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Biological Characteristics of Mesenchymal Stem Cells during Ex Vivo Expansion

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Research Article

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ABSTRACT

Mesenchymal stem cells (MSC) have been widely used in different areas of medicine because of their immunosupressive properties and influence on regeneration of the damaged tissues. The objective of this study was to examine the kinetics of growth (MSC from 4-3 passages had significantly higher proliferative activity compared with cultures of 10-12 passages), changes in immunophenotypic characteristics of human MSC in early (3-4) and late (10-12) passages when cultured in vitro as well as study of genetic stability. In the analyzed cultures of MSC was found normal karyotype (46, XY or 46, XX). In most of cultures studied MSC karyotype and the level of aneuploidy remained unchanged even after prolonged culturing.
The author's experience of ex vivo expansion of MSC using both standard medium DMEM with the supplementation of fetal bovine serum (FBS) and serum-free medium MesenCult MSC Basal Medium (Human) (StemCell technologies Inc.) with addition of Mesenchymal Stem Cell Stimulatory Supplements (Human) is presented. The influence of the type of medium on the growth and immunophenotype of MSC is analyzed. The study found that when cultured in serum-free medium proliferative potential of MSCs was lower.

Keywords: Mesenchymal stem cells; phenotypic profile; genetic stability; expansion ex vivo; FCS-free culture;

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1. INTRODUCTION

Bone marrow stromal stem cell with the capacity of forming colonies of fibroblast-like cells in culture was first identified by Friedenstein et al. (Fridenstain et al., 1970; Fridenstain and Luriia, 1980; Vladimirskaya and Koshel, 1990). These cells were called colony-forming unit fibroblasts (CFU-F). The stem cell nature of these cells including the ability to self-renewal and differentiation into various mesenchymal elements has been demonstrated in numerous studies (Fridenstain et al., 1970; Fridenstain and Luriia, 1980; Vladimirskaya and Koshel,1990; Fridenstain, 1982; Owen and Fridenstain, 1998; Owen, 1995; Minguell, 2001; Minguell and Conget, 2000; Prockop, 1997; Castro-Malaspina et al., 1980). Due to their capacity to differentiate into cells of connective tissue lineage, including bone, fat, cartilage and muscle later these cells were called mesenchymal stem cells (MSC) (Caplan, 1994; Pittenger et al., 1999). In recent years, MSC are increasingly used in clinical practice for cell therapy (Minguell and Conget 2000; Le Blanc and Ringden, 2007; Miura et al., 2005; Stenderup et al., 2003). However, lack of standardized methods of isolation, expansion and immunophenotype characterization of MSC requires a well defined criterion for cells expanded in vitro for clinical purposes.

The most commonly used supplements to standard medium for in vitro expansion of MSC are fetal bovine serum (FBS) and fetal calf serum (FCS). Both of them contain a lot of factors that stimulate cell growth (nutrients, growth factors, etc.).

Most preclinical studies and more than 10 clinical protocols used MSC expanded in medium containing 10-20% FCS (Caterson and Nesti, 2002; Sotiropoulou et al., 2006; Ringden et al., 2006).

Nevertheless, animal serum could be undesirable source of xenogeneic antigens and bear the risk of transmission of animal viruses, prions when MSC used in clinical settings. In addition, bovine proteins and peptides could be conjugated to MSC, and subsequently induce an immune response, especially after repeated injections of MSC as well as subsequently cause rejection of transplanted cells (Horwitz et al., 1999). Therefore, alternative serum-free culture medium has been implemented for better safety of MCS used in clinical practice. Chemically standardized composition of serum-free medium could be a good alternative during the expansion of MSC. But at the present time ex vivo expansion of MSC in serum-free medium is still under discussion.

One of the important issues of the clinical application of MSC is oncogenic safety. The risk of carcinogenesis from the adult stem cells after transplantation is indicated by the fact that they express common markers of embryonic cell lines (Tai et al., 2004; Rubio et al., 2005). In cultures of human MSCs isolated from subcutaneous adipose tissue and bone marrow, after prolonged cultivation in vitro (20 divisions) revealed colonies of actively dividing cells with multiple chromosomal abnormalities characteristic of malignant cells, these cells acquired the ability to form tumors when administered to SCID-mice (Miura et al., 2005; Kassem et al., 2005). Other authors have not identified signs of malignant transformation in long-term cultures of MSC (Stenderup et al., 2003).

