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Study of Growth Parameters and Potentialities of Differentiation of Multipotent Mesenchymal Stromal Cells from Rat Bone Marrow *In Vitro*

N. S. Sergeeva, I. K. Sviridova, V. A. Kirsanova, S. A. Akhmedova, N. V. Marshutina, V. S. Sergeeva*, and D. Yu. Bednik**

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The dynamics of growth and proliferative activity of the population of multipotent mesenchymal stromal cells from rat bone marrow was studied during 7 passages. The efficiency of colony formation, the morphology of multipotent mesenchymal stromal cells, and the possibility of spontaneous and induced differentiation were studied. The rat bone marrow fibroblast-like multipotent mesenchymal stromal cells are capable of clonal growth; their proliferative activity and the yield remained high until passage 4, but then decreased. Induction of osteo- or adipogenic differentiation of bone marrow multipotent mesenchymal stromal cells increased the percentage of morphologically modified cells carrying specific markers.

Key Words: *multipotent mesenchymal stromal cells; passage characteristics; differentiation*

Studies of bone marrow multipotent mesenchymal (nonhemopoietic) stromal cells (MMSC) were carried out by the present time, including studies of their plasticity [3,5,7,8] and possible use in replacement cell therapy in experimental animals [2,4,9, 12]. The growth dynamics of bone marrow MMSC population, its morphology, spontaneous and induced differentiation (osteo- and adipogenic) during culturing and passaging remain little studied [9,11].

We studied growth dynamics, morphology, and spontaneous and induced osteo- and adipogenic differentiation of rat bone marrow MMSC during culturing by passages.

Department of Prediction of Conservative Treatment Efficiency, P. A. Hertzen Oncological Institute, Russian Ministry of Health; *N. I. Pirogov Russian State Medical University; **Biological Faculty, M. V. Lomonosov Moscow State University. ***Address for correspondence:*** mnioi@mail.ru. I. K. Sviridova

MATERIALS AND METHODS

Isolation of MMSC from rat bone marrow. Stromal cells were isolated from the scapular and ileac bone marrow of 10 Wistar rats weighing 180-200 g (Nursery of Research Center of Biomedical Technologies, Russian Academy of Medical Sciences, Kryukovo, Moscow region). Biological material was placed in medium 199 with antibiotics (100 mg/ml streptomycin, 100 U/ml penicillin), thoroughly cleansed from soft tissues, and washed in phosphate buffer saline (pH 7.2-7.4) on cold. The bone marrow was then removed from spongy bone tissue by repeated washing in phosphate buffer saline. Bone marrow cell suspension was prepared by mechanical disaggregation of tissue and precipitated by centrifugation (1000 rpm, 10 min). The cell precipitate was diluted with complete growth medium containing DMEM and glutamate (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences),

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10% fetal calf serum (FCS; Gibco), and standard solution of antibiotic/antimycotic (Sigma). Mononuclears were counted in a Goryaev chamber. Isolated cells were then cultured (inoculation density 5×10^5 cell/cm^2) in flasks (Sarstedt) at 37°C and 5% CO_2 for 17-21 days. The medium was replaced for the first time after 24 h, then every 3 days.

Culturing of rat bone marrow MMSC by passages. Primary cultures attaining a preconfluent monolayer state, the cells were routinely removed from culture plastic with trypsin-EDTA (Sigma) and transferred into culture flasks at a density of 1×10^3/cm^2. In parallel, the cultures were inoculated at the same density into 6-well plates onto slides and cultured in complete growth medium for 4 weeks for morphological evaluation. On days 3, 7, 14, 21, and 28 the slides were removed, washed in phosphate buffer, fixed in May-Grunwald solution, and stained with Azur and eosin.

