Human Umbilical Cord-Derived Mesenchymal Stem Cells Do Not Undergo Malignant Transformation during Long-Term Culturing in Serum-Free Medium

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Abstract

Background: Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are in the foreground as a preferable application for treating diseases. However, the safety of hUC-MSCs after long-term culturing in vitro in serum-free medium remains unclear.

Methods: hUC-MSCs were separated by adherent tissue culture. hUC-MSCs were cultured in serum-free MesenCult-XF medium and FBS-bases DMEM complete medium. At the 1st, 3rd, 5th, 8th, 10th, and 15th passage, the differentiation of MSCs into osteogenic, chondrogenic, and adipogenic cells was detected, and MTT, surface antigens were measured. The telomerase activity of hUC-MSCs at passage 1–15 was analyzed.

Results: Flow cytometry analysis showed that very high expression was detected for CD105, CD73, and CD90 and very low expression for CD45, CD34, CD14, CD79a, and HLA-DR. MSCs could differentiate into osteocytes, chondrocytes, and adipocytes in vitro. There was no obvious chromosome elimination, displacement, or chromosomal imbalance as determined from the guidelines of the International System for Human Cytogenetic Nomenclature. Telomerase activity was down-regulated significantly when the culture time was prolonged. Further, no tumors formed in rats injected with hUC-MSCs (P15) cultured in serum-free and in serum-containing conditions.

Conclusion: Our data showed that hUC-MSCs can be safely expanded in vitro and are not susceptible to malignant transformation in serum-free medium, these cells are suitable for cell therapy.

Introduction

The presence of MSCs has been demonstrated in various fetal and adult tissues, including bone marrow, fetal blood and liver, cord blood, amniotic fluid and, in some circumstances, peripheral blood in adults. hUC-MSCs hold great promise as therapeutic agents in regenerative medicine [1],[2]. MSCs from all of the above sources can undergo extensive proliferation in vitro and, when cultured under specific conditions, they retain the ability to differentiate into multiple lineages, including bone [3], cartilage, fat, muscle [4] and stromal cells. These cells have attracted considerable interest both because of their value as a model for studying the molecular basis of differentiation and for their therapeutic potential in tissue repair and immune modulation [5].

However, the use of MSCs requires large-scale in vitro expansion, which increases the probability of malignant transformation. Some researchers have found that stem cells may naturally undergo malignant transformation during a long culture period [6]. Further, hUC-MSCs may also become carcinogenic after transfection or other modifications. Hence, the safety of these stem cells for clinical applications has begun to be examined more closely. Mouse bone marrow-derived MSCs have been shown to undergo spontaneous transformation after long-term in vitro culturing [6],[7]. Thus, for the clinical application of MSCs in different fields of medicine, their biosafety must be carefully investigated through appropriate and sensitive tests. The absence of transformation potential in cultured MSCs must be documented before these cells may be considered for treating patients, particularly immunocompromised subjects, in whom the failure of immune surveillance mechanisms might further favor the development of tumors in vivo.

The components of the culture medium are considered to be the main factors affecting the biological characteristics of in vitro cultured MSCs. The basic medium composition does not seem significant, and Dulbecco’s modified Eagle medium (DMEM) or α-minimum essential medium (α-MEM) can be used for in vitro culture of MSCs. However, the two pivotal compounds in the medium that could be responsible for malignant transformation...
are serum, of either animal or human origin (fetal calf serum (FCS) or human serum or plasma), and growth factors. Classically, the optimal conditions for MSC expansion require FCS-supplemented media, the standard being 10% FCS [8]. The FCS needs to be carefully tested to ensure the best expansion rate. Although FCS may be carefully tested for viruses, the risk of transmission of infectious diseases cannot be excluded. Moreover, in such a culture medium, MSCs retain some FCS proteins in their cytoplasm, which may elicit immunologic responses in vivo. Some serum-free media have been developed for the purpose of research, but media suitable for clinical-scale production of MSCs in accordance with good manufacturing practice guidelines have yet to be formulated and characterized. Thus, the aim of the present study was to investigate the potential susceptibility of hUC-MSCs to malignant transformation in vitro in serum-free medium and to ascertain whether the biological properties of these cells after expansion remain appropriate for their use in cell therapy.

