Chromosome Variability of Human Multipotent Mesenchymal Stromal Cells

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We elaborated a method of preparing cytogenetic preparations of cultured multipotent mesenchymal stromal cells from the adipose tissue. It was found that karyotypic changes (monosomy, translocations) appear in some samples during culturing. Clones with changed karyotype were detected in 11-14-passage cultures from 2 of 7 individuals. The percent of aberrant cells in cultures from different individuals varied from 1.5 to 5.95 per 100 cells, which attested to karyotype instability. These data substantiate the need for cytogenetic control of cells before their transplantation into donor organism and further investigation of chromosome variability in stem cells.

Key Words: *multipotent mesenchymal stromal cells; karyotyping; aneuploidy; chromosome aberrations*

Stem cells cannot be taken from the organism in amounts sufficient for therapeutic purposes; therefore their transplantation to the recipient is usually preceded by culturing aimed at increasing cell number and elimination of non-stem cells from the aspirate. Culturing can led to the appearance of cells with aberrant karyotype and selection of cells with higher proliferation rate, which can enrich the transplant with transformed cells.

Karyotyping and evaluation of biomarker expression are very important and most widely used methods for detection of genetic instability in long-term cell culture and allow elimination of cultures with stable chromosome aberrations. Multipotent mesenchymal stromal cells (MSC) [2] have great potentialities for clinical application [1], because of the possibility of *ex vivo* culturing, capability to multilineage differentiation (into osteocytes, adipocytes, and chondrocytes), and due to the existence of methods for their isolation from the bone marrow, adipose tissue, and cartilage [5]. In some reports, no karyotypic changes were detected by cytogenetic analysis in MSC isolated from the bone marrow on passages 2-10 [3,4]. At the same time, it is well known that MSC can spontaneously transform after long-term culturing *in vitro* and gain the properties of tumor cells [6,8], which is accompanied by chromosome instability [6]. Transplantation of transformed cells to laboratory mice led to the formation of multiple tumors. Immortalized MSC strains from human bone marrow can also induce *in vivo* tumor growth in mice [7]. Thus, the evidence on possible chromosome instability and potential transformation of MSC during their long-term culturing *in vitro* attests to the necessity of cytogenetic control of MSC cultures.

The aim of the present study was the development of methodical conditions for obtaining cytogenetic preparation of MSC and evaluation of chromosome variability in cultures of human MSC from the adipose tissue.

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MATERIALS AND METHODS

Isolation of MSC cultures and conditions of culturing. Cell cultures for therapeutic transplantation were prepared routinely. Fat samples from the anterior abdominal wall were obtained during cosmetic liposuction or other surgical intenventions. Donor age varied from 26 to 55 years. The tissue was washed 3-4 times with PBS (pH 7.4) and suspended in an equal volume of the same buffer containing 1% BSA and 0.1% type 1 collagenase (Worthington Biochemical Corp.). Deaggregation was performed on a magnetic stirrer at 37°C for 1 h followed by 5-min centrifugation at 300-500*g*. The supernatant containing mature adipocytes was discharged. The cell pellet containing cells of the stroma and vessels (fibroblasts, pericytes, endothelium, *etc*.) was cultured. The cells were seeded to 90-mm plastic Petri dishes (Corning) in DMEM/F12 growth medium containing 10% FCS (HyClone) and 1% insulin-transferrin-selenite (Paneko). The medium was replaced after 48-h incubation in a CO_2 incubator (5% CO_2, 37°C), *i.e.* after the cells adhered to the plastic. Then the cultures were grown to 75-90% confluence, harvested with trypsin solution, and seeded onto new dishes. Seeding density was 1.5×10^3 cells/cm^2.

Technique of preparing cytogenetic preparations. Colchicine in a final concentration of 0.5 μg/ml was added to Petri dishes 1.5 before fixation. Then the cells were harvested with Versene (1 min) and then with 0.25% trypsin (3-7 min at 37°C), after that 5 ml DMEM was added and the suspension was transferred into centrifuge tubes. After centrifugation (10 min at 1000g) the supernatant was discharged. The pellet was resuspended by gentle pipetting. Hypotonization was performed with 0.55% KCl (12 min at 37°C), 3-5 drops of a fixative was added to stop hypotonization. The cells were fixed with methyl alcohol-glacial acetic acid mixture (3:1). The first fixative was added gradually and mixed with the pellet by gentle pipetting. Then, the fixative was twice replaced with fresh portions.