Due to the conflicting results of malignant potential of ex vivo expanded MSC tin long-term cultures, the problem of genetic safety of MSC used in clinical settings is relevant and requires further investigation.

The objective of this study was to examine the kinetics of growth, changes of immunophenotypic characteristics of human MSC in early (3-4) and late (10-12) passages when cultured in vitro, to study genetic stability, as well as MSC expansion in medium supplemented with animal serum and in serum-free medium.

2. MATERIALS AND METHODS

MSC were isolated from healthy bone marrow transplantation donors (n = 77) in Federal Clinical Research Center of Pediatric Hematology, Oncology and Immunology.

Expansion of bone marrow stromal fibroblasts was performed by the method proposed by Friedenstein et al. (1973). Mononuclear cells isolated from 20-40 ml of bone marrow were seeded in air culture flasks with bottom area 75 cm^2 at a concentration of 30-40 10^6 per flask in DMEM with low glucose concentration supplemented with 20% FCS. After 1-3 days nonadherent cells were washed out and the plastic adherent MSC were further expanded. At the day 14 MSC were removed from plastic using trypsin-EDTA and passaged every 7 days at a concentration of $0.5x10^6$ per flask before the end of expansion. Cultivation was performed at 37 °C in a humidified atmosphere containing 5% CO2 and 95% air.

We used three types of media for the expansion of MSC:

1. Standard DMEM (“Biolot ") supplemented with 20% FCS (Fetal calf serum,“Biolot”).
2. Ferum-free MesenCult MSC Basal Medium (Human) (StemCell technologies Inc.) supplemented with Mesenchymal Stem Cell Stimulatory Supplements (Human).
3. Ferum-free MesenCult MSC Basal Medium (Human) (StemCell technologies Inc.) supplemented with Mesenchymal Stem Cell Stimulatory Supplements (Human) and 5% FCS.

Kinetics of MSC expansion were defined as the ratio of number of cells obtained from this passage to the number of cells seeded in the previous passage.

The following panels of antibodies were used for immunophenotyping: CD3, CD13, CD14, CD19, CD25, CD29, CD31, CD34, CD38, CD44, CD45, CD73, CD90, CD105, CD106, CD166, and HLA-DR. We determined both relative number of MSC expressing a particular antigen in culture (in %) and the relative fluorescence intensity of the studied antigens (in rMFI).

Cytogenetic analysis. Colchicine at a final concentration of standard (0.5 ug / ml) was injected into the culture flasks for 1.5 hours before fixation. After graduating kolhitsinizatsii cells were removed from the wall of the vial with a solution of trypsin-EDTA. Gipotonizatsiyu spent 0.55% solution of KCl (8 min at 37 ºC) before centrifugation was added 3-5 drops striker to stop gipotonizatsii. Fixation was carried out with a mixture of methanol and glacial acetic acid (3:1) in a standard way, using a 3-shift striker. Karyotyping. Cytogenetic differentially stained preparations for karyotyping were prepared by G-staining. For each MSC culture at least 15 metaphases were analyzed. Analysis of the drugs was carried out according to Shaffer and Tommerup (2005).

Aneuploidy analysis. Centromere-specific DNA probes for chromosomes X, Y, 6, 8, 11 (Vysis, Inc) were used to perform interphase FISH-analysis. Denaturation, hybridization and washing off were carried out according to the standard protocol. For contrasting nuclear staining DAPI were used. FISH-preparations were analyzed under AxioImager microscope

with a set of interference filters with FISH-analysis software (Fish View System, Applied Spectral Imaging, GmbH). At least 1000 interphase nuclei were analyzes in each culture. Using the method of multi-color interphase FISH-analysis allowed us to exclude from the analysis nucleus with ineffective hybridization.

Statistical data processing was carried out in Biostat programs, Excel and Statistica, 6. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

MSC cultures had monomorphic appearance and consisted of spindle-cells from the third passage regardless of the medium used for expansion (Figure 1).