Materials and Methods

Isolation and culture of hUC-MSCs

Ten human umbilical cords were obtained from healthy full-term and naturally delivered newborns after written informed consent was obtained from their mothers and family members. The study protocols were reviewed and approved by the hospital review board and ethics committee of Taizhou People’s Hospital. hUC-MSCs were separated using adherent tissue culture. For this, the cord tissue was transferred to a Petri dish containing 20–40 mL sodium chloride using sterile forceps and washed (to remove blood). Wharton’s jelly was teased out of the cord and collected in another Petri dish, and the arterial blood vessel, venous blood vessel, and amnion were discarded. The Wharton’s jelly was sliced into small fragments around 1 mm in diameter and added to culture flasks. They were covered with glass slides to prevent them from floating. After the hUC-MSCs adhered to the flasks, the medium was changed every 3–4 days. The hUC-MSCs were cultured in serum-free MesenCult-XF medium and and FBS-bases DMEM complete medium (Stemcell, Vancouver, Canada) at 37°C in a humidified atmosphere with 5% CO2. At this point, cells were considered to be at stage 0 (P0). Once the cells reached 70–80% confluence, they were scraped off using 0.05% trypsin/EDTA and passed at a density of 1×10^4 to 4×10^4 cells/cm^2 [8]. The cells were observed under a microscope and their morphology recorded. The time span from seeding Wharton’s jelly to harvesting P0 was assessed in serum-free and in serum-containing conditions.

Immunophenotyping of hUC-MSCs

hUC-MSCs (1×10^6 cells) were harvested with trypsin and washed twice with PBS. After they were filtered through a 200-mesh screen, the cell concentration was adjusted to 2×10^6/mL. The surface molecules on the hUC-MSCs were then examined by flow cytometry with the following antibodies: CD34-PE, CD45-FITC, CD73-PE, CD14-FITC, CD79a-APC, HLA-DR-PE, CD90-APC, and CD105-PE.

Differentiation potential of MSCs

Osteogenesis Differentiation. MSCs (6×10^6 cells/well) were seeded in 24-well plates and cultured at 37°C in a humidified atmosphere with 5% CO2 in DMEM/F12 supplemented with 0.10 volume fraction of fetal bovine serum (FBS), 1.0×10^{-6} mol/L dexamethasone, 2.0×10^{-6} mol/L ascorbic acid, and 7.0×10^{-7} mol/L β-glycerophosphate. The medium was changed every 3–4 days. After 21 days of culture, the cells were processed for Alizarin Red S staining, to detect osteogenesis. The cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM Alizarin Red S (pH 4.2) for 10 min at room temperature, washed five times with deionized water, and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was then observed at different magnifications under a microscope [9].

Adipogenesis Differentiation. MSCs (6×10^6 cells/well) were seeded in 24-well plates and cultured at 37°C in a humidified atmosphere with 5% CO2 in DMEM/F12 supplemented with 0.10 volume fraction of FBS, 1.0×10^{-6} mol/L dexamethasone, 10 mg/L insulin, 100 mg/L 1-methyl-3-isobutyl xanthine, 100 mg/L indomethacin, 100 U/mL penicillin, and 100 mg/L streptomycin. This adipogenesis differentiation medium was replaced every 3–4 days. After 14 days of culture, the cells can be processed for Oil Red O staining, to detect adipogenesis. The staining process was similar to that used for detection of osteogenesis.

Chondrogenic Differentiation. For this test, 7.5×10^6 MSCs were seeded in 6-well plates and cultured at 37°C in a humidified atmosphere with 5% CO2 in DMEM/F12 supplemented with 0.10 volume fraction of FBS, 1.0×10^{-7} mol/L dexamethasone, 6.25 mg/L insulin, 50 µg/L vitamin C, 6.25 mg/L transferrin, and 10 µg/L transforming growth factor β. This chondrogenic differentiation medium was changed every 3–4 days. Chondrogenic pellets were harvested after 28 days of culture. The pellets were formalin-fixed and paraffin-embedded for Alcian blue staining.

Growth curve analysis using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Proliferation of hUC-MSCs was assessed on days 1, 2, 3, 4, 5, 7, and 8 after initiation of growth, by mitochondria-dependent reduction of MTT (Beyotime, Shanghai, China). Cell monolayers were washed twice with two kinds of medium. MTT (100 µL of 5 mg/mL MTT in PBS; final concentration, 0.3 mg/mL) was then added to each well and the cells incubated at 37°C for 6 hours before overnight solubilization in 500 µL sodium dodecyl sulfate (10% wt/vol in 0.01 M HCl). A sample (150 µL) from each duplicate well was then transferred to a 96-well microplate and the optical density was determined using an automated dual wavelength microplate reader against a reagent blank, sample containing all components except for the cells, at a test wavelength of 490 nm and a reference wavelength of 630 nm. Growth curve analysis by MTT assay at P0 of MSCs from the ten donors was two kinds of conditions. Data shown are the mean ± SEM of values for the ten donors.

