Assessment of in vitro aging of mesenchymal stem cell

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Abstract
Mesenchyml stem cell (MSC) are receiving much attention in treatment of various diseases. The low frequency of MSCs in bone marrow (BM) necessitates their in vitro expansion prior to clinical use. We evaluated the effect of long term culture on the senescence of these cells.
BM cells were taken from 11 transplant donors with mean age of 25 years. In different passages, MSC were examined for different aging indicators including: telomere length assay, differentiation ability, immunophenotyping of CD13, CD44 and CD34 antigens, determination of cumulative population doublings (CPDs), and study of morphological characteristics of MSC cultures.
The mean long term culture was 118 day and the mean passage number was 9. The average number of PD decreased from 7.7 to 1.2 in the 10^{th} passage. The mean telomere length decreased from 9.19 Kbp to 8.7 kbp in the 9^{th} passage. Differentiation potential dropped from the 6^{th} passage on. The culture's morphological abnormalities were typical of the Hayflick model of cellular aging.
We believe that MSC enter senescence almost undetectably from the moment of in vitro culturing. Simultaneously these cells are losing their stem cell characteristics. Therefore, it is much better to consider them for cell and gene therapy early on.

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Introduction
Mesenchymal stem cells (MSCs) are of great therapeutic potential because of their ability to self-renew and differentiate into multiple tissues.(1) They enhance engraftment of donor hematopoietic cells after cotransplantation in animal models.(2) In humans, MSCs have been used to regenerate the marrow microenvironment after myeloablative therapy.(3) Possible clinical application for MSC in stem cell transplantation,(4) stem cell strategies for the repair of damaged organs(5) and gene therapy(6) have been proposed. Friedenstein first described(7) MSCs in bone marrow (BM) as a very rare population (0.01% to 0.001%), and Wexlel et al(8) reported a 1 in 3.4×10^4 frequency for these cells. Their level is even lower in cord blood(9) and peripheral blood. Therefore, it is essential to culture and populate MSCs in vitro before putting them to therapeutic use. We know that culture and division of cells cause a gradual senescence due to the shortening of the telomere during each division cycle.

The fact that environmental treatment can significantly increase or decrease culture life span suggests that telomeres can last at different rates under different conditions. Van Zglinicki and et al reported that cells incubated in 40% oxygen soon stopped growing and, at the same time, had shortened telomeric DNA. They showed that telomeric DNA can be lost without cell division, perhaps by single-strand breaks in DNA.(10)

With this in mind, we designed a study to evaluate the effect of long term invitro culture on the proliferation, differentiation, telomere length, phenotype, and morphology of these cells.

Materials and Methods
Sample collection and MSC cultures
Ten ml of BM was obtained from 11 healthy hematopoietic stem cell transplant donors at the time of collections. Informed consent according to an approved protocol by the internal medicine ethics committee was obtained. The mean age of BM donors was 25 years (range:2.5 to 63).

The mononuclear cells (MNCs) were isolated from the collected samples by the Ficoll density

gradient method. Then, they were washed in RPMI 1640 and were resuspended at 1×10^6 cells/ml in culture medium [Dulbecco's modified Eagle medium, low glucose (DMEM-LG), 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (all from Gibco)]. Twenty-one ml of Cell suspension was plated in a 75 cm^2 flask (Greiner bio-one) for primary culture.

Flasks were incubated at 37°C in a humidified atmosphere of 5% CO2 and were fed by complete medium replacement every 4 days. When fibroblast-like cells at the base of the flask reached more than 90% confluence, adherent cells were detached using 0.25% trypsin EDTA and replated (passaged) at a density of 1×10^4 cells/ml in two 75 cm^2 flasks. On reaching confluence, all cultures were passaged sequentially up to 120 days. For every even passage, some of the expanded MSC was separated to examine their ability to form differentiaed adipocyte and osteocyte, and immunophenotyping. For every odd passage, the cells were examined for telomere length.

Immunophenotyping

Surface expression of CD44, CD13 and CD34 was determined on culture-expanded MSCs. The monoclonal antibodies used were anti-CD44 fluorescein isothiocyanate (FITC), anti-CD13 phycoerythrin (PE), and anti-CD34 FITC (all from Dako). Relevant isotope control antibodies were also used. Flow cytometry was performed on a FACScan (Becton Dickinson), and data were analyzed with Cellquest software.

