Alterations of Cytological and Karyological Profile of Human Mesenchymal Stem Cells during *in Vitro* Culturing

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Transplantation of human bone marrow mesenchymal stem cells is considered as a promising therapeutic approach to the therapy of many diseases. However, the problem of possible alterations of the properties of mesenchymal stem cells during their expansion in *in vitro* cultures before transplantation is not solved. In our study, one of two hundred examined cultures of mesenchymal stem cell cultures derived from donors without bone marrow pathologies and developed under standard culturing conditions demonstrated spontaneous disturbances in morphology, proliferation, and karyotype at early passages. The cells of this abnormal culture retained immunophenotype characteristic of normal mesenchymal stem cells, but some of them (15-25%) had numerous numerical and structural chromosome aberrations.

Key Words: *mesenchymal stem cells; chromosomal aberrations; malignant transformation*

Transplantation of autologous or allogeneic mesenchymal stem cells (MSC) isolated from the bone marrow is a promising therapeutic approach to the therapy of some diseases such as graft-versus-host disease, diabetes, various neurodegenerative pathologies, *etc*. [7,10,12]. According to the reports of National Institute of Health of USA, nearly one hundred MSC research projects are now in progress in various countries [www.ClinicalTrials.gov].

The amount of MSC isolated from the bone marrow punctate is insufficient for transplantation, which explains the need for their *in vitro* expansion. Some demonstrative experiments showed that human MSC retain stable morphological, phenotypic, and genome features during expansion [5], although the problem of possible negative effects of routine culturing technique on the properties of MSC remained unsettled [6]. Changes in the genome can be expected in MSC that are deprived of physiological environment and immune system control, which can augment the risk of malignant transformation [11,14]. There are papers reporting accumulation of mutations in MSC genome followed by malignant transformation of the cells. These data originate mostly from mouse MSC [3,8,11,13-15]. Human MSC demonstrated spontaneous alterations in the adhesion properties and appearance of tumorigenic features only after a large number of passages *in vitro* (>50) [11].

This study is aimed at the analysis of morphology, proliferation characteristics, and karyotype of human MSC during short-term culturing (<7 passages) performed before transplantation. The study showed that changes in MSC karyotype appear even after short-term culturing under standard conditions without changes in the immunophenotype characteristic of this cell type.

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MATERIALS AND METHODS

We examined 200 MSC cultures derived from the bone marrow of donors (men and women), which had no clinical or laboratory signs of hematological, oncological, or other diseases of the bone marrow.

MSC were isolated from the bone marrow punctate derived from iliac bone (punctate volume 25-30 ml) or sternum (punctate volume 2-8 ml). The punctate was diluted 2 times with α-MEM (HyClone), fractionated by centrifugation in Ficoll density gradient (1:1) to isolate a layer of mononuclear cells. These cells were centrifuged at 1500 rpm in serum-free α-MEM. The pellet was suspended in this culture medium supplemented with 20% FCS (HyClone) and 100 μg/ml penicillin/streptomycin (HyClone) and inoculated into T-75 culture flasks (Sarstedt). After 48 h, MSC and bone marrow islets were twice washed with PBS (20 mM phosphate buffer, pH 7.4, 0.1 M NaCl) from blood cells and placed again in culture medium. Subculturing was performed using trypsin solution with EDTA (HyClone). The MSC culture was first subcultured 8-10 days after explantation and then every 2-3 days with initial density of 1.27×10^3 cell/cm^2. The cells were cultured through 7 passages with daily morphological characterization.

The number of cells during each passage was counted at a Brite-Line hemocytometer. The doubling time of each MSC population was also determined. The data were processed statistically using StatGraphics software (Stat Soft Inc.).

Immunophenotyping of all MSC cultures was performed on passages 4 and 6 by flow cytometry using antibodies to CD34, CD44, CD45, CD90, CD105, and CD106 surface cell markers (Becton Dickinson). To this end, the cells were harvested from dishes with trypsin-EDTA (HyClone), washed twice with PSB (20 mM phosphate buffe, pH 7.4, 0.1 M NaCl) and incubated for 1 h with fluorochrome-conjugated monoclonal antibodies in a dilution of 1:20. Then the cell were twice washed with PBS and analyzed in an Epics XL flow cytofluorimeter (Beckman Coulter).