Population doubling time (PDT) was calculated by the following formula:

\[ \text{PDT} = \left( \frac{1}{C} \times \ln \frac{N_f}{N_i} \right), \]

where \( C \) is cell culture time, \( N_i \) is the initial number of cells and \( N_f \) is the final number of cells.

Telomerase activity assay

Using a telomerase detection kit (TRAPEZE Telomerase Detection Kit, Chemicon, S7700), the telomerase activity of cultured MSCs cells was detected and quantified using a software program [10],[11].

Karyotype analysis

For this analysis, 2×10^6 cells were harvested and 0.1–0.4 µg/mL colchicine was added to the culture medium. The cells were collected after 12 h, and 0.075 M KCl was added to them, after which they were placed in a 37°C water-bath. Next, 1 mL fixative
nude mice were randomly divided into five different groups. Next, pooled, and SGC7901 human gastric cancer cells. For this, 40 of the mice were stained with hematoxylin-eosin. 0.2 mL of this cell suspension was injected subcutaneously into the right forelimb of each nude mouse. The control group was administered 0.2 mL saline. Subcutaneous tissue from the armpit was washed with distilled water, and observed under an electron microscope after drying at room temperature. Chromosome analysis was carried out by using G-bands following the guidelines of the International System for Chromosome Nomenclature 2009 (ISCN 2009). On average, 20 metaphases were evaluated, and cells from passage 1 and 15 were tested. If the karyotype of passage 0 cells was abnormal, the sample was discarded.

Tumorigenicity test
Healthy, 4-week-old male nude mice were provided by the Animal Center of Second Military Medical University. The procedure for the animal experiment was approved by the Institutional Animal Care and Use Committee at the Animal Center of the Second Military Medical University. In vitro tumorigenicity studies were carried out with the blank controls, hUC-MSCs in two culture medium (P15 from ten donors were pooled), and SGC7901 human gastric cancer cells. For this, 40 nude mice were randomly divided into five different groups. Next, 1x10^6 cells were suspended in 2 mL physiological saline, and 0.2 mL of this cell suspension was injected subcutaneously into the right forelimb of each nude mouse. The control group was administered 0.2 mL saline. Subcutaneous tissue from the armpit of the mice were stained with hematoxylin-eosin.

Statistical analysis
Data were expressed as mean +/- SEM. Comparisons of mean values among the passages were analyzed using a Tukey’s multiple comparison test. “Serum-free” vs “serum-containing” were compared using Student’s t test. A five percent probability (P<0.05) was used as the level of significance. Differences were considered as statistically significant with P<0.01.

Results

Morphologic observation
Morphologic observation of MSCs is the most intuitive method for clearly differentiating cell types. We examined every cell passage of cells from the 10 donors and found no typical morphological changes. Figure 1 shows the morphological results of MSCs derived from donor 2. There was no visible difference in morphology of MSCs between donor 2 and other donors. But the time span from seeding Wharton’s jelly to harvesting P0 was different between different donors in the same culture medium. Donor 2 displayed longer time span, compared to one from donor 5 (P<0.05). The time span of serum-containing conditions was shorter than serum-free cultured ones (P<0.05).

Immunophenotype analysis by flow cytometry
We investigated the immunophenotypes of the MSCs using immunofluorescence flow cytometry, since this is a very important biochemical method used to understand cell types. We tested passage 1, 3, 8, 10 and 15 cells. As shown in Figure 2, very high expression was detected for CD73, CD90, and CD105, and very low expression was detected for CD14, CD45, CD34, CD79a, and HLA-DR. There was no visible difference in immunophenotype of MSCs between 10 donors in two conditions. These results meet the International Society for Cellular Therapy (ISCT) criteria for MSC definition (Figure 2).

Multilineage potential
An important characteristic of MSCs is their differentiation into osteoblasts, adipocytes, and chondroblasts. To investigate the feasibility of inducing the differentiation of hUC-MSCs into osteoblasts, adipocytes, and chondroblasts in vitro, we induced cells from passage 1, 3, 8, 10, and 15 under different culture conditions. We found that all the samples kept multipotent differentiation potential according to ISCT standards (Figure 3). In serum-free and in serum-containing conditions hUC-MSC have comparable differentiation potential from different donors.

