±6.0	25.0±2.5*
MSC yield, per CFU-F	2078±663	1148±46	8770±2133*
Number of doublings of cell population	10.3±0.4	10.0±0.5	13.0±0.5*
Cell diameter, μ			
small cells (<20 μ)	28.6±11.0	32.7±16.5	33.4±4.9
medium cells (20-40 μ)	64.2±10.8	54.4±19.0	65.0±5.0
large cells (>40 μ)	7.0±2.8	12.9±7.2	1.6±1.0*
Cell phenotype, %			
CD3	3.9±1.5	7.3±6.9	6.6±3.2
CD14	5.6±2.3	21.1±20.5	0.15±0.15
CD16	5.6±1.7	1.2±0.1	N.d.
CD20	3.5±2.6	9.8±9.0	3.0±2.0
CD34	0.7±0.3	1.2±0.9	4.7±3.1
CD73 (SH3, SH4)	88.0±3.7	71.3±28.2	96.8±1.4
CD90 (Thy1)	83.0±2.6	72.0±17.9	54.0±0.6
CD105 (SH2)	90.0±6.3	98.0±0.8	95.7±2.1
HLA—DR	11.1±1.6	5.6±4.2	11.6±5.3

Note. N.d.: no data. *p<0.05 compared to bone marrow (Mann—Whitney *U* test).

For evaluation of the intensity of osteogenic differentiation of isolated MSC we used original approach proposed by us (patent application No. 2007143233, November 21, 2007) and based on measurements of optical density of MSC cultures in cell concentration range from 625 to 10,000 cells per well after their culturing in osteogenic induction medium and staining of calcium deposits by the method of van Kossa. Optical density of control MSC cultures isolated from the adipose tissue was about 0.100 extinction units irrespective of the initial cell concentration, which can be explained by the formation of cell monolayer on the well bottom during long-term culturing (Table 2). The presence of factors inducing osteogenic differentiation of MSC increased optical density of experimental cul-

TABLE 2. Optical Density (Ext.Units) and IOD for MSC Isolated from Adipose Tissue and Placenta

MSC cultures	Number of MSC per well				
	625	1250	2500	5000	10,000
MSC from adipose tissue (*n*=6)					
control	0.118	0.117	0.102	0.098	0.091
experiment	0.127	0.127	0.162	0.179	0.149
IOD	0.97	1.09	1.01	1.51	1.57
	0.91±0.07	1.05±0.06	1.15±0.15	1.68±0.37	1.61±0.14
MSC from placenta (*n*=4)					
control	0.101	0.124	0.111	0.113	0.129
experiment	0.076	0.117	0.139	0.150	0.111
IOD	0.74	1.17	1.39	1.23	1.08
	0.80±0.18	1.18±0.16	1.38±0.18	1.28±0.9	0.97±0.16

Note. The data are presented as medians and *M*±*SE*.

tures due to the formation of black calcium deposits. The increase in optical density was maximum (51-57%) in cultures with initial MSC content 5000-10,000 cells per well. MSC isolated from the placenta were also capable to osteogenic differentiation (Table 2), but in contrast to MSC from the adipose tissue, the maximum increase in optical density was observed at the initial MSC concentration of 2500/well (IOD=1.39). In cultures with higher content of placental MSC (5000/well), the intensity of production of calcium deposits decreased and attained the basal level in wells containing 10,000 cells. However, the osteogetic potential of placental MSC was lower than that of MSC from the adipose tissue even at optimal cell concentration (IOD 1.39-1.23 *vs.* 1.51-1.57, respectively).

At the next stage, we used the proposed approach for the analysis of the effect of culturing duration and conditions on the osteogenic potential of MSC. In contrast to cells cultured for 20 days (1-2 passages) before the start of osteogenic differentiation, MSC from the adipose tissue obtained after 9 passages (120 day in culture) were resistant to osteogenic signals and produced no calcium deposits (Table 3), which is probably related to the known phenomenon of cell aging [6,7].