Differentiation of MSC

Adipogenic differentiation was assessed by incubating the cells with DMEM-LG and 10% FBS supplemented with 0.5 μM hydrocortisone, 0.5 μM isoboutyl methylxanthine, 60μM indomethacin (Sigma-Aldrich), and 10μg/ml insulin (R&D) for 2-3 weeks. Adipocyte is recognized by the accumulation of lipid-containing vacuoles which stain red with Oil red O.

Osteogenic differentiation was assessed by incubating the cells with DMEM-LG and 10% FBS supplemented with 0.1 μM dexamethasone, 10 μM β-glycero-phosphate, and 50 μM ascorbate (all from Sigma-Aldrich) for 2-3 weeks. To assess mineralization, cultures were stained with silver nitrate (Von Kossa's staining).

Determination of population doubling level and morphological characteristics of long term culture

Long –term cell growth in vitro is a more sensitive method to detect subtle changes in the kinetics of proliferation of the cell population. For determining the number of cumulative population doublings, BM mononuclear cells were seeded 1×10^6 cell/ml (28×10^4 cell/cm^2) in T-75 flasks. The adherent MSC were counted after 3-4 days to quantify the initial number. At confluence, the cells were trypsinized, counted and reseeded at a density of 1×10^4/ml (2.8×10^3 cell/cm^2). The numbers of PDs were calculated using the formula logN/log2 [11], where N is the cell number of the confluent monolayer divided by the initial number of cells seeded. This procedure was repeated in every passage.

To study the morphological characteristics, cell culture flasks were observed every time at medium re-feeding intervals (every 4 day), to detect any abnormalities in cell morphology and medium. When any variation was observed, the data of changes were recorded in the culture files.

Determination of telomere length

For telomere length assay, we used the "Telo TAGGG Telomere Length Assay" kit (Roche Molecular Biochemical) according to the manufacturer's recommendations. Briefly the test principle is as follows: The genomic DNA was isolated and digested using restriction enzymes *RsaI* and *HinfI* for 2 h at 37ºC. The DNA fragments were separated by gel electrophoresis for 2-4 h at 70 V on a 0.8% agarose gel and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments are hybridized to a digoxigenin (DIG)-labeled probe specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline phosphate. Finally, the immobilized telomere probe is visualized by virtue of alkaline phosphatase metabolizing CDP-*Star*, a highly sensitive chemiluminescence substrate. The average TRF length can be determined by comparing the signals relative to a molecular weight standard. After exposure of the blot to an X-ray film, the mean TRF length were scanned and

Table 1. The result of the mean and SD of BM-MSC culture.

passage number	Mean number of harvested cell ($\times 10^5$) ±SD	Mean time of counfluency (day) ±SD	Mean number of PD ±SD	Mean number of telomere length (Kbp) ±SD
1th passage	17.0 ±105.26	15± 4.23	7.7 ±.55	9.2 ±.60
2th passage	40.4 ±190.72	10 ±4.78	3.1 ±.67	–
31th passage	28.0 ±148.52	12 ±4.10	2.6 ±.68	9.0 ±.56
4th passage	28.3 ±103.55	12 ±1.75	2.6 ±.58	–
5th passage	25.8 ±109.73	14 ±4.76	2.5 ±.56	8.9 ±.58
6th passage	23.2± 122.26	16 ±5.94	2.3 ±.61	–
7th passage	20.6 ±152.95	14 ±5.27	2.0 ±.81	8.8 ±.56
8th passage	17.5± 77.57	14 ±4.71	1.9 ±.69	–
9th passage	21.0± 134.06	17 ±4.65	2.1 ±.82	8.7 ±.75
10th passage	10.9± 61.68	15 ±3.35	1.2 ±.73	–

calculated by the multianalizor (Bio-rad) software.

Statistical analysis

All data are presented as mean ±SD. The data collected in ten passages was compared by Repeated Measurement analysis of variance test. P- values of less than P‹ 0.05 were considered significant.

Results

The results of MSC culture

Three days after plating the mononuclear cells of BM in culture flasks, the mean number of fibroblast like cells were about 6800 (90 cell/cm^2) of total heterogeneous cells. Gradually the fibroblast like cells (MSC) increased and the heterogeneous cells decreased in the culture.

The predominant cell after 2 weeks of culture was fibroblast like (Fig.1) and dislodged readily on trypsinization in 3 minutes.