Induction of osteogenic and adipogenic differentiation of the rat MMSC. The differentiation potential of rat bone marrow MMSC was studied by culturing these cells on slides in 6-well plates in osteogenic or adipogenic media by previously described methods [10,13] for 28 days; the medium was replaced twice a week. MMSC cultured in complete growth medium served as spontaneous differentiation control. Slides with cells were removed from the wells on days 3, 7, 14, 21, and 28 of the experiment and washed in phosphate buffer. Induction of osteogenic differentiation in the control and experiment was evaluated by expression of alkaline phosphatase in cells (measured by method of azocombination, Kit No. 85, Sigma). Adipogenic differentiation was evaluated by accumulation of neutral fat drops in cells (staining with Oil Red O after Lilly, Sigma) [1].

Evaluation of rat bone marrow MMSC colony formation. The efficiency of bone marrow stromal cell colony formation (ECF) was evaluated by the method of maximum dilutions with decreasing inoculation density: 210 cell/cm^2, 104 cell/cm^2, 52 cell/cm^2, 26 cell/cm^2, 13 cell/cm^2, and 7 cell/cm^2 with subsequent 14-day culturing of cells in 6-well plates. The resultant colonies (>50 cells) were stained with Azur and eosin and counted using a stereomagnifier (Opton). ECF was calculated as the ratio of colony number to inoculation density (in %).

Evaluation of bone marrow MMSC proliferative activity. The percentage of proliferating cells was evaluated by indirect immunofluorescent method. Murine monoclonal antibodies to rat proliferating cell nuclear antigen (Ki-67) served as primary antibodies (1:30; Dako). FITC-labeled goat antibodies to mouse immunoglobulins served as second antibodies (1:40; Dako). The percentage of cells with specific fluorescence of the nuclei was determined. The results were processed using standard methods of variation statistics (Statistika 5.0 software).

RESULTS

Primary culture (stromal cells cultured for 17-21 days) was passaged (up to 7 passages). Every time the cells were grown to 70-80% confluent monolayer. The number of isolated and percentage of adherent mononuclears in primary culture, ECF in passage 1, MMSC increment coefficient in passages, and proliferative activity of stromal cells were evaluated.

The percentage of adherent (in primary culture) fibroblast-like cells from different bone marrow samples varied more than 10-fold (0.47-6.90%), the mean value being 2.5% (Table 1).

TABLE 1. Percentage of Adherent Fibroblast-Like Cells in Primary Rat Bone Marrow (BM) Culture

Rat No.	Number of mononuclears isolated from BM and inoculated	Number of adherent mononuclears	Adherent mononuclears, %
1	45×10^6	1.98×10^6	4.4
2	42×10^6	2.14×10^6	5.1
3	82.6×10^6	1.32×10^6	1.59
4	99.2×10^6	1.24×10^6	1.25
5	148.2×10^6	3.3×10^6	2.2
6	137.5×10^6	2.64×10^6	1.92
7	186.6×10^6	12.9×10^6	6.9
8	60×10^6	0.29×10^6	0.48
9	190×10^6	0.86×10^6	0.47
10	112.5×10^6	1.33×10^3	1.17
M±*m*	$110.12\times10^6\pm19.2$	$2.8\times10^6\pm1.15$	2.50±0.68

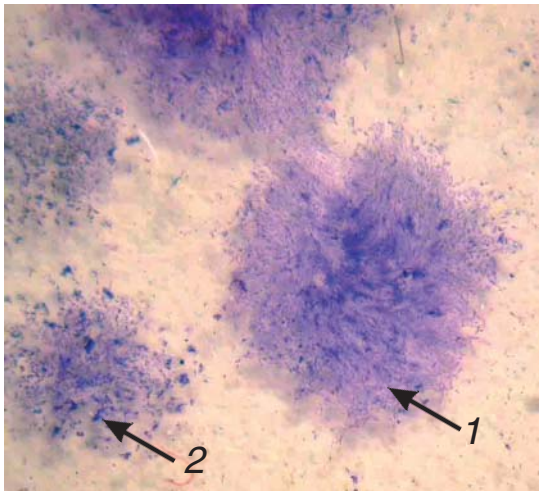


Fig. 1. Compact (*1*) and diffuse (*2*) colonies of rat bone marrow multipotent mesenchymal stromal cells (MMSC). Azur and eosin staining, ×100.