The cell suspensions were dripped onto cold slides. Dried preparations were stained by the method of G-staining for karyotyping and by routine technique for the analysis of chromosome aberrations.

For aneuploidy analysis, the preparations were prepared routinely without colchicine. Double-color interphase FISH was performed using probes purchased from Vysis, Inc. Denaturation, hybridization, and washout were performed routinely. DAPI was used for contrast staining of nuclei.

Karyotyping and analysis of aneuploidy. Not less than 15 metaphases were analyzed for each MSC culture during karyotyping. For the analysis of chromosome aberrations single and paired fragments and chromosome and chromatide exchanges were counted. Gaps were not taken into account. From 100 to 600 metaphases were analyzed for each end-point.

FISH preparations were examined under an Axioplan 2 microscope equipped with a set of interference filters (Zeiss) using FISH analysis system and appropriate software (Isis, Metasystems GmbH). A total of 3800 nuclei (not less than 900 nuclei in each hybridized preparation) were analyzed.

RESULTS

Karyotyping and analysis of chromosome aberrations in all cultures were performed at early passages (1-5 passages), but some cultures were also karyotyped during passages 10-14. In 3 of 7 cultures, the number of mitosis during late passages was insufficient for cytogenetic analysis.

The results of karyotyping of MSC cultures are presented in Table 1. At early terms of culturing

TABLE 1. Karyotyping of MSC Cultures

Culture No.	Early passages			Late passages		
	passage	cell number	karyotype	passage	cell number	karyotype
1	4	15	46,XX	10	No mitoses	
2	4	15	46,XX	14	21	mos45,XX,der(21;22)(q10;q10)[18]/46,XX[3]
3	5	15	46,XX	11	20	46,XX
4	4	19	46,XX	10	No mitoses	
5	5	29	46,XX	10	38	mos45,XX,-6[30]/46,XY[8]
6	3	30	46,XX	10	No mitoses	
7	3	37	46,XX	10	16	46,XX

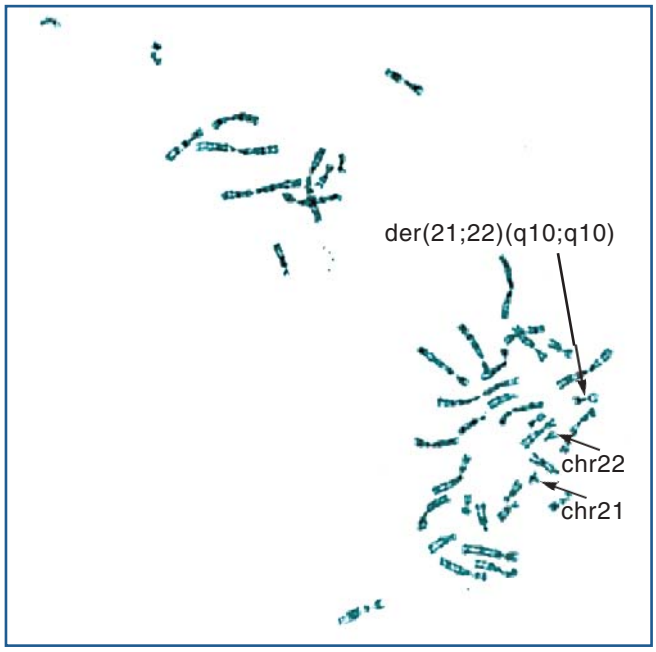

Fig. 1. Metaphase plate of culture No. 2 during the 14th passage. Arrows show normal chromosomes 21 and 22 and Robertson translocation between chromosomes 21 and 22.

(passages 1-5), the chromosome set in 7 cultures of MSC obtained from the adipose tissue corresponded to normal female set (46,XX).