Karyotyping of MSC culture was carried out after passage 6 according to the method described elsewhere [2]. To this end, 6 MSC cultures with normal morphology and immunophenotype were randomly selected. One culture with abnormal morphology was also analyzed. To obtain the preparations of metaphasic chromosomes, colchicine solution in a final concentration of 0.05 mg/ml was directly added to 40-ml culture flasks containing 3.5 ml α-MEM (HyClone) and incubation was carried out at room temperature for 4-6 h. MSC culture was then treated with trypsin-versene solution (1:3) and the cell suspension was transferred into centrifuge tubes with 3 ml 0.55% KCl and incubated for 20 min at room temperature for hypotonic treatment. The cells were then prefixed (75 μl fixative) with ethanol:glacial acetic acid (3:1) mixture and centrifuged for 10 min at 1000 rpm. The supernatant was discharged leaving 0.3-0.5 ml remnant fluid over the precipitate. The precipitate was thereafter pipetted, 3 ml fresh cold fixative (ethanol:glacial acetic acid, 3:1) was added, and the suspension was stirred.

The cells were fixed at 4°C for 20 min. Then the fixative was replaced, and the suspension was centrifuged for 10 min at 1000 rpm. The supernatant was discharged leaving 0.3-0.5 ml fluid over the precipitate. The suspension was then dropped onto slides previously cooled in water to 4°C (30-50 μl per slide dropped from a height of 30-50 cm). The slides we dried on air at room temperature.

The chromosome preparations were stained with fluorochrome solution (Hoechst 33258), contrasted with actinomycin D, and placed into solution prepared on the basis of phosphate-citrate McIlwaine buffer. The preparations were examined under LEICA DM LS microscope equipped with FLUOTAR objective lens (×20/0.40 and ×100/1.30-0.60), automatic microscope camera adapter, and a Leica DFC 320 color camera. The photos were processed with Leica DFC Twain software.

Karyotyping of MSC was carried out at the resolution of 400-450 chromosome segments per haploid genome.

RESULTS

During *in vitro* culturing, only one of 200 MSC cultures had cells with a shape different from the fibroblast-like appearance typical of MSC culture (Fig. 1, *a*, *b*). Typical morphological changes were observed as early as on passage 2. The cells grew in size and became more branched. Their nuclei contained 1-2 clearly detected large nucleoli. These cells lost the property of contact inhibition and formed round multilayer clusters on the flask surface. On passages 3-4, some cells of this atypical culture lost adhesion properties, detached from the flask surface, and persistently divided in the suspension (Fig. 1, *c*). MSC cultures derived from the repeated punctate of the same donor demonstrated similar spontaneous alterations.

Proliferation activity of MSC populations derived from various donors was highly variable, although normal doubling time of cultured cells was 2-3 days. In contrast, unique MSC culture with drastically abnormal morphology differed also by the proliferation rate. While doubling time of cells retaining adhesion properties was not less than 8 days, other cells of this abnormal culture that lost adhesiveness on passages 3-4 actively proliferated with doubling time <1 day

