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Cytogenetic heterogeneity and their serial dynamic changes during acquisition of cytogenetic aberrations in cultured mesenchymal stem cells

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ABSTRACT

To minimize the risk of tumorigenesis in mesenchymal stem cells (MSCs), G-banding analysis is widely used to detect chromosomal aberrations in MSCs. However, a critical limitation of G-banding is that it only reflects the status of metaphase cells, which can represent as few as 0.01% of tested cells. During routine cytogenetic testing in MSCs, we often detect chromosomal aberrations in minor cell populations. Therefore, we aimed to investigate whether such a minority of cells can expand over time or if they ultimately disappear during MSC passaging. We passaged MSCs serially while monitoring quantitative changes for each aberrant clone among heterogeneous MSCs. To investigate the cytogenetic status of interphase cells, which represent the main population, we also performed interphase FISH analysis, in combination with G-banding and telomere length determination. In human adipose tissue-derived MSCs, 4 types of chromosomal aberrations were found during culturing, and in umbilical cord MSCs, 2 types of chromosomal aberrations were observed. Sequential dynamic changes among heterogeneous aberrant clones during passaging were similar to the dynamic changes observed in cancer stem cells during disease progression. Throughout all passages, the quantitative G-banding results were inconsistent with those of the interphase FISH analysis. Interphase FISH revealed hidden aberrations in stem cell populations with normal karyotypes by G-banding analysis. We found that telomere length gradually decreased during passaging until the point at which cytogenetic aberrations appeared. The present study demonstrates that rare aberrant clones at earlier passages can become predominant clones during later passages. Considering the risk of tumorigenesis due to aberrant MSCs, we believe that our results will help to establish proper safety guidelines for MSC use. In particular, we believe it is critical to test for chromosomal aberrations using both G-banding and FISH to ensure the safety of human stem cells that are manufactured *in vitro* for clinical applications.

Abbreviations: MSCs, mesenchymal stem cells; array CGH, array comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; hAD-MSCs, human adipose tissue-derived mesenchymal stem cells; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; α-MEM, alpha-minimum essential medium; FBS, fetal bovine serum; hUCB-MSCs, human umbilical cord blood-derived mesenchymal stem cells; HBSS, Hank's balanced salt solution; ISCN, International System for Human Cytogenetic Nomenclature; DAPI, 4′,6-diamidino-2-phenylindole; BACs, bacterial artificial chromosomes; Q-FISH, quantitative FISH; TRAP, telomeric repeat amplification protocol; QTD, quantitative telomerase detection kit; PCR, polymerase chain reaction; NSG, NOD/SCID/IL-2Rg−/−; gDNA, genomic DNA; FDA, Food and Drug Administration; CMC, chemistry manufacturing and control; INDs, investigational new drug applications; PB, peripheral blood.

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1. Introduction

Stem cell therapy is a promising treatment for many chronic diseases. However, prolonged exposure to stressful conditions during the cell enrichment and differentiation processes has raised concerns about the safety of stem cell therapy [1]. The accumulation of genetic mutations during cell culturing and the subsequent risk of cell transformation is another drawback of stem cell therapy. With respect to these concerns, the International Society for Stem Cell Research has created "Guidelines for the Clinical Translation of Stem Cells." These guidelines strongly warn against potentially cancerous changes that can occur in stem cells, such as aneuploidy, mutations in oncogenes and tumor-suppressor genes, and epigenetic changes [2]. The guidelines for stem cell therapy recommend that related tests be performed to ensure the safety of the stem cells, including conventional G-banding, array comparative genomic hybridization (array CGH), fluorescence *in situ* hybridization (FISH), cDNA arrays, whole-genome sequencing, whole-exome sequencing, and global methylation studies [2]. Molecular genetic techniques are often performed using DNA extracted from intermingled populations of cells containing both normal and abnormal cells. Therefore, the results of such molecular genetic studies represent an average of the examined cells, and considering a typical lower sensitivity of 20% or 30%, such experiments are not suitable for detecting minor populations of cells with cytogenetic aberrations among mesenchymal stem cells (MSCs). However, the practical application of these tests can be difficult, and there are no clear criteria for interpreting the results. Furthermore, obtaining adequate numbers of stem cells to perform these safety checks is a practical hurdle for stem cell providers, and the sensitivity and specificity of the recommended tests can vary considerably between methods and even between laboratories.