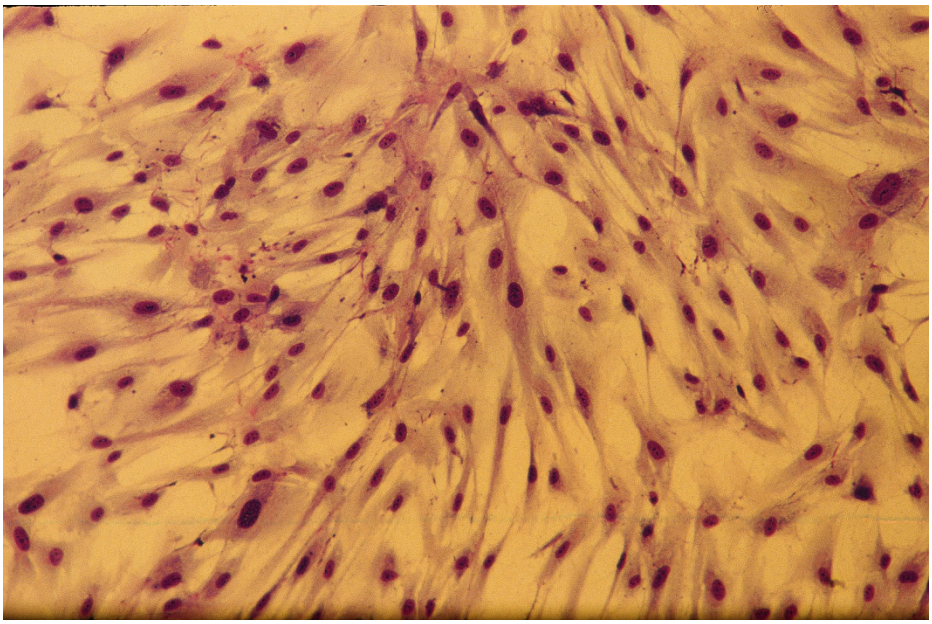

Fig. 1. Morphology of MSC in culture

We found that proliferative activity of MSC gradual decreased during the long-term in vitro cultivation. Data are presented in Table 1. Thus, MSC from 4-3 passages had significantly higher proliferative activity compared with cultures of 10-12 passages. Expansion rate was 5.88 and 2.03, respectively.

Table 1. MSC expansion rate in long-term cultures

№ passage	**3,4 passage n=32**	**5,6 passage n=32**	**7,8,9 passage n=32**	**10,11,12 passage n=25**
Growth rate median	5,88	4,81	2,63	2,03
p	$P_{3,4\text{-}10,11,12}$ =0.012			

Thus, the rate of MSC expansion in vitro is maximal at 3-4 passages, slightly decreased to 5-6 passages and significantly decreased in the later (10-12) passages.

Comparative analysis of MSC expansion in different culture medium showed that after the first passage 4.58 x10^6 cells per sample (range 0,2-14,1) were obtained in the medium 1, 2.08 x10^6 cells per sample (range 0,1-8,1) in medium 2 and 5.04 x10^6 cells per sample (range 0,1-16) in medium 3 (Figure 2). Data depicted on the diagram are not statistically significant due to small sample size.

