CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Methodological Guidelines for Genetic Safety Testing of Cell Transplants

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A combination of karyotyping and aneuploidy analysis by interphase fluorescent *in situ* hybridization is a sensitive method for evaluation of genetic stability of stem cell cultures. The methodology and specific features of preparing and analyzing the cytogenetic preparations are described as exemplified by human multipotent mesenchymal stromal cells.

Key Words: *methodological guidelines; cell transplant; multipotent mesenchymal stromal cells; karyotyping; aneuploidy*

An obligatory condition of cell therapy is to provide its safety. The development of universal production standards for the use of cell preparations is in progress now [3]. However, evaluation of genetic stability was never before regarded as an obligatory condition of pre-transplantation testing. On the other hand, many examples of chromosome aberrations are known, leading to the development of predisposition to cancer or directly causing malignant transformations [12]. In addition, introduction of a cell transplant containing abnormal clones to a patient can lead to immune complications associated with elimination of genetically modified cells. In each case it is essential to evaluate the balance between therapeutic characteristics and potential risk of the destructive effect of the cell transplant [14]. These factors determine the need to rule out the use of cell strains carrying chromosome aberrations, because of the known regularities of chromosome instability and its significance in the oncogenesis.

The methodological problems of cytogenetic studies of stem cells (SC) do not receive due attention. However, cytogenetic studies of SC are a special sphere. Experience of SC research, namely, evaluation of the incidence of abnormal cells and interpretation of the results of analysis from the viewpoint of genetic safety of the cell culture, has been gained at the Laboratory of Mutagenesis of Medical Genetic Center.

Here we present methodological bases of cytogenetic studies of human multipotent mesenchymal stromal cells (MSC).

MATERIALS AND METHODS

Isolation and culturing of MSC from the bone marrow and adipose tissue. Culturing of bone marrow stromal fibroblasts is carried out by the method proposed by A. Ya. Friedenstein *et al.* [6]. Mononuclear cells isolated from the bone marrow or adipose tissue are inoculated in ventilated culture flasks with DMEM containing 10-20% FCS, addition of 1% insulin-transferrin-selenite is optional. After 24-48 h, nonadherent cells are removed and the medium is replaced. The

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cultures are then incubated until 75-90% confluence. The cells are then removed with trypsin and reinoculated in new flasks. Culturing is carried out at 37°C at absolute humidity and 5% CO_2 in the air. Starting from passage 3, the MSC cultures are monomorphic and consist of spindle-shaped cells.

Preparation making. It is better to carry out comprehensive evaluation of genetic safety after 2-3 passages of MSC culture. The MSC are fixed for cytogenetic analysis on days 2-3 after the latest passage. The culture density on the day of fixation should not exceed 60% confluence.

Colchicine or colcemide in final concentrations of 0.5 and 0.25 μg/ml, respectively, are added to the culture flasks. The total duration of colchicine (or colcemide) treatment should not exceed 2 h. For evaluation of the incidence of aneuploidy in interphase nuclei, addition of substances arresting mitotic division at the metaphase stage is not obligatory, but possible if the exposure to these substances does not exceed the time of one cell division.

The cells are removed from the flask in two stages. Mitotic cells collected at stage 1 are used for karyotyping. MSC in this case are not treated with trypsin which can modify the quality of metaphase chromosomes and impede differential staining. All methodological steps (removal of cells from flasks, hypotonization, fixation) for making the cytogenetic preparations should sparing. The incidence of damaged cells with scattered chromosomes is much higher than in preparations from lymphocyte cultures.

At stage II, the rest cells are collected from the flask after trypsin treatment. The cells collected at this stage (interphase) are used for evaluating the incidence of aneuploidy in interphase nuclei by FISH technique (fluorescent *in situ* hybridization).