For late passages karyotyping was performed only for 4 MSC cultures. In other 3 cultures that number of mitoses was insufficient for the analysis of chromosome set. Further improvement of this technique is required in order to obtain cytogenetic preparations from the majority of cultures at late terms of culturing.

Two of 4 analyzed cultures (Nos 3 and 7) retained normal chromosome set. In culture No. 2 during passage 14 the majority of cells (85.7%) had Robertson translocation between the 21st and 22nd chromosomes (Fig. 1). In culture No. 5 we found a

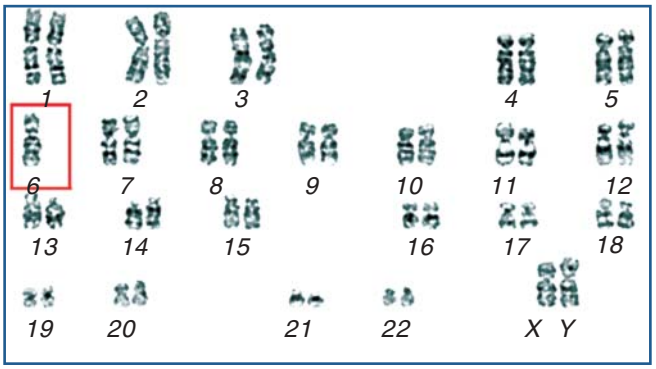

Fig. 2. Karyotype of culture No. 5 during the 10th passage. Chromosome 6 monosomy.

cell clone with monosomy 6 (Fig. 2). Thus, cell clones with abnormal karyotypes were detected in 2 of 4 cultures, which deserves special attention, because these cells can transform into tumor cells.

We analyzed autosome aneuploidy (chromosomes 7 and 11) in 2 MSC cultures at different stages of culturing (passages 1 and 3; Table 2). Double-color interphase FISH allowed us to exclude nuclei with ineffective hybridization and polyploidy from the analysis and to detect nuclei with nullisomy (Fig. 3). The incidence of aneuploid nuclei (monosomy, trisomy, and tetrasomy) varied from 0.80 to 2.62%. No cells with nullisomy were detected. The incidence of cells with monosomy varied from 0.60 to 2.52% and was higher than that of cells with trisomy (0.10-0.31%). The difference in the incidence of aneuploid cells between passages 1 and 3 was insignificant. It is difficult to determine is it normal or not. We found no published data on the incidence of aneuploidy in interphase stem cells, but one paper [9] reported increased incidence of aneuploidy in cultured embryonic brain cells (1.3-7.0%) compared to intact fetal brain cells (0.6-3.0%).

TABLE 2. FISH-analysis of aneuploidy

Chromosome	Sample		Incidence of aneuploidy, %	Number of fluorescent signals				χ^2, *P*
	culture No.	passage		1	2	3	4	
7	8	1	1.57	12	938	3	0	χ^2=2.48; *P*>0.05
		3	2.62	25	966	1	0	
	9	1	1.25	9	948	3	0	χ^2=0.70; *P*>0.05
		3	1.71	14	973	3	0	
11	8	1	1.05	9	943	1	0	χ^2=0.51; *P*>0.05
		3	1.41	13	978	1	0	
	9	1	1.04	7	950	2	1	χ^2=0.29; *P*>0.05
		3	0.80	6	982	2	0	