TABLE 2. Mean Values of Cell Growth Coefficient in Each Passage

Passage No.	Growth coefficient, *M*±*m*	Number of samples
1	2.17±0.35	6
2	2.29±1.17	7
3	1.56±0.44	6
4	1.26±0.58	4
5	0.73±0.37	4
6	2.33±0.38	3
7	0.63±0.15	3

By days 10-14 of culturing adherent fibroblast-like bone marrow cells formed colonies of different density: compact (with radial growth of cells) and diffuse (Fig. 1). No strict relationship between ECF and cell inoculation density (7-210 cell/cm^2) was detected: ECF varied from 0.7 to 4.8% (on average of 1.9%).

The dynamics of cell population growth was evaluated by the increment coefficient in successive (1-7) passages. For each passage the increment coefficient was calculated for 3-7 culture samples with the same cell inoculation density. The results (Table 2) in general indicate predominance of proliferation over cell death during the first 4 passages and predominance of cell death over proliferation during subsequent culturing (except passage 6).

These data were confirmed by studies of proliferative activity of MMSC culture during different passages (always on day 3 of culturing). The percentage of proliferating cells with specific green fluorescence in the nucleus (Fig. 2) gradually decreased by passage 7, *i.e.* the dynamics of this parameter during passages coincided with the dynamics of MMSC population growth (Fig. 3, *a*).

In parallel, the morphology of bone marrow stromal cells during passages was studied. During the first 3 passages the cells retained predominantly fibroblast-like phenotype. During further culturing round and cube-like cells appeared, then polygonal cells with processes appeared (Fig. 4). The latter were also present during first passages, particularly with increasing monolayer confluence. Dynamic evaluation of MMSC proliferative activity during one passage (on days 3, 7, 14, 21, and 28 of culturing) showed that the percentage of proliferating cells was maximum on days 3-7 of culturing, but then gradually decreased (virtually to zero) by day 21 because of contact inhibition of the culture, when the cells formed a compact monolayer (Fig. 3, *b*). On the other hand, the culture contained cells morphologically different from fibroblast-like ones. For this reason the cells should be subcultured long before the monolayer formation in order to preserve the monomorphic properties of the culture and its high proliferative activity.

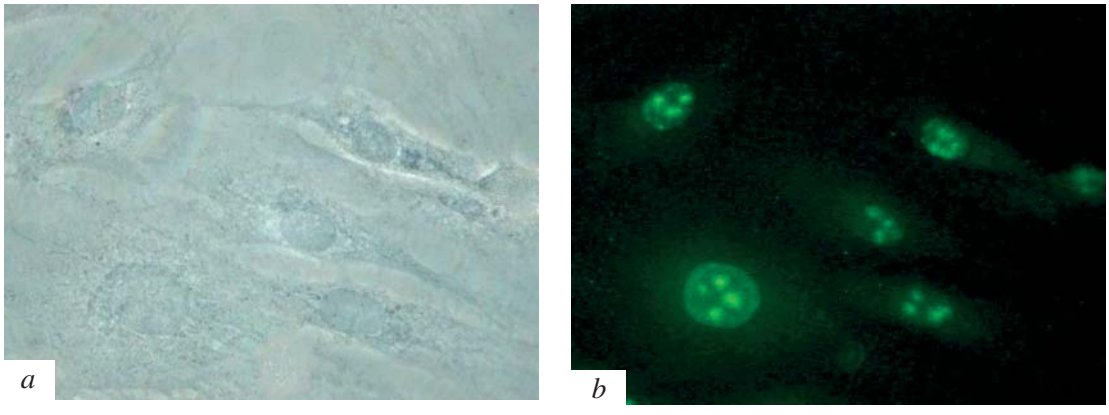


Fig. 2. Fluorescent reaction to Ki-67 in rat MMSC culture (day 3 of culturing, ×400). *a*) phase contrast; *b*) cells positively stained for Ki-67.