Primary culture took between 11 and 25 days (mean: 15.6 day). The total count of harvested cells from the primary culture was between 5.4×10^5 and 39.0×10^5 (mean: 17.0×10^5 cell). The mean number of harvested MSC and the mean time of confluency from the second culture on is shown in table 1.

As can be seen in table 1, the average number of cells that proliferate during each passage and the average confluency time in the various passages are different. Therefore, to analyze these differences we performed the Repeated Measurement Analysis of Variance statistical test, but we did not find any significant difference between the different passages.

The goal of this study was keeping the MSC in the culture by multiple passages up to 120 days. Nine of the 11 samples survived between 100 and 164 days and were passaged between 8 to 10 times. Two samples survived up to 63 and 95 days, and were passaged 5 and 6 times respectively, and didn't finish the procedure because of contamination.

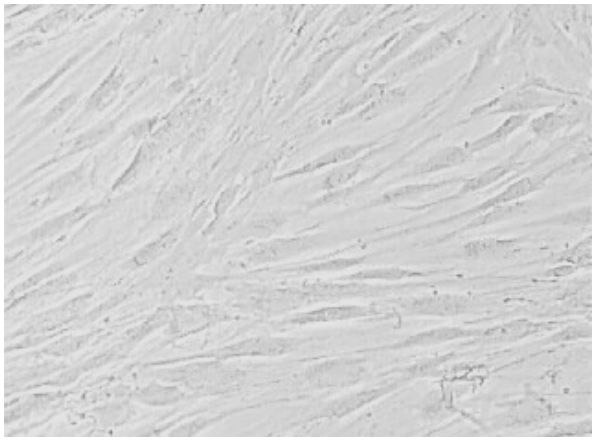

Figure 1. Bone marrow MSC culture (10X magnification, Nikon Eclipse TS100)

Immunophenotype of MSCs

The mean percent of expression of the three CD antigens in 5 different passages was compared using Repeated Measurement Analysis of Variance test and results demonstrate no significant difference on their expression. These cells stained positively for CD44 and CD13 but negative or very low positive for CD34.

Differentiation of BM MSCs

MSC were differentiated along adipogenic and osteogenic lineages. Oil red O staining for adipocyte and Van Kossas and Alkaline phosphatase staining for osteocyte were positive. Adipogenic differentiation of MSC after 2-3 weeks incubation in adipocyte culture media demonstrated scattered aggregates of oil red o positive staining cells. But within the same time limit, the osteocyte culture media exhibited a homogeneous and relatively abundant number of Van Kossas positive cells.

The differentiation ability of MSC varied in the 5 different passages. Every one of the samples cultured in osteogenic and adipogenic mediums differentiated to osteocyte and adipocytes by the 4th culture. In the 8th passage 25% (2 of 8), and in the 10th passage 20% (1 of 5) of the samples lost their osteogenic differentiation potential. For adipocytes in the 6th passage 10% (1 of 10), in the 8th passage 50% (4 of 8), and in the 10th passage 60% (3 of 5) of the samples lost their adipogenic differentiation potential.

Long-term growth kinetics

To examine long-term growth kinetics of MSC culture, we measured cumulative population doublings (PDs), with respect to the passage number in multiple donors. The average number of cumulative PD in 10 passages was about 30 (range 26-32). MSCs underwent an average of 7.7 PDs prior to the first passage. The mean PD and growth kinetics of different samples upon subsequent passages is shown in table 1 and Figure 2, respcetivly.

Morphological characteristics of BM MSCs in long-term culture

After a long period of normal growth, MSC culture showed abnormalities typical of the Hayflick model of cellular aging known from cultured human fibroblasts.[12] The cells varied in size and shape, the cytoplasm began to be granular with many cell inclusions, and debris was formed in the medium. The presence of granules in the cytoplasm was on average 84 days (range: 62-122) after primary cultures, and the presence of debris in the medium formed on average, in about 72 days (range: 35-97) after the onset of cultures. However, in the late stages, from day 120 in some cultures, the granular cell began to be vacuolated, elongated cells changed to rounded cells and finally detached from the base of the flasks.

Telomere length of MSC

The range of the mTRFL varied between 10.2 kbp to 7.8 kbp in different BM samples and in different passages. mTRFL was decreased from an average of 9.19 kbp in the first passage to 8.75 kbp in the 9th passage, giving a telomere shortening of about 0.44 kbp during 120 days of expansion (Table 1).