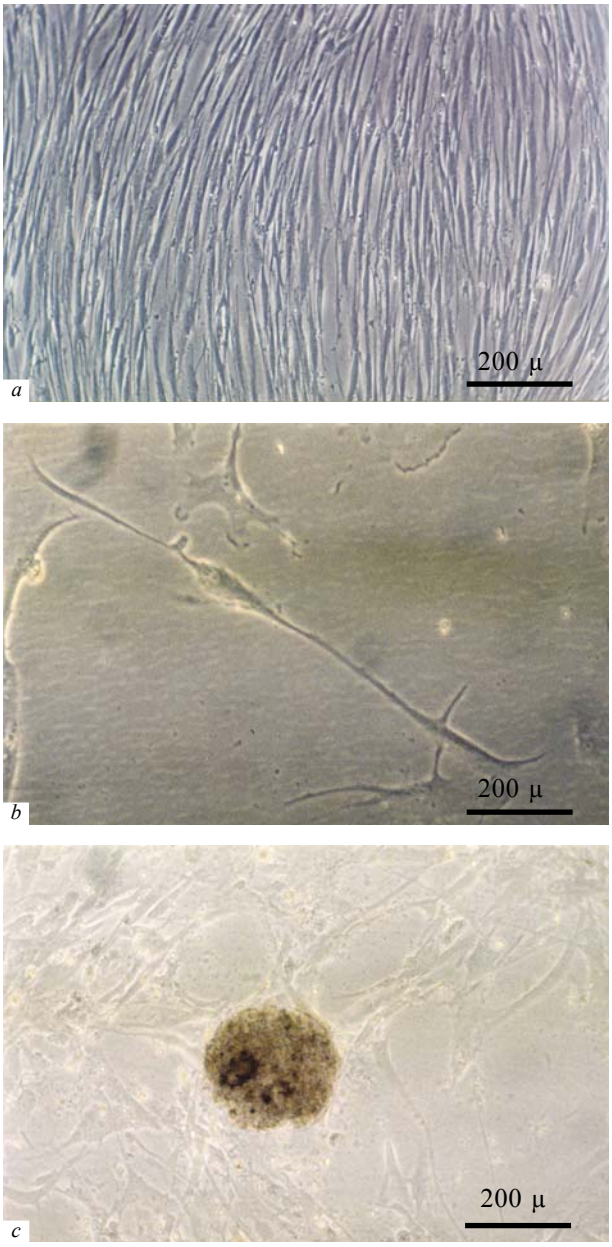

Fig. 1. Morphology of MSC culture on passage 6 examined under phase contrast light microscope. *a*) normal MSC culture; *b*) the adhesive cells in abnormal MSC culture; *c*) formation of a cell conglomerate in the suspension fraction of abnormal culture.

(Fig. 2). Cells of the repeated punctate derived from the same "abnormal" donor were also characterized by active proliferation.

Immunophenotype of all examined MSC cultures was stable through six passages. MSC cultures comprised the following cells with the corresponding surface markers: $CD106^+$ (2-3%), $CD34^+$ (<1%), $CD45^+$ (<1%), $CD105^+$ (~50%), $CD44^+$ (95-99%), and $CD90^+$ (>99%), which corresponded to immunophenotype of human MSC cells [4]. Abnormal MSC cultures derived from the first and second bone marrow punctate of the same donor demonstrated no significant alterations in the expression profile of the surface markers in comparison with normal cultures and maintained stable immunophenotype throughout the entire culturing period (Table 1). By surface markers, single peculiarity of MSC with abnormal morphology was up-regulated expression of CD106 (VCAM, receptor to CD49d/CD29), which according to its biological role is a leukocyte adhesion molecule mainly characteristic of activated endothelium and dendritic cells. Nevertheless, despite unique character of abnormal MSC culture and variability of surface marker expression in normal cells, it cannot be excluded that up-regulation of CD106 expression on the cell surface is a characteristic sign of disturbance in cell properties.

We previously showed that human MSC retain normal karyotype 46,XX or 46,XY through the first 7 passages [2]. The present study showed that MSC culture with aberrant morphology and abnormal proliferation activity contains cells with abnormal karyotype (Table 2, Fig. 3). In most examined cells, changes in chromosome structure (translocation, deletion, insertion, chromosomal markers) and chromosome number (monosomy 8 and 17; tetraploidy) were detected. No repeatability of genome and chromosome mutations was observed, which attested to their low incidence.