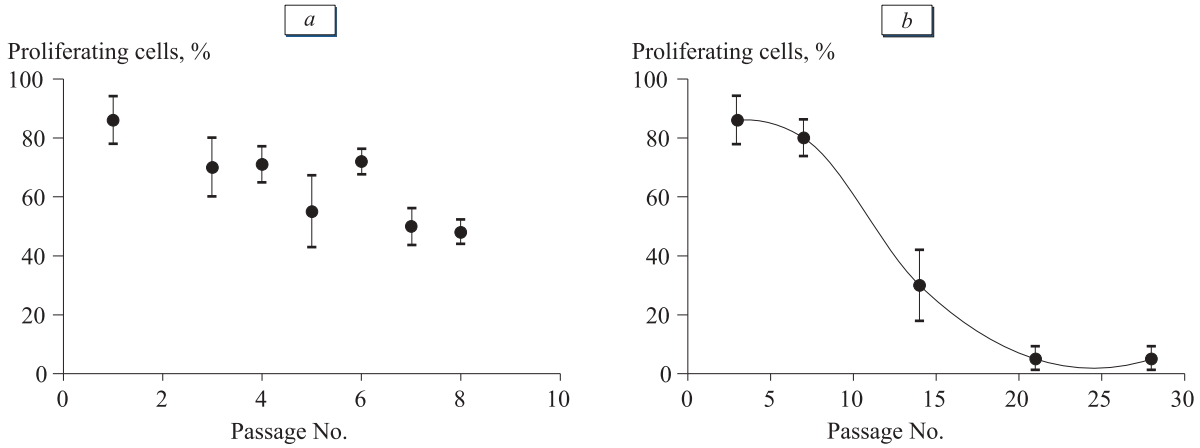


Fig. 3. Proliferative activity of rat bone marrow MMSC in the course of culture growth during passages (*a*) and during passage 1 (*b*).

