CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Statistical Analysis of Clone Formation in Cultures of Human Stem Cells

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We performed a statistical analysis of clone formation from aneuploid cells (chromosomes 6, 8, 11, X) in cultures of bone marrow-derived human multipotent mesenchymal stromal cells by spontaneous level of aneuploidy at different terms of culturing (from 2 to 19 cell cycles). It was found that the duration of cell cycle increased from 65.6 h at passages 2-3 to 164.5 h at passage 12. The expected ratio of aneuploid cells was calculated using modeled 5, 10, 20 and 30% selective preference in reproduction. The size of samples for detecting 10, 25, and 50% increased level of aneuploidy was calculated. The presented principles for evaluation of aneuploid clone formation may be used to distinguish clones of any abnormal cells.

Key Words: *mesenchymal stromal cells; aneuploidy; clone formation; sample volume*

Cell clones with chromosome and genome mutations can appear during SC culturing for transplantation purposes [1,8]. For ensuring genetic safety of cell therapy, special criteria for sorting out the cultures with selective multiplication of cells with abnormal chromosome sets (clone formation) should be developed. Similar task was solved in clinical cytogenetics for detection of mosaicism [4,6,9]. Statistical analysis in these cases was based on standard verification of hypothesis on equality of the means in steady-state cell populations. This approach can not be applied for the analysis of SC cultures, because in this case with speak about the dynamics of cell populations.

The dynamics of selective proliferation of the clone-forming population of abnormal cells is described by a mathematic model with a possible variant of its practical realization. At the same time, culture clonality can be evaluated on the basis of calculation experiments employing the data on spontaneous aneuploidy level in human SC.

The aim of the present study was to substantiate the criteria of clone formation in cultures of human SC on the basis of experimental data on the count of aneuploid cells passing different number of divisions.

MATERIALS AND METHODS

Analysis of clone formation was based on previous experimental data on evaluation of aneuploidy in cultures of multipotent mesenchymal cells (MSC) from the bone marrow (BM) at different passages [1,5,7] and statistical analysis was carried out.

For calculation of the dynamics of cell populations, cell cycle was used as a unit and its duration was evaluated by cell multiplication factor during passages [7]. The duration of each passage was 7 days (168 h).

The duration and the number of cell cycles in cultures passing different number of passages, the expected ratio of aneuploid cells under conditions of

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selective preference in reproduction, and the volume of cell samples for the analysis of clone formation were calculated.

RESULTS

Basic experimental parameters for the analysis of clone formation. The ratio of cell lines (normal or aneuploid) detected on fixed preparations is a function of cell cycle duration for each variant and proliferation rate. The cell cycle duration for a cell with abnormal chromosome set can be equal, shorter, or longer than in normal cells. Abnormal cells with normal or reduced proliferation rate reflect spontaneous level of variability and, hence, are safe for transplantation. These cultures do not require clone formation prognosis. In turn, cells with accelerated cycle have selective preference and their relative content in the total population increases during culturing (clone formation). Hence, the following parameters are required for statistical analysis of clone formation: cell cycle duration, number of passed cycles, and mean spontaneous level of aneuploid cells.

Cell cycle duration can be calculated by cell multiplication factor [7]; during *in vitro* culturing this parameter was 5.88 at passages 3-4, 4.81 at passages 5-6, 2.63 at passages 7-9, and 2.03 at passages 10-12 (n=32). Cell multiplication factor is a ratio of the cell number at a certain passage to that at the previous passage ($k=n_i/n_{i-1}$).

From the presented data, the number of cell cycles at different passages can be calculated (Table 1). The number of cell divisions (m) by passage i was calculated by the formula: $m_i=\log_2 k_i$, where: m_i the number of cell division at passage i, i is number of passage; k is cell multiplication factor for a single passage.

From the number of cycles (Table 1) and duration of culturing, *i.e.* number of passages (duration of each passage was 7 days, or 168 h) we can calculate the duration of cell cycles (in hours) at different terms of culturing (Fig. 1).