Stage I. One hour after colchicine (colcemide) addition, the flasks are placed onto a platform of an orbital shaker (100 rpm) for 30 min, this providing sparing rotation shaking. Dividing cells poorly adhere to the substrate and rotation shaking promotes their separation from the flask. After 30 min, the culture medium containing mitotic cells is collected into 15-ml centrifugation tubes. The material is centrifuged at 1000 rpm for 8 min. The supernatant is collected so that about 0.5 ml medium is left in the tube above the precipitate, the volume is adjusted to 10 ml with DMEM, and centrifugation at 1000 rpm for 8 min is repeated. The supernatant is collected.

Stage II. Trypsin treatment is needed to separate nondividing cells from the flask. To this end, the flask is washed from serum with saline (7-10 ml depending on the flask size), which is discarded during careful shaking. This procedure is repeated 2 times. Versen solution is added to the flask and after 1 min 0.25% trypsin solution is added. The solutions should just slightly cover the bottom of the flask. Commercial trypsin-EDTA solution can also be used; 3 ml is the optimal volume for 75-cm^2 flasks. After 3-7-min incubation in a thermostat at 37°C, the cells separate from the flask bottom, which can be seen under an inverted phase microscope. The trypsin is neutralized with 3 ml FCS and then DMEM is added to a volume of 15 ml. The suspension is thoroughly dispersed, collected into a centrifugation tube, and centrifuged at 1000 rpm for 8 min. The supernatant is collected.

Solution of KCl (0.55%) is added to the tubes prepared at stages 1 and 2 and the material is incubated at 37°C for 8-9 min. Hypotonization is arrested before centrifugation by adding 3-5 drops of fixative (3:1, methanol and glacial acetic acid). The tubes are centrifuged at 1000 rpm for 8 min. The preparations are fixed in 3 portions of methyl alcohol–glacial acetic acid (3:1) mixture by the standard method. The cell suspensions fixed by this method can be stored for a long time at temperutures from -20 to +4°C.

Before pipetting the cell suspension onto the slides, the fixative is replaced one more time. The suspension is pipetted onto cold wetted slides and dried on the jet flame. The quality of the preparation is evaluated by phase contrast microscopy. If the preparation contains compact nuclei and metaphase plates surrounded by a compact layer of cytoplasmic material, pipetting should be repeated on a fresh slide, because the use of poor-quality preparations impedes the cytogenetic and molecular cytogenetic analysis and even makes it impossible.

The following steps are essential for improving spreading of chromosomes and interphase nuclei on the slide. The humid camera or water bath is warmed to 60-70°C. A plastic container for slides can serve as the humid camera. A cloth or a piece of filter paper abundantly impregnated with water is put onto the bottom of the container.

The cell suspension (3-5 droplets) is pipetted onto cold slide, after which 2-3 droplets of the fixative or glacial acetic acid are pipetted, and the slide is placed into warm humid camera or is held above the water bath (60-70°C) for 10-20 sec, after which it is dried on a solid plate at 55-65°C or above the jet flame. This treatment leads to more rapid evaporation of methanol and to an increase in the acetic acid concentration in the cell. The hydrophilic properties of acetic acid promote penetration of greater volumes of water into fixed cells and hence, to increase of their volume and flattening. The processes in cell suspension during its pipetting onto slides were described in detail [4,9].

Preparation of specimens to staining. Differential staining of preparations for karyotyping is to be

carried out best of all on the next day after they have been made. If analysis of the preparations has to be carried out earlier, the slides are warmed at 60°C for 2-3 h after pipetting the suspensions.

Preparations for FISH *in situ* are recommended to be prepared at least 7-10 days before the experiment. If fresh preparations are used, additional prehybridization treatment is to be carried out. The preparations in this case are dehydrated in ascending (70, 95, 100%) alcohols (3-5 min in each), after which the slides are warmed at 60°C for 2 h. If another method of pre-hybridization treatment is used, the preparations after dehydration in ascending (70, 95, 100%) alcohols are incubated in 2× saline sodium citrate (SSC) for 2 min at 73°C or for 1 h at 37°C.