Discussion

Since the MSCs can differentiate into multiple tissues in vitro and in vivo, they attract a lot of attention in cell and gene therapy. The low frequency of MSCs in BM[7,8] necessitates their in vitro expansion prior to clinical use. It is known that when the cells become senescent, they are unable to proliferate further. As a result, it is necessary to evaluate the proliferative capacity of expanded MSCs to maintain long-term tissue regeneration before re-infusion.

The key feature of the senescence of dividing cells is the fact that a long period of normal growth is followed by cessation of growth. So, events must be occurring and accumulating throughout the life span, all of which culminate to bring about senescence. Our study has shown a number of changes in physiological, functional, and molecular parameters that occurred during our long-term cultures. These changes included:

- Typical Hayflick Phenomenon of cellular aging.
- Gradual decreasing proliferation potential.
- Telomere shortening.
- Impairment of functions.

The actual age of a culture is normally recorded in population doublings (PDs). Colter et al [13] recorded that the single-cell-derived colonies of MSC can be expanded through as many as 50 PDs in about 10 weeks. But we found that MSC can be expanded as many as 30 PDs, similar to Kassem et al [11], in about 130 days (18 weeks).

The curve relationship between cumulative PD and passage number demonstrates a relatively linear decreasing PD rate with the progression of passages. Furthermore, an appreciable decrease in the number of PD was seen in the last passage (P10= more than 130 days in culture), suggesting that MSC proliferative potential decrease faster after 120 days in vitro expansion. To investigate the aging of MSC morphologically, we studied the culture for the presence of any abnormalities in cell morphology. Production of debris in the medium and granules in the cytoplasm has been used as markers for aging of fibroblasts in vitro [14]. In the present study, granulated cytoplasm and debris increased from early to late-passage cells.

We investigated another marker for cellular senescence, normally the mean telomere length.

In the absence of the enzyme telomerase, telomeres gradually get shorter as cell division proceeds, and shortening of telomere throughout the life span is well-documented.[15] The consequence maybe the inactivation of genes closest to the telomere sequences, either directly or indirectly by a position effect, perhaps involving the formation of heterochromatin. However, maintenance of telomere length is observed in germ cells, some stem cells and malignant cells due to activation of the telomerase enzyme.[16]

Our present study shows that MSC senescence is associated with telomere shortening during in vitro expansion. The rate of telomere shortening was 100 bp in every two passage. Previous studies,[17,18] like our study, have shown that the telomere length of MSCs shortens in culture expansion. However the rate of telomere shortening in Kassem et al[11] study was 100 bp/PD, which is very high in comparison to our study. This difference maybe due to the long duration of their long-term culture (> 500 days) in comparison to our study (120 days). They showed that mTRFL decreased from an average of 10.4 kb in early passage cells to 7.1 kbp in late passage cells. However losing mTRFL in later passage is high but the mean amount of shortening was divided to the cumulative PD number. So it can be the reason of this difference.

In addition to the telomere length shortening, we investigated the osteogenic and adipogenic differentiation capacity of MSC. MSC differentiation into other lineages has been used as a marker for the multipotential nature of these cells.[19] In our study the differentiation potential to adipocyte and osteocyte dropped in the late-passages, which is similar to what has been reported previously.[20,21] Our study also demonstrated that, in the later passages, a greater number of samples lose their adipocyte differentiation potential in comparison to osteocytes in the same condition. At the level of one culture plate, the percentage of cells that differentiated into adipocyte were lower than those differentiating into osteocytes.

It is generally agreed that senescent cells are irreversibly blocked in cell division, but they are still capable of many other cell functions. According to the telomere theory of cellular senescence, the shortening of chromosome ends would itself trigger a cell cycle block.

Conclusion

We believe that MSC, like all other cells, enters senescence almost undetectably from the moment of in vitro culturing. Simultaneously, these cells are slowly losing their stem cell characteristics (self-renewal, differentiation potential). Therefore, it is a much better alternative to consider these cells for cell therapy and gene therapy early on (the younger, the better). We believe the best time is between the 2nd and 6th passage. This is because they have the highest PD rate, lowest confluency time, almost complete differentiation potential to adipocyte and osteocytes, and they have a reasonable cell population yield.

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