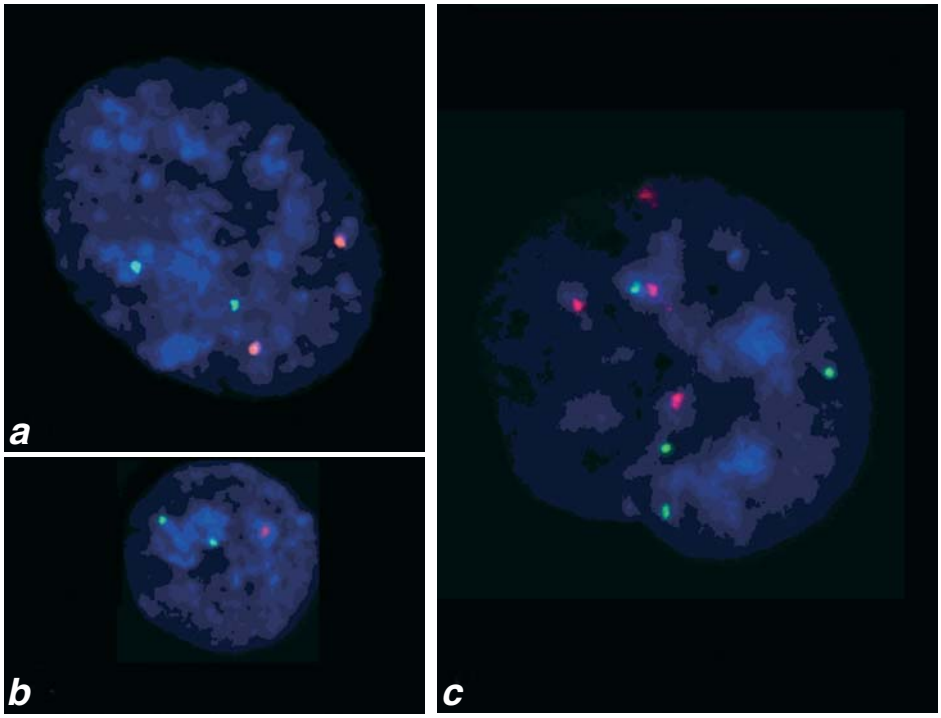

Fig. 3. Interphase nuclei from MSC cultures, simultaneous hybridization with two centromere probes. *a*) diploid cell with two chromosomes 7 and two chromosomes 11, *b*) chromosome 7 monosomy, *c*) tetraploid cell.

The results of the analysis of spontaneous level of chromosome aberrations in 7 MSC cultures at early terms of culturing are presented in Table 3. The mean incidence of cells with chromosome aberrations was 2.95%. The percent of aberrant cells in cultures varied from 1.50 to 5.95%. A total of 3.33 aberrations per 100 analyzed cells were found. Most aberrations were single fragments. It means that cells with chromatide aberrations die and new chromatide aberrations appear during subsequent mitosis, otherwise they would transform into chromatide aberrations. The incidence of chromosome aberrations in the analyzed cultures during the early passages compared to peripheral blood lymphocyte culture is at the upper limit of normal.

Experiments on stem cell cultures showed that the procedures of preparing cytogenetic preparations (harvesting, hypotonization, fixation) should be carried out under mild conditions. Disrupted cells with scattered chromosomes were more incident in stem cell cultures than in lymphocyte cultures. This is probably why fewer reports are devoted to the analysis of chromosome sets in cultures of regional stem cells. However, our study demonstrated the appearance of considerable karyotypic changes after several passages. This fact is very important from the viewpoint of safety of cell therapy and therefore detailed investigation of regularities of chromosome aberrations of stem cells is required.

Chromosome variability in MSC manifests as instable chromosome aberrations, chromosome translocations, and aneuploidies. The role of these changes is still unclear, but it is unlikely that they are

TABLE 3. Incidence of chromosome aberrations in MSC cultures during early passages

Culture No.	Number of analyzed cells	Number of aberrant cells	Percent of aberrant cells	Number of aberrations per 100 cells
1	340	11	3.24	4.71
2	202	6	2.97	3.47
3	600	9	1.50	1.67
4	117	3	2.56	2.56
5	129	7	5.43	5.43
6	168	10	5.95	5.95
7	277	8	2.89	2.89
Total	1833	54	2.95	3.33

inessential for cells intended for transplantation into the organism after culturing. The methods applied in our study allow evaluation of only crude chromosome abnormalities and aneuploidies, but the use of molecular cytogenetic methods makes it possible to detect more changes.

Our experiments demonstrated selective propagation of cells with abnormal karypotype. This attests to the necessity of cytogenetic control of stem/progenitor cells before their transplantation as a part of cell therapy safety system. In case of dynamic cytogenetic control, cell propagation should be stopped after the appearance of clones with chromosome abnormalities.

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