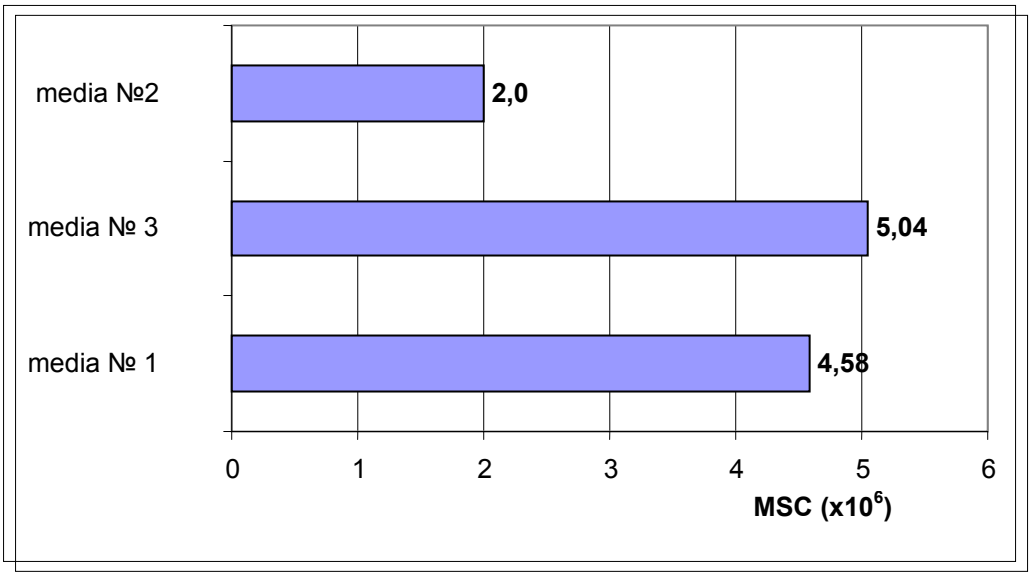

Fig. 2. Number of cells (x10^6) obtained after 14 days expansion in different media

Analysis of MSC number after each passage showed that the lowest number of cells to passage 4 (P4) was obtained in serum-free medium.

One of the limitations of our study is the small number of samples, not allowed us to reach statistical significance in number of MSC expanded in different media. Nevertheless, there was a trend towards lower expansion rate in serum-free media compared to medium supplemented with FBS. However, several authors successfully used serum-free media for MSC expansion obtaining amounts of MCS required for clinical purposes. Further studies are needed to determine the place of serum-free medium in the protocols of ex vivo MSC expansion, including clinical protocols. Significant differences of cell surface antigen profile of MSC were observed in number of studies. Several factors could contribute to that disagreement, including different media used for MSC expansion, enzymatic solutions for washing cells from plastic, different time intervals between the processing of cells and flow cytometric analysis, differences in MSC cell age (the number of passages). Nevertheless, the minimal panel for immunophenotypic characterization of MCS after 3-4 passages has been proposed recently (Le Blanc and Ringden, 2007; Delorme, 2008).

Our results are consistent with these data. After 3-4 passages the high (>60%) expression of the following markers was observed on MSC: CD90, CD105, CD166, CD44, CD73. CD13

and CD29 were intermediately expressed (30-60%). Stromal bone marrow cells did not carry the following markers CD45, CD34, CD133, CD3, CD19, CD25, CD38, CD45, CD106, CD31 (% positive cells <5%). The data are presented in Table 2.

Table 2. Immunophenotype of MSC during long-term in vitro cultivation

Antibody	Positive cells (%)		Significance
	MSC 3-4 passage (n=77) median	MSC 10-12 passage (n=77) median	
CD3	0,48	0,26	p=0.22
CD13	24,54	13,19	p=0.48
CD14	0,58	0,155	↓p=0.07
CD19	0,29	0,3	p=0.90
CD25	0,23	0,29	p=0.63
CD29	53,82	35,59	p=0.59
CD31	2,22	1,1	↓p=0.06
CD34	0,46	0,64	p=0.67
CD38	0,55	0,33	p=0.16
CD44	81,94	82,14	p=0.34
CD45	2,75	1,3	↓p=0.049
CD73	97,5	98,5	p=0.34

The data presented in Table 2 showed that with an increase of MSC cultivation to 10-12 passages only the number of cells expressing CD90 and CD166 was significantly reduced. In contrast to the data reported by Kassem et al., 2005, we did not observe increase in the number of CD133 + cells during passaging MSC to 10-12 passages. Along with the increase number of passages of MSC disappear admixtures of hematopoietic cells (CD45 +) and endothelial cells (SD31 +).

The results of the intensity of expression of cell surface antigens on MSC during long-term cultivation in vitro are presented in Table 3. With increasing period of cultivation MSC to 10-12 passages, despite the decline in the number of CD90+ cells and CD31+ cells, the intensity of expression of these antigens was increased. Our data revealed no statistically significant differences in the intensity of the expression of other antigens studied during in vitro expansion of MSC.