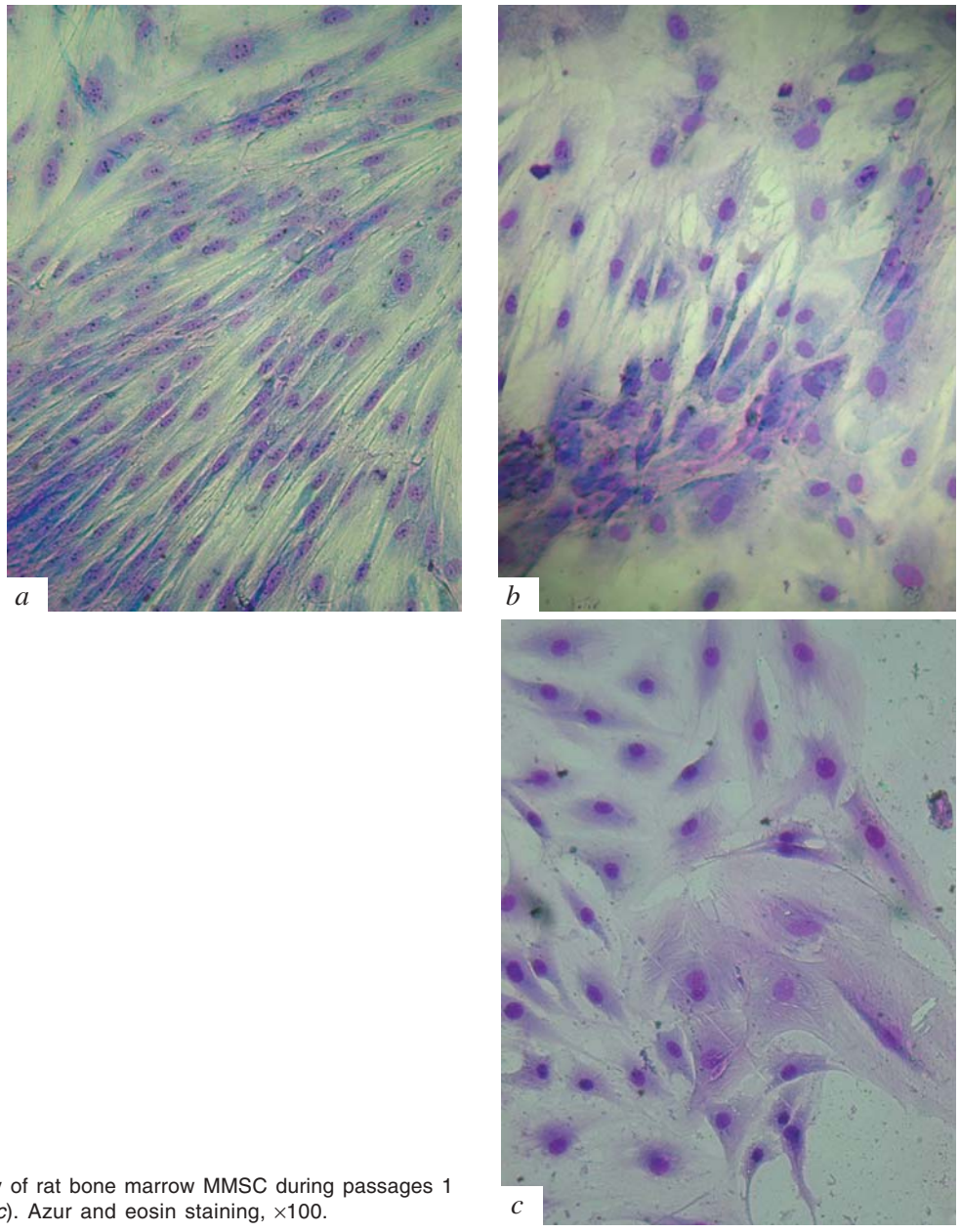
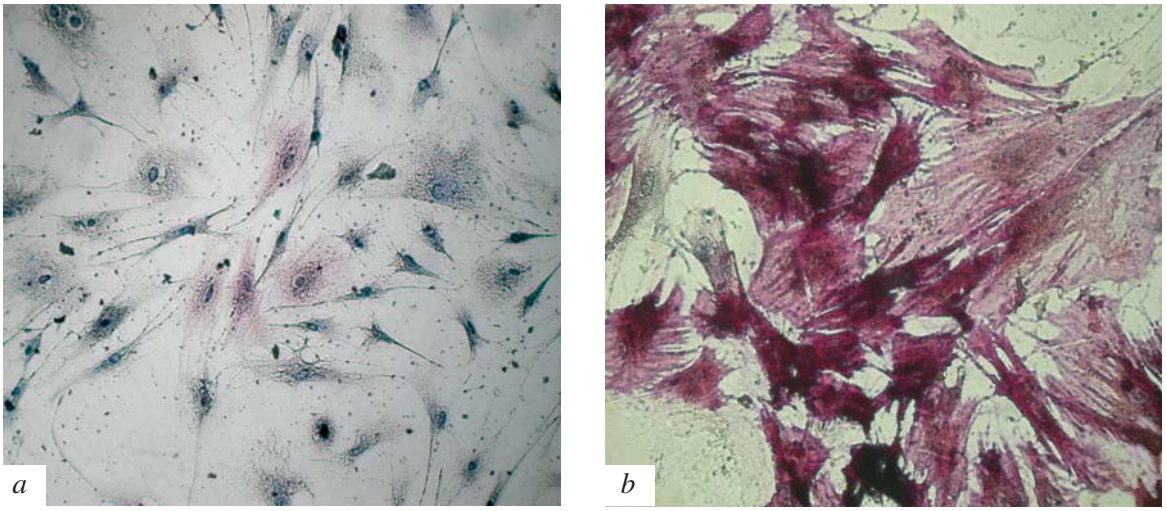


Fig. 4. Morphology of rat bone marrow MMSC during passages 1 (*a*), 4 (*b*), and 7 (*c*). Azur and eosin staining, ×100.