The frequency of spontaneous aneuploidy is one of the basic experimental parameters for the analysis of clone formation. In Table 2, the data from previous reports [1,5,7] are summed up for different BM cultures, passages, and chromosomes.

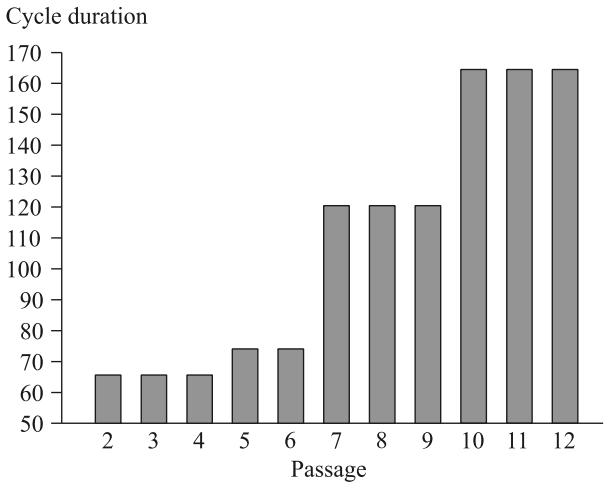

Fig. 1. Cell cycle duration at different terms of culturing

TABLE 1. Number of Cell Cycles by the Moment of Fixation at Different Passages

Passage No.	Multiplication factor (k)	Number of cell cycles (m)	
		in previous passages*	by the moment of fixation+
2	5.88	2.56	2.56
3	5.88	2.56	5.11
4	5.88	2.56	7.67
5	4.81	2.27	9.93
6	4.81	2.27	12.20
7	2.63	1.40	13.60
8	2.63	1.40	14.99
9	2.63	1.40	16.39
10	2.03	1.02	17.41
11	2.03	1.02	18.43
12	2.03	1.02	19.45

Note. *$m=\log_2 k_i$, +$m_{sumi}=m_{sumi-1}+m_i$.

Calculation of expected ratio of aneuploid cells at selective preference in reproduction. These calculations allows us to detect clone formation. Cell population with a frequency of aneuploid cells exceeding two standard deviations is considered as a clone-forming population.

Previous reports [1,5,7] showed that the control level of aneuploid cells in MSC culture is about 1.0%. In the analyzed cultures [1,5,7], the aneuploidy rate by chromosomes 6, 8, 11, and X (*i.e.* per one conventional chromosome, M) was 1.21±0.37. The confidence interval for M was calculated with the assumption on normal distribution of the total population; the confidence probability was taken as 95%.

This value can be taken as a boundary between the normal and clone-forming MSC cultures. Hence, cell cultures containing 2 aneuploid cells per 100 cells should be considered as potentially clone-forming cultures.

The expected ratio of aneuploid cells was calculated by the formula:

$$L_k=[(v_n\times(1+L_a))^k\times P_a]/[v_n{}^k\times(1-P_a)+v_n\times(1+L_a))^k\times P_a],$$

where L_k is the ratio of abnormal cells after k cycles, k is the number of division cycles of normal cell, P_a is control level of aneuploid cells, v_n is the number of doublings of normal cell over one cycle (in our case v_n is 2, *i.e.* all cells underwent one division), and L_a is shortening of cell cycle duration.

TABLE 2. Summarized Mean Frequencies (%) of Aneuploid Cells in MSC

Chromosomes	Early passages	Late passages
6	1.14	1.12
8	1.16	0.94
11	1.39	1.82
X (women)	1.51	0.58

Table 3 shows the results of calculation for cultures passing 2, 4, 6, and 10 cycles at different initial control level of aneuploid cells (0.005, 0.01, and 0.05, or 0.5, 1.0, and 5.0%).