By light microscopy MSC both at early and late passages represented a homogeneous population of large-sized cells, distinct from hematopoietic. The observed changes in the expression of cell surface antigens on MSC during expansion could be explained by the clonal heterogeneity in the population of cultured cells.

Cell surface antigen profiles were studied at early and late passages of MSC expanded in different media (standard DMEM medium supplemented with FCS and in serum-free medium).

Table 3. Intensity of the expression of cell surface markers on MSC during long-term in vitro cultivation

Antibody	Intensity of the expression (rMFI)		Significance
	MSC 3-4 passage (n=77) median	MSC 10-12 passage (n=77) median	
CD13	422	647	p=1,0
CD29	400	727	p=0.89
CD31	13	34	p=0.045
CD44	5309	4725	p=0.34
CD73	2577	1538	p=0.74
CD90	496	703	p=0.047
CD105	1507	2060	p=0.54
CD133	13	20	p=0.64
CD166	354	374	p=0.26

All cells expressed surface markers characteristic of MSCs. However, contamination of hematopoietic cells was less pronounced in the expansion in serum-free medium both in the early and late passages (data not significant).

We have analysed the karyotype of 21 culture of MSC and found normal karyotype (46, XY or 46, XX) in 1 cultures. The karyotype didn't change during expansion. In two cultures clones were detected, which was confirmed by FISH-analysis.

Average frequency of aneuploidy for sex chromosomes is shown in Table 4. Spontaneous frequency of loss of the Y chromosome in a male donor MSC was higher than the X chromosome ($p < 0,05$). This is because nullisomy on chromosome X is fixed by us as a very rare phenomenon, which may be associated with the identification of single events only of the X chromosome or hybridization artifact. Frequency of disomy for chromosomes X and Y are not changed after prolonged cultivation and did not differ among themselves (see Table. 4). In the analysis of X chromosome aneuploidy in cultures of MSC women, as well as for the autosomes, the frequency of observed excess over monosomy trisomy ($p < 0,05$). When comparing the frequencies of aneuploidy of sex chromosomes at early and late passages, the difference was not revealed. Aneuploidy frequency in autosomes and sex chromosomes were not different and, apparently, is characterized by spontaneous level of aneuploidy in the MSC.

Table 4. Average frequency of aneuploidy for sex chromosomes in MSC

Passages	Early passages		Later passages	
Male cells	Nulisomy	Disomy	Nulisomy	Disomy
chromosome X	0,13±0,05*	1,36±0,19	0,01±0,01	2,00±0,43
chromosome Y	0,56±0,15*	0,97±0,18	0,32±0,17	1,68±0,40
Female cells	Monosomy	Trisomy	Monosomy	Trisomy
chromosome X	1,30±0,31	0,21±0,10	0,68±0,29	0,19±0,03

During the analysis of the frequency of aneuploidy in cultures of MSC 25 000 nuclei were analyzed. Table 4 presents the results of the frequency of X chromosome aneuploidy in cultures of men - bone marrow donors in the early and late passages.

The average number of normal cells with one X chromosome was 99,40 ± 0,12% at early and 99,54 ± 0,14% at late passages. The frequency of nuclei with two X chromosomes varied from 0,1 to 1,07% in different cultures, and the average was 0,52 ± 0,10% (early) and 0,46 ± 0,14% (late passage). Nulisomy on chromosome X was extremely rare (0,08 ± 0,04%). This finding may be associated with the identification of single events only loss of the X chromosome due to nondisjunction or lagging in the process of cell division, or may be viewed as an artifact of hybridization. According to the literature nulisomy on X chromosome is not compatible with the survival of cells. Such cells can be identified by the sensitivity of FISH-analysis on interphase nuclei and is likely to have already launched the mechanism of apoptosis.

The frequency of aneuploidy for sex chromosomes did not change during cultivation, and corresponds to data obtained in cultures of human lymphocytes (Nazarenko and Timoshevskii, 2004).

Data on the frequency of aneuploidy for chromosomes 6, 8, 11 and sex chromosomes in the MSC are presented in Tables 5. In total at different passages about 60000 interphase nuclei were analyzed.