Deprivation of serum factors in the culture medium considerably modulated the capacity of MSC to osteogenic differentiation (Table 4). For instance, production of calcium deposits by MSC isolated from the adipose tissue inversely correlated with the presence of FCS in the culture medium, *i.e.* the

TABLE 3. IOD for MSC from Adipose Tissue with Different Time in Culture

MSC cultures	Number of MSC per well				
	625	1250	2500	5000	10,000
MSC (*n*=6) after 1-2 passages					
IOD	0.97	1.09	1.01	1.51	1.57
MSC (*n*=1) after 9 passages					
IOD	1.17	1.11	1.0	1.05	1.17

Note. Here and in Table 5: The data are presented as medians and individual values.

TABLE 4. IOD for MSC Isolated from Adipose Tissue and Placenta under Conditions of FCS Deprivation in Culture Medium

MSC cultures		Number of MSC per well			
		1250	2500	5000	10,000
MSC from adipose tissue (*n*=1)	FCS 20%	0.78	1.04	1.53	2.18
	FCS 10%	0.91	1.10	1.74	2.31
	FCS 5%	0.91	1.13	2.28	3.20
MSC from placenta (*n*=1)	FCS 20%	1.50	1.53	1.52	0.52
	FCS 10%	1.07	1.13	1.10	1.12
	FCS 5%	0.90	0.96	1.09	1.04

TABLE 5. Osteogenic Differentiation Potential of MSC Isolated from Bone Marrow of Patients with Various Pathologies

Group of patients		Number of MSC per well				
		625	1250	2500	5000	10,000
ST (*n*=3, adults)	IOD	0.90	0.88	0.94	1.01	1.26
ST (*n*=2, children)	IOD	1.27	1.64	1.32	1.04	1.08
CL (*n*=4)	IOD	1.14	1.38	1.38	1.27	2.05
OLL (*n*=1)	IOD	0.96	1.08	0.97	0.98	0.90
SLE (*n*=1)	IOD	1.1	0.97	0.98	1.04	1.18

maximum intensity of osteogenic differentiation was observed in the presence of FCS in the lowest concentration (5%). The efficiency of osteogenic differentiation of placental MSC directly depended on the content of serum factors in the culture.

The proposed approach for evaluation of the osteogenic potential of MSC can be used not only in fundamental biology for the analysis of functional activity of MSC from different tissue sources an/or under various culturing conditions, but also can be practically applied for MSC characterization under various pathological conditions. MSC isolated from the bone marrow aspirate of patients with spinal trauma (ST, 3 patients aging 38-40 years and 2 children aging 4 and 9 years), patients with cirrhosis of the liver (CL, *n*=4, 36-48 years), acute lymphoblast leukemia (OLL, *n*=1, 31 years) and systemic lupus erythematous (SLE, *n*=1, 41 years) differed significantly by their capacity to osteogenic differentiation (Table 5). MSC from adult patients with ST were characterized by moderate osteogenic activity only in cultures with high initial cell density (IOD=1.26 at 10,000 cells per well). At the same time, in children with ST this level of osteogenic differentiation was observed at minimum cell density (IOD=1.27 at 625 cells per well), while peak values were recorded in cultures with initial MSC content of 1250 cells per well (IOD= 1.64). Cells from patients with CL were characterized by high osteogenic potential and actively produced calcium deposits in a wide cell concentration range (from 1250 to 5000 cells per well), in cultures with high cell density (10,000 cells per well) functional activity of MSC was maximum (IOD=2.05). In contrast, MSC from patients with OLL and SLE were resistant to osteogenic inductors and produced no calcium deposits under specified culturing conditions, which probably attests to functional defects of bone marrow stromal cells determined by systemic nature and severity of the disease and/or intensive drug therapy.

Our findings suggest that the developed approach can be used for semiquantitative evaluation of the intensity of osteogenic differentiation of MSC. Preliminary analysis of functional activity of MSC and evaluation of conditions required for the realization of their osteogenic potential are important for improving the efficiency of MSC transplantation for stimulation of reparative osteogenesis.

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