Thus, acceleration of the cell cycle by 20% and more can lead to the appearance of a clone with progressing ratio of aneuploid cells (start of clone formation) as soon as after 4 cycles (passage 3, Table 1), while by the 10th cycle (passage 5-6, Table 1) it attains 5.89%. It is natural that the higher is cycle acceleration, the higher is the rate of clone-formation (Table 3).

Table 3 presents the results of calculation at the initial level of aneuploidy of 5.0%. This may concern cells entering the phase of accelerated proliferation. In this case, acceleration of the cell cycle by 20-30% leads to an increase in the content of aneuploid cells by 20-40%.

TABLE 3. Expected Ratio of Abnormal Cells with Different Selective Preference

Shortening of cell cycle, %	Control level of aneuploid cells, %	Ratio of abnormal cells, %			
		after 2 cycles*	after 4 cycles*	after 6 cycles*	after 10 cycles*
5		0.55	0.61	0.81	
10	0.5	0.60	0.73	0.88	1.29
20		0.72	1.03	1.48	3.02
30		0.84	1.41	3.37	6.48
5		1.10	1.21	1.34	1.62
10	1.0	1.21	1.46	1.76	2.55
20		1.43	2.05	2.93	5.89
30	1.68	2.80	4.65	12.22	
5		5.48	6.01	6.59	7.90
10	5.0	5.99	7.15	8.53	12.01
20		7.05	9.84	13.58	24.58
30		8.17	13.07	20.26	42.05

Note. Single cell cycle duration of normal cells is taken as a unit (see Table 2)

TABLE 4. Volume of Analyzed Cell Samples for Detection of Increased Aneuploidy Level

Control level of aneuploidy, %	Aneuploidy frequency increase		
	by 10%	by 25%	by 50%
0.5	76448	12232	3058
1.0	38032	6085	1521
2.0	18824	3012	754

Volume of analyzed cell samples for detection of increased aneuploidy level. Our aim was to evaluate the number of cells to be analyzed for detection of changes in aneuploidy level during cell proliferation. The initial level of aneuploidy is supposed to be set within the limits of spontaneous level (0.5-2.0%). During calculation, the excess in this parameter, which should be detected during the experiment with a certain probability, was also set. Similar task has been solving in cytogenetic screening of workers for detection of occupational hazard [2].

In this case, we used integral Laplace theorem [3] for solving the problem. The probability of detection of aneuploid cells in the sample was taken as 95%. Table 4 shows the calculated sample volumes depending on the control level of aneuploidy and preset aneuploidy excess.

Thus, the higher is the level of aneuploidy, the lower is sample volume required for its detection (Table 4). The samples can be reduced in case of considerable elevation of aneuploidy frequency. Increasing the sample is required for proving the reliability of the negative response >95%, detection of minor (<10%) effects, and for detection of dynamic regularities of aneuploidy.

We analyzed calculations of the total cell sample volume without consideration of individual cell culture origin. The question on the ratio of individuals (cell cultures) and the number of analyzed cells from each individual requires special study based on the analysis of individual variations of spontaneous level of aneuploid cells.

Three main problems were studied from the viewpoint of clone formation detection in MSC cultures: 1) cell cycle duration at different passages; 2) expected ratio of abnormal cells with selective preference in reproduction; 3) sample volume of analyzed cells for detection of elevated aneuploidy level. They all are related to the analysis of aneuploidy in whole. However, the frequency of aneuploid cells is a sum of trisomic (result of chromatid nondisjunction) and monosomic cells (result of chromatid nondisjunction and chromosome lagging). It cannot be excluded that such an analysis in some cases should be done separately for trisomic and monosomic cells. There are no methodical limitations for using the described principles of clone formation evaluation in trisomic, monosomic, and other abnormal clone-forming cells. At the same time, the population dynamics of abnormal cells in culture can greatly vary. For evaluation of the nature of population cell processes, experimental methods should be supplemented by mathematic and simulation methods.

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