Growth curve analysis by MTT assay
hUC-MSCs exhibit robust proliferation properties in vitro. The cells were in the latent period during the first couple of days after incubation and proliferation was not evident. Three to seven days into the logarithmic phase, cell proliferation was accelerated, and after 7-8 days the growth plateaued. There were two cell multiplication cycles, one was between the days 3-4 and the other was between days 4-7. The PDT of hUC-MSCs at P8 from donor 2 was different from the one from donors 5 (P<0.05) in the same culture medium. The PDT of serum-containing conditions was shorter than serum-free cultured ones (P<0.05) (Fig. 4).

Quantitative assay for telomerase activity
To investigate the effects of long-term in vitro culture on the genotype of MSCs, we examined telomerase activity. We found that telomerase activity was significantly down-regulated as the culture time increased. The telomerase activity in the MSCs gradually decreased after numerous passages were cultured in vitro. The calculations are based on the average values of the 10 donors for each passage (Fig. 5).

Karyotype analysis
Cytogenetic karyotype analysis was performed on cells at passage 0, 8, and 15. As shown in Figure 6, no abnormal chromosome phenomenon was found. Further, there was no chromosome elimination, or displacement or chromosomal imbalances, as determined according to the International System for Human Cytogenetic Nomenclature (Fig. 6).

Tumorigenicity test
Finally, we carried out a tumorigenicity assay. We compared tumor formation in mice injected with cells (P15) of two culture conditions. After three months of normal feeding, no solid tumor formation was noted in the hUC-MSC (P15 from both conditions) or control group. The mice remained in the healthy survival mode and were followed up for 6 months. Hematoxylin-eosin staining showed subcutaneous tissue from the hUC-MSCs group were normal. However, 8 of the 10 mice in the SGC7901 group developed tumors after 3 weeks, while the other 2 developed tumors after 4 weeks (Fig. 7).

Discussion
Many studies have shown that stromal cells with stem cell potency can be isolated from human umbilical cord mesenchymal tissue, namely, Wharton’s jelly [12],[13], and hUC-MSCs are a promising tool for disease treatment. MSCs support the expansion of other stem cells, such as hematopoietic stem cells, are well-tolerated by the immune system, and have the ability to home to tumors [14],[15]. In contrast to bone marrow MSCs, hUC-MSCs have greater expansion capability, faster growth in vitro, and more readily available. Although hUC-MSCs have been proven to be therapeutic in several different pre-clinical animal models of...
human disease, such as neurodegenerative disease, cancer, and heart disease, the effects of their proliferation during long-term in vitro culturing remain unclear. Culture of hUC-MSCs is a dynamic process, and improper procedures during this process will result in adverse changes in the cells’ inherent properties [16],[17]. Thus, the requirement of cell expansion in vitro may cause some specific risks. Several studies on MSCs from different sources have highlighted how genomic instability could lead to spontaneous immortalization and malignant transformation. Spontaneous malignant transformation of mouse BM-MSCs has been described following long-term culturing in vitro [18]. Further, some publications have reported spontaneous transformation of hUC-MSCs as well [6],[7],[19]. Further studies are urgently needed in this area to ensure the long-term safety of hUC-MSCs in vitro.

In the present study, 10 human umbilical cords were obtained from healthy full-term naturally delivered newborns. The cords were selected according to the America Association of Blood Banks technical manual. Special care was taken not to collect the perivascular cells in the cord (pericytes), which have different characteristics despite being MSCs [20]. MSCs were separated from the umbilical cords. Conventional karyotyping was performed at passage 0 on cells from the 10 donors. If the results at passage 0 were normal, the umbilical cord was included in the study; if not, it was discarded. To objectively compare and contrast study outcomes, we analyzed the morphology, immunophenotype, and multi-lineage differentiation potentials of the MSCs according to ISCT standards. In 2006, the ISCT proposed the following minimal criteria for defining human MSCs [21]: First, the cells must be plastic adherent when maintained under standardized

![Figure 1. Microscope morphological observation of MSCs at passage 1, 8, and 15 from in serum-free condition.](doi:10.1371/journal.pone.0098565.g001)