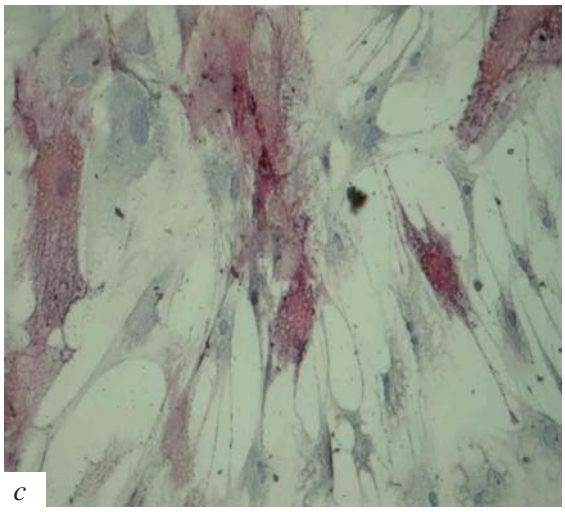


Fig. 5. Expression of alkaline phosphatase in rat bone marrow MMSC culture on days 3 (*a*), 14 (*b*), and 28 (*c*) after induction of osteogenic differentiation. Hematoxylin poststaining, ×100.

Fig. 6. Lipid inclusions in bone marrow MMSC culture 14 days after induction of adipogenic differentiation. Oil Red O staining, hematoxylin poststaining, ×100.

The capacity of rat bone marrow MMSC to osteogenic differentiation was studied during different passages (2-6) and over the course of one passage at different periods of culturing by inducing differentiation as described previously. Expression of alkaline phosphatase and changes in cell morphology were evaluated.

After induction of osteogenic differentiation of bone marrow MMSC the percentage of alkaline phosphatase-positive cells increased during one passage: solitary specifically stained cells appeared during the first days of culturing after induction, later groups of cells were seen, and then the majority of cells in the culture were stained (Fig. 5, *a*-

c). This was paralleled by morphological changes in MMSC culture: the cells were enlarged, acquired a shape close to polygonal or stellate (with axons). This trend persisted in further passages.

The capacity of rat bone marrow MMSC to adipogenic differentiation was studied after its induction by a previously method described; the appearance of lipid inclusions in cells was observed (Oil Red O staining). The first fat droplets were detected in solitary cells one week after induction of adipogenic differentiation, later they were seen in groups of cells of different shape (fibroblast-like, polygonal, spread, axonal; Fig. 6). Hence, rat bone marrow MMSC exhibited an *in vitro* capacity to specifically responded to inductors of both osteogenic and adipogenic differentiation.

In the control solitary cells were wealky stained for alkaline phosphatase and neutral fats in some experiments during different passages. The number of these cells increased to 10% with increasing monolayer confluence. These data suggest that the population of MMSC includes cells initially committed to osteo- and adipogenic differentiation.

Hence, we studied the parameters of rat bone marrow MMSC growth and capacity to induced differentiation. Fibroblast-like MMSC constitute about 2.5% rat bone marrow mononuclears in primary culture. These cells are capable of clonal growth in culture. Their proliferative activity (and increment) remains high until passage 4 and then decreases. Proliferative activity of the rat bone marrow MMSC decreases during one passage with increasing monolayer confluence and is restored after subculturing. As the monolayer density increases, morphologically modified cells containing alkaline phosphatase or lipid inclusions appear, which indirectly attests to the beginning of spontaneous osteo- or adipogenic differentiation; in other words, an initially committed cell pool is present among MMSC. Induction of osteo- or adipogenic differentiation of bone marrow MMSC *in vitro* by special cocktails leads to increase in the percentage of morphologically changed cells, positive for alkaline phosphatase or containing lipid droplets.

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