Thus, one MSC culture derived from a donor without bone marrow pathology demonstrated enhanced percentage of cells with abnormal karyotype detected at early passages. However, this observation cannot attest to malignization of cells or their tumorigenic properties because there are no relevant *in vivo* data. Still, similar alterations in the genome are characteristic of cells with tumorigenic properties [9], which is an alarming resemblance. Most probably, the observed alterations in the morphology and karyotype resulted not from specific culturing environment, but

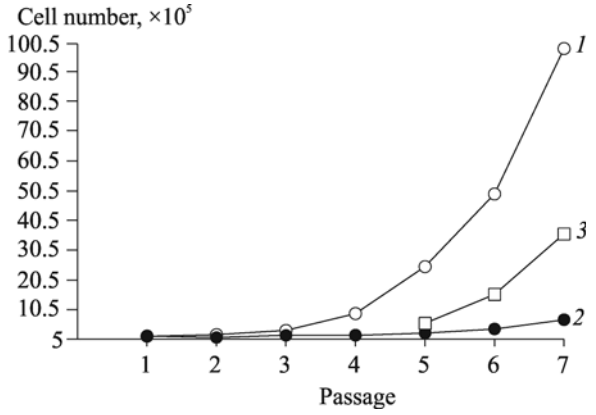

Fig. 2. MSC proliferation in the abnormal culture derived from the first bone marrow punctate in comparison with normal culture. The passages were performed every 2-3 days. *1*) normal MSC culture; *2*) adhesive fraction of the abnormal MSC culture; *3*) suspension fraction of the abnormal MSC culture.

TABLE 1. Expression Profile of Surface Markers in Abnormal MSC Culture in Comparison with Normal Passage 4 MSC Culture

Index	Cells, %					
	$CD106^+$	$CD34^+$	$CD45^+$	$CD105^+$	$CD44^+$	$CD90^+$
Normal MSC culture	2.14	0.01	0.01	53	96.5	99
The range of marker expression in normal MSC culture	1-10*	0-2	0-2	50-70	95-100	95-100
Abnormal MSC culture						
first BM punctate	21.7	1.07	1.42	53	96.7	98.8
repeated BM punctate	21	0.49	0.56	50	95	99.6

Note. *In rare normal cultures, expression of CD106 increased to 20-50%. Here and in Table 2: BM: bone marrow.

from individual features of the donors. It is an established fact that the early passages of MSC cultures can reveal aberrant clones with selective advantage in proliferation [1]. In that study, 3 of 7 MSC cultures examined on passages 2-4 demonstrated aneuploidy such as 45,X/46,XY mosaicism, X(45,X) monosomy, and chromosome 8 trisomy. Out study revealed a dramatic increase in proliferative activity of abnormal cells and their transition into suspension and a decrease in proliferative activity of adherent cells. These features indicate acquirement of selective advantage of some abnormal MSC.

Despite pronounced morphological alterations, disturbances in proliferation activity, and numerous aberrations in the karyotype, the immunophenotype of these cells did not differ from the normal. Presently, immunophenotyping by flow cytometry remains the basic method of routine control over the parameters of MSC intended for transplantation, while genetic stability is not taken into consideration in assessment

TABLE 2. Karyotyping of MSC Culture with Abnormal Morphology and Immunophenotype in Comparison with Normal Passage 6 MSC Culture

Index	Normal karyotype	Aberrant karyotype	Total number of metaphases	Cells with aberrations, %
Normal MSC culture	46, XY [91]	No	15	0
Abnormal MSC culture				
the first BM punctate	46, XY [30]	81,?X?Y,inc [1]	40	25
	88,?XYY,inc [1]			
	92,XXYY,inc [1]			
	46,XY,der(13),inc [1]			
	47,XY,der(9),+mar [1]			
	45,XY,–8 [1]			
	45,XY,der(3),–17,inc [1]			
	46, XY,del(2)(q36~37) [1]			
	46,XY,r(4) [1]			
	46,XY,der(2),inc [1]			
the repeated BM punctate	46, XY [15]	83,YY,inc [1]	18	16.7
	40,X,t(7;17)(q31;q13), –14,–15,–16,–21,–21,–Y [1]			
	46,XY,add(7)(q?),inc [1]			

Note. *The square brackets show the number of metaphases.