![Figure 2. Analysis of MSCs for the expression of surface markers by flow cytometric analysis.](doi:10.1371/journal.pone.0098565.g002)
culture conditions. Second, they must express CD105, CD73, and CD90 but not CD45, CD34, CD11b, CD79a, or CD19, or HLA-DR surface molecules. Third, they must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro, as MSCs have multiple differentiation capacities. These standards were adopted by other investigators examining adipose tissue-derived MSCs [22],[23]. In these studies, aside from the surface molecules mentioned above, CD44, CD166, CD80, CD86, and CD4 were also detected. In our study, at the 3rd, 5th, 8th, 10th, and 15th passages, MSCs were tested for osteogenic, chondrogenic, and adipogenic differentiation, and surface antigens were measured by flow cytometry. The expression of surface antigens met the standards set by the ISCT. In a previous study performed by Dah-Ching Ding et al., 10–16% of human adipose-derived stem cells were positive for the CD34 surface marker, which is often expressed on hematopoietic stem cells and may adhere to the adipose-derived stem cells. The different outcomes among studies could be attributed to differences in cell preparations, culture medium, and the timing and method of isolation. In our study, although the MSCs could differentiate into osteoblasts, adipocytes, and chondroblasts in vitro, they showed a weaker ability to differentiate following an increase in the frequency of subculture. In serum-free and in serum-containing conditions hUC-MSC have comparable differentiation potential and immunophenotypic. This finding seems in agreement with the phenotypic and functional properties reported by others[24]. Compared between cells of different generations from one donor and between cells of different donors at the same generation. It took different time to harvest P3 from different donors (P<0.05). After subculture, cells exhibited similar biological characteristics. The population doubling time of serum-containing cultured hUC-MSCs was significantly shorter than serum-free cultured ones (P<0.05).

In human chromosomes, telomeres consist of thousands of copies of 6-base repeats (TTAGGG). Telomere length is progressively shortened with each cell division both in vivo and in vitro, due to the inability of the DNA polymerase complex to replicate the very 5' end of the lagging strand. The progressive shortening of the telomeres has been proposed as being the main trigger for replicative senescence, because it functions “as an internal clock”, with every cell division the number of telomere repeats decreases[25]. In our study, karyotype analysis by G-banding showed no translocation or losing of chromosomes in any MSC groups. The biosafety of hUC-MSCs should be further investigated using molecular karyotyping methods, such as array-CGH, classic cytogenetics, and subtelomeric fluorescence in situ hybridization. Because of the high resolution it affords and in light of the difficulty in obtaining cultured MSC metaphases, array-CGH may be considered as the method of choice for characterizing the genomic situation of MSCs expanding in vitro. In the present study, we compared tumor formation in mice injected with early- and

Figure 3. Differentiation of hUC-MSCs from donor 3 was showed at the 15th passage. (a) Alizarin Red S staining to detect osteogenesis differentiation. (b) Oil Red O staining to detect adipogenesis differentiation. (c) Alcian Blue staining to detect chondrogenic differentiation.

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Figure 4. The proliferation capacity of hUC-MSCs: (a). Growth curve analysis by MTT assay at P8 of MSCs from the ten donors. Data shown are the mean ± SEM of values form ten donors. (b). The PDT of hUC-MSCs at P8 from donor 2 was different from the one from donors 5(P<0.05) in the same culture medium. The PDT of serum-containing conditions was shorter than serum-free cultured ones (P<0.05)

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late-passage MSCs. These tumorigenicity assays showed that no malignant transformation was noted in mice injected with cells from either passage. Previous studies [26],[27] also showed that MSCs do not spontaneously undergo malignant transformation. There have been few reports on spontaneous human MSC in vitro transformation, of which two turned out to be caused by contamination by tumour cell lines and were retracted afterwards[28-31]. In contrast to mouse MSC studies, four of the induced human MSC transformation studies consist of the exogenous expression of human telomerase reverse transcriptase(hTERT) in human cells. This may be attributed to the much shorter telomeres in human MSCs than their mouse counterparts,
the much shorter life span of mice than human and the difference in telomere damage signaling pathways between mouse and human. Malignant Transformation Potentials of Human Umbilical Cord Mesenchymal Stem Cells Both Spontaneously and via 3-Methyldanolnethrene Induction[27].

In conclusion, our data showed that although the differentiation ability of and telomerase activity in hUC-MSCs show a trend of gradual decreasing with culture duration, these cells do not show an aptitude for spontaneous transformation and can be safely expanded in vitro in serum-free medium without any sign of immortalization or development of chromosomal abnormalities.

Author Contributions
Conceived and designed the experiments: GC AY. Performed the experiments: ZR YY RW YR. Contributed reagents/materials/analysis tools: GC AY LZ. Wrote the paper: GC AY.

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