Staining of preparations for karyotyping. Differential staining of preparations for karyotyping is carried out using the GTG method (G-bands by trypsin using Giemsa) [12]. The preparations are treated for 10-20 sec with trypsin solution and then in GKN solution. Each slide is treated in a fresh portion of stain (Giemsa in PBS) for 1-2 min. The stain is then washed with cold tap water and the preparation is dried in the air.

Preparation of specimens for FISH. Cytogenetic preparations are treated with pepsin at 37°C for 10 min and then in PBS for 5 min. The next step is postfixation in 1% paraformaldehyde solution applied under coverslip for 10 min. The preparations are then plunged in PBS (5 min) at constant stirring, washed in distilled water, and dehydrated in ascending alcohols (70, 95, and 100%). The preparations are then denatured with 70% formamide at 73°C for 3 min, after which the slides are rapidly plunged in cold (-20°C) 70% ethanol and dehydrated in ascending (95 and 100%) alcohols. The hybridization mixture consists of dextrane sulfate, 4-fold SSC, formamide, DNA samples, or commercial DNA probes. The probes are denatured at 75°C for 5 min, after which the mixture is cooled to 0°C.

The preparation, hybridization mixture, and humid chamber are warmed to 37°C directly before the start of hybridization. Hybridization mixture (3-10 μl) is pipetted onto a pre-selected area of the slide containing sufficient number of nuclei not touching each other and is covered with a coverslip. In order to provide hermetic sealing, the edges of the coverslip are glued with rubber glue. Hybridization is carried out in a humid chamber at 37°C. The duration of hybridization varies from 2 to 24 h.

After hybridization the glue and coverslip are carefully removed, the preparations are washed in 0.4-fold SSC at 70°C in a water bath and then in 4-fold SSC-Twin at constant stirring and ambient temperature. After dehidratation in ascending (70, 95, 100%) alcohols, the glasses are dried in the darkness.

The nuclei are contrasted with DAPI applied under the coverslip.

Cytogenetic preparations are analyzed under light and fluorescent microscopes with a set of interference filters and an immersion objective.

Karyotyping. At least 15 metaphases are analyzed for each MSC culture. The preparations are analyzed in accordance with the International nomenclature [12]. The preparations are examined under a light microscope with an immersion objective. If abnormal karyotype is detected, the number of analyzed metaphases should be increased to 30 and more.

Evaluation of aneuploidy in interphase nuclei. Due to the use of dual- or three-color interphase FISH analysis, the nuclei with ineffective hybridization can be excluded from registration. Only well-spread non-overlapping interphase nuclei with intact membranes are analyzed. It is recommended to examine at least 800-1000 interphase nuclei.

Nuclei with 1, 2, 3, and more signals are registered. Cells without signals (nullisomy) are excluded from the analysis because of high probability of inefficient hybridization. When analyzing the nuclei with 4 signals, it is important to pay special attention to interpretation of the results, because it is hardly possible to differentiate tetrasomy in a diploid cell from tetraploidy or from cells in the late S or early G_2 stage of the cell cycle by FISH analysis of 2-3 chromosomes.

The signals located closer to each other than one nuclear diameter, but equal to other signals in the visual field by their size and fluorescence intensity, should be registered as two signals. Two signals at a distance of one nuclear diameter, but connected to each other are considered as the replica of one chromosome.

All data are recorded in the protocol of the study. Two protocols are presented: one for dual-color, the other for three-color FISH (Figs. 1, 2).

Cells with abnormal numbers of signals are registered in Table A of the protocol of incidence of aneuploidy and polyploidy by two chromosomes (Fig. 1). Each line and column in the table have titles showing the number of signals from each of the studied chromosomes (chromosomes a and b can be presented by 0, 1, 2, 3, 4, *etc.* signals in the cell). One dot denotes one analyzed nucleus. For example, crossings of lines titled 1, 2, 3, and 4 (one, two, three, four signals of chromosome b) and columns titled 1, 2, 3, 4 (one, two, three, and four signals of chromosome a) represents all monosomic (haploid), disomic (diploid), trisomic (triploid), and tetrasomic (tetraploid) nuclei, respectively. Crossing of the line titled 1 (one signal of chromosome b) and columns titled 2, 3, 4 (two, three, and four signals of chromosome a) and of the line titled 2 (two signals of chromosome b) with columns titled 1, 3, 4 (one, three, and four signals of chromosome

Protocol of Aneuploidy and Two Chromosome Polyploidy Incidence

Operator:

Date of study:

Slide No.