Table 5 presents data on the frequency of mono- and trisomy of chromosomes 6, 8 and 11 in the MSC in the early passages. The frequency of monosomy and trisomy of chromosomes 6, 8 and 11 did not differ ($P > 0,05$) and was 1% and 0.3%, respectively. Nuclei with monosomy prevailed over nuclei with trisomy. These data correspond to results obtained previously in the study of MSC isolated from adipose tissue. (Bochkov et al., 2008). The frequency of aneuploidy of autosomes didn't varied during cultivation of MSC ($p>0,05$) . However, comparison of the level of aneuploidy between chromosomes 8 and 11 in the late passages revealed that the frequency of monosomy of chromosome 11 was higher than the corresponding value of chromosome 8. Since the frequency of trisomy for these chromosomes did not differ, the data do not allow to conclude that individual chromosomes contribute differently in the formation of chromosomal aberrations and are likely to indicate a need for more material on the frequency of aneuploidy in different chromosomes. According to another chromosome aneuploidy frequency did not differ.

Table 5. Frequency of aneuploidy on chromosomes 6, 8 and 11 in MSC at early and at late passages

Passages	Chromosome 6		Chromosome 8		Chromosome 11	
	Monosomy, % (M+м)	Trisomy, % (M+м)	Monosomy, % (M+м)	Trisomy, % (M+м)	Monosomy, % (M+м)	Trisomy, % (M+м)
Early passages	0,92±0,14	0,48±0,14	0,81±0,12	0,22±0,07	1,20±0,24	0,27±0,08
late passages	0,92±0,23	0,37±0,10	0,77±0,12*	0,16±0,05	1,56±0,32*	0,27±0,06

Thus, in most of cultures studied MSC karyotype and the level of aneuploidy remained unchanged even after prolonged culturing. In part of the cultures even at early passages clones with an abnormal karyotype were revealed. Immunophenotype of these cultures didn't changed, but the capacity of colony formation, inherent in MSC resulted into the formation of clones.

It is well known that chromosomal abnormalities characterize the genetic instability of cultures, which can lead to malignant transformation. However, there is a belief that culture with karyotypic changes are not dangerous for transplantation. Clones carrying the various chromosomal aberrations are likely to differ in their potential danger. Thus, clones with one sex chromosome X identified by us can be attributed to benign in terms of carcinogenic potential. In transplantation, these cells are likely to restore the capacity for hematopoiesis, or compensate a deficiency of the enzyme. The clone with trisomy of chromosome 8 is potentially carcinogenic. Trisomy 8 - one of the most frequent aberrations in myeloid malignancies and as the sole chromosomal abnormality occurs in about 8-15% of patients (Wolman et al., 2002). Clones with trisomy 8 are resistant to chemotherapy (Serakinci et al., 2004). The survival probability in patients with trisomy 8 is lower than in patients with normal karyotype (Wolman et al., 2002). The biological role of trisomy of chromosome 8 in the pathogenesis of tumors may be associated with an increase in gene copy number and increased the expression of oncogene C-MYC, located on 8q.

Currently, genetic stability assessment of MSC is not mandatory procedures before transplantation. Further research in this area will allow to establish methodical approach for rapid comprehensive assessment of genetic stability of cell transplants, the identification and characteristics of abnormal clones with potential oncogenic properties before their clinical application.

4. CONCLUSION

For the purpose of cell therapy it is critically important to transplant well-characterized, homogenous population of MSC cells (Minguell and Conget, 2000; Le Blanc and Ringden, 2007; Stenderup et al., 2003; Delorme et al., 2008). Based on our results we can conclude that linear homogeneity of ex vivo expanded MSC observed at 3-4 passage, preserved up to 10-12 passages. However, observed changes of surface phenotype, kinetics of growth rate and the analysis of aneuploidy in cultured cells showed clonal heterogeneity of the population of MSC and selective advantage of certain clones during the expansion. Ex vivo

expansion protocol used in our study allowed us to obtain sufficient for clinical application amounts of well-characterized MSC after at 3-4 passage. However, the role of serum-free medium in MSC expansion protocols remains to be clarified. Cell surface phenotype and genetic stability of MSC obtained at late passages for therapeutic purposes should be closely evaluated, this in turn help to avoid undesirable long-term effects of MSC in the future.

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