Chromosomal aberrations are hallmarks of cancer [3], and sporadic reports of chromosomal changes occurring during passaging highlight the risk of cancerous transformation in stem cells grown *in vitro* [1,4]. The majority of these reports have focused on embryonic stem cells, although several reports have focused on adult human MSCs [5,6]. An increased risk of chromosomal aberrations has also been reported in culture, warning against extended passaging for the enrichment of stem cells [7]. Safety checks on manipulated stem cells and the evaluation of chromosomal aberrations typically involve conventional chromosome analysis, also known as G-banding. Conventional G-banding is not routinely performed on stem cells prior to infusion into patients due to the need for substantial numbers of cells (approximately 10^6 cells). Conventional G-banding can detect chromosomal aberrations in cells that have entered metaphase; however, the number of cells in metaphase represents only a small proportion (approximately 0.1%) of the tested cells. Indeed, the majority of cells are in interphase, and these cells cannot be examined by G-banding. When preparing cells for chromosomal analysis, we generally count at most 200 metaphase cells. Among these cells, karyotyping is performed on only 20 metaphase cells, representing 0.01% of the total cells. In other words, the results of G-banding analysis likely do not accurately reflect the status of the cell population as a whole. To overcome the limitations of G-banding, we also used FISH to detect chromosomal aberrations in interphase cells. By analyzing both interphase and metaphase cells, there is the potential to assess chromosomal aberrations across the entire cell population. Using these techniques, we were able to detect minor cell populations containing chromosomal changes that would be missed by other molecular genetic techniques, such as array CGH or cDNA arrays.

Next, we asked whether minority cells with cytogenetic aberrations within MSC populations can expand or if they ultimately disappear during passaging. We previously reported the frequencies of chromosomal aberration among 68 different MSC lines using *in situ* karyotyping [8]. In this manuscript, we describe the effects of serial passaging on heterogeneous clones in two types of MSCs that were previously found to have chromosomal aberrations among the 68 MSCs. To assess the cytogenetic status of both interphase and metaphase cells, we performed interphase FISH combined with G-banding analysis at each passage and tracked the quantitative changes in clones with chromosomal aberrations.

2. Materials and methods

2.1. Isolation and culture of human adipose tissue-derived mesenchymal stem cells and human umbilical cord blood-derived mesenchymal stem cells

Human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) were kindly provided by Seong Who Kim (Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul, Korea). Briefly, adipose tissue was isolated from young healthy women undergoing liposuction. The tissues were washed to remove skin and then treated with 0.075% collagenase type I (Worthington Biochemical Corp, Lakewood, NJ, USA) in phosphate-buffered saline (PBS; Amresco, Solon, OH, USA) for 30 min at 37 °C. The collagenase was inactivated by the addition of an equal volume of alpha-minimum essential medium (α-MEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone). The suspension was then centrifuged at 3000 rpm for 10 min to separate the floating adipocytes and remove the debris. The cells were plated and incubated in α-MEM with 10% FBS and antibiotics (penicillin-streptomycin, Gibco, USA) at a density of 1×10^4 to 5×10^4 cells/cm^2 in T75 flasks. After 24 h, the non-adherent cells were removed and washed with PBS. Spindle-shaped cells were obtained by day 4 of culture. Subculturing was performed when the cells reached 80% confluence.

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) were kindly provided by SW Kim and were maintained as previously described [9]. The cells were incubated in medium (α-MEM, 10% FBS, 1× antibiotics); when the cells reached 70% confluence, 5×10^5 cells/cm^2 were passaged into T175 flasks. The cells were grown for seven passages.

After both types of MSCs were prepared, they were maintained in α-MEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were passaged when they reached 50–70% confluence. The cells were treated with 1 mL 0.25% Trypsin/ethylenediaminetetraacetate (EDTA) (Gibco) and washed with culture medium, pelleted by centrifugation at 400–600 g for 5 min at room temperature, and plated at a density of 4×10^5 to 5×10^5 cells/cm^2 in T75 flasks.

2.2. In situ culture and karyotyping

In situ culturing and karyotyping were performed as previously described [10]. Briefly, approximately 1×10^6 MSCs were grown on 22×22 mm^2 coverslips (Paul Marienfeld, Germany) in 35×10 mm^2 Petri dishes (Corning, NY, USA) containing medium (α-MEM). The media was exchanged at 16–24 h after seeding, and the cells were harvested after reaching 50–70% confluence. Colcemid solution (KaryoMAX Colcemid Solution, Invitrogen, Grand Island, NY, USA) was added, and the cells were incubated in a CO_2 incubator at 37 °C for 40–50 min. The media was removed, and 2 mL of prewarmed 0.6% sodium citrate hypotonic solution was added. The cells were incubated in a 37 °C incubator with CO_2 for 30 min. Then, 8–10 drops of fresh fixative (methanol/acetic acid, 2:1) were added to the dish, mixed, and then incubated at room temperature for 10 min. The supernatant was discarded, and 2 mL