TABLE A. Number of cells with different numbers of signals

Chromosome b \ Chromosome a	0	1	2	3	4
0					
1		●●● (3)	●● (2)		
2		●● (2)	986	● (1)	
3				● (1)	
4					●●●●● (5)

TABLE B. Cells with two signals from both chromosomes

10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	6	—

TABLE C. Number of cells with different numbers of signals from each of the chromosomes

Number of signals	**0**	**1**	**2**	**3**	**4**
Chromosome a	0	5	988	2	5
Chromosome b	0	5	989	1	5

Conclusion: Three cells with monosomy, one with trisomy, five with tetrasomy by chromosomes a and b were detected. Two signals from chromosome b and only one from chromosome a were detected in two cells. Two cells had one signal from chromosome b and two from chromosome a. One cell had two signals from chromosome b and three from chromosome a. A total of 986 cells contained 2 signals from both analyzed chromosomes. A total of 1000 cells were analyzed.

Fig. 1. A protocol of aneuploidy and two chromosome polyploidy studies.

Protocol of aneuploidy and three chromosome polyploidy incidence

Operator:

Date of study:

Slide No.

TABLE A. Number of cells with different numbers of signals

Chromosome a / Chromosome b	0	1	2	3	4
Chromosome c: 0 signals					
0					
1					
2					
3					
4					
Chromosome c: 1 signal					
0					
1		•(1)			
2					
3					
4					
Chromosome c: 2 signals					
0					
1					
2			996		
3					
4					
Chromosome c: 3 signals					
0					
1					
2			•(1)		
3					
4					
Chromosome c: 4 signals					
0					
1					
2					
3					
4					••(2)

TABLE B. Cells with two signals from all chromosomes

10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	6

TABLE C. Number of cells with different numbers of signals from each of chromosomes

Number of signals:	**0**	**1**	**2**	**3**	**4**
Chromosome a	0	1	997	0	2
Chromosome b	0	1	997	0	2
Chromosome c	0	1	996	1	2

Conclusion: One nucleus monosomic by each of the studied chromosomes, one aneuploid nucleus containing three chromosomes c, two chromosomes a, and two chromosomes b; two tetrasomic cells with four replicas from each of analyzed chromosomes were detected. A total of 1000 cells were analyzed.

Fig. 2. A protocol of aneuploidy and three chromosome polyploidy incidence.

a); line titled 3 (three signals of chromosome b) and columns titled 1, 2, 4 (one, two, and four signals of chromosome a); line titled 4 (four signals of chromosome b) and columns titled 1, 2, 3 (one, two, and three signals of chromosome a) present all aneuploid cells. The number of all diploid nuclei is shown in the cell at the crossing of line 2 (two signals of chromosome b) and column 2 (two signals of chromosome a). Since the number of these cells is very high after analysis of 1000 nuclei, they are shown in a separate table (B). Table B consists of 100 sections, with 10 cells with two signals from both chromosome in each of them.

Table C shows the number of cells aneuploid by each chromosome. The percentage of cells without signals, with one, two, three, and four signals is calculated from these data.

Protocol 2 (Fig. 2) is used for simultaneous analysis of three chromosome (a, b, c) aneuploidy. The protocol is filled in similarly as for two chromosome analysis.

The protocols presented here give an idea of the primary events in the cell and help evaluate the possible combinations of aneuploidies for different chromosomes in each abnormal cell, which is essential for verifying the hypotheses on the appearance of abnormalities of chromosome number.

RESULTS

When discussing the criteria of genetic stability of cell transplants one should take into account the summary data of karyotype and aneuploidy analysis in the corresponding cell types.