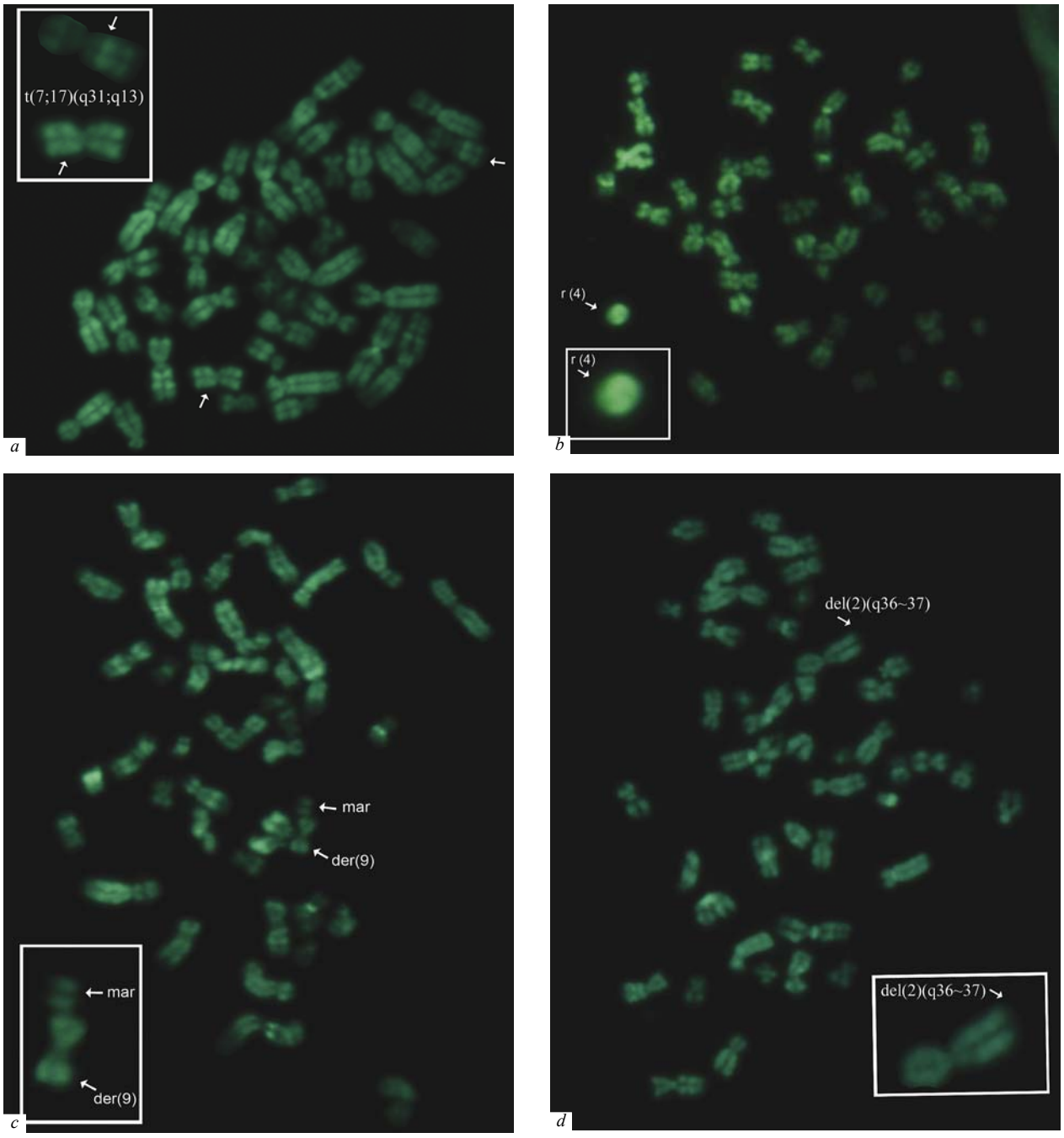

Fig. 3. Metaphase plates in MSC with abnormal karyotype. *a*) 40,X,t(7;17)(q31;q13), –14, –15, –16, –21, –21, –Y; *b*) 46,XY,r(4); *c*) 47,XY,der(9),+mar; *d*) 46,XY,del(2)(q36~37).

of the transplantation risks. Our data agree with some reports [1] and indicate the necessity to develop more strict system of the control over MSC parameters incorporating the cytogenetic methods.

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