According to our data, abnormal clones were detected in about 30% adipose tissue MSC and in 11% bone marrow MSC cultures [1,2]. Initial aspirated bone marrow or adipose tissue can be the sources of abnormal clones. For example, a clone with 45,X karyotype (95%) was detected in an MSC culture derived from a donor with mosaic karyotype of peripheral blood lymphocytes (95% 46,XY and 5% 45,X). Obviously, the abnormal clone in this culture originated from donor cells which had a selective advantage during culturing.

A specific cell clone, appearing *de novo* and characterized by high chromosome instability and proliferative advantages, can become the cause of malignant transformation of cultured cells. For example, we detected MSC cultures in which clones with the Robertson's translocation (21;22), chromosome 6 monosomy, and chromosome 18 trisomy appeared during culturing.

Hence, even short culturing (2-4 passages) can drastically increase the probability of malignant transformation at the expense of appearance of *de novo* genome mutations and as a result of positive selection of abnormal cell clones existing in the body and characterized by higher multiplication rate in comparison with normal cells.

The problem of evaluating clone formation in mosaic cultures, discussed previously [2], deserves special attention. The presence of a clone in a culture is confirmed by the presence of a group of cells with the same chromosome abnormality, but the problem – which, specifically, incidence of aneuploid cells in the culture implies that this culture should be classified as mosaic culture with clones – deserves a special study. The difficulty of the problem consists in heretofore not studied regularities of the time course of growth of cell populations in which abnormal cells appear. As the conditions of culturing and reinoculations of cell cultures are usually similar, the appearance of abnormal cell clones can be explained by their selective advantages in multiplication.

Mutant cells with abnormalities can die during cell division, can multiply at the same rate as normal cells, or multiply more rapidly, forming modified cell clones. It remains unclear, which abnormalities modify the cell growth potential. All three variants are most likely probable, and hence, it is obvious that cytogenetic study should register separately the chromosome and genome variability which should be differentiated from the formation of clones with chromosome abnormalities.

Clinical use of cultures with chromosomal and genomic changes can lead to untoward delayed effects, while genetic screening of prospective cell transplants using various DNA probes for detection of aneuploidies or other markers of genetic instability of cultures can prevent them.

Opposite viewpoints on the hazards of karyotypic changes in cultures used for cell therapy have been expressed. The majority of authors think that the appearing chromosome abnormalities characterize the genetic instability of culture, which can lead to malignant transformation [17,20].

Chromosome abnormalities appearing at different stages of fetal SC culturing have been described [5,8,10,15,19]. A recent report describes the development of a multifocal neuroglial tumor of the brain in a 13-year-old boy suffering from ataxia-telangiectasia receiving repeated intracerebellar and intrathecal injections of fetal neural SC [7]. It was shown that these tumors formed from donor cells, to be more exact, from cells obtained from at least two donors.

Though no karyotype changes were detected in the majority of studies on MSC [13,16,22], it was shown that they could spontaneously transform during *in vitro* culturing, acquiring the characteristics of tumor cells [17,21]. Injection of these cells to laboratory mice resulted in the formation of numerous tumors [17]. Immortalized MSC strains from human bone marrow

also can induce *in vivo* tumor growth in mice [18]. All authors are unanimous that MSC culturing for transplantation should not be long. Some authors think that genetic transformation of MSC can be a result of different approaches to cell culturing, while long-term culturing and transfection of cells can lead to negative consequences because of genetic instability.

Hence, our results and published data indicate that clones with chromosome restructuring can appear during delayed passages of cell cultures. These clones effectively multiply and are characterized by more rapid proliferation and better survival after cryopreservation. An abnormal clone can originate from initial bone marrow or adipose tissue aspirates. A specific cell clone, characterized by high chromosomal instability and proliferative advantage, pre-existing or appearing during early stages of culturing, can become the cause of malignant transformation of cultured cells. Hence, cytogenetic testing of SC before their transplantation should be a component of the cell therapy safety provision system. The method for rapid comprehensive evaluation of genetic stability of cultures, proposed in this paper, will provide the safety of cells for transplantation before their clinical application.

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