Original Article

An Investigation on Mitochondrial DNA Deletions and Telomere Shortening during Multiple Passages of Adult Stem Cells

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Abstract

Background: Limited resources for adult stem cells necessitate their *in vitro* culture prior to clinical use. Investigating mitochondrial DNA (mtDNA) and telomere shortening has proved to be important indications of stem cell validity. This study was designed to investigate these indicators in multiple passages of three adult stem cell lines which were produced in our stem cell laboratory.
Methods: In this study, Dental Pulp Stem Cells (DPSCs), Periapical Follicle Stem Cells (PAFSCs) and Human Foreskin Fibroblast (HFF) cell lines were expanded for 20 passages. After 1, 5, 10, 15 and 20 passages, expanded cells were harvested and DNA was extracted for further studies. Common mtDNA mutation was detected by multiplex PCR and telomere shortening was tested by Southern blot analysis.
Results: The common deletion was not detected in any of the stem cells or cell lines after several passages. In addition, Southern blot analysis indicated that the mean difference of telomere length between first and last passage was 0.25 *kb* in DPSC, 0.1 *kb* in PAFSC and 0.32 *kb* in HFF which indicates that the mean telomere length in various passages of the samples showed insignificant changes.
Conclusion: Absence of mtDNA mutations in adult stem cell lines indicates good mitochondrial function even after 20 passages. In addition, absence of telomere shortening indicates stem cells validity after multiple passages. It is hoped this information could pave the way for using *in vitro* expansion of adult stem cells for future clinical applications.

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Introduction

Stem cell therapy is a new method for the treatment of many different diseases. Recently, the stem cells originating from adult tissues have been used for this purpose. Limited resources for these kinds of stem cells necessitate their *in vitro* culture prior to clinical use. The researchers use some indicators in the cell lines to investigate whether expanded adult stem cells have a long enough life span for future division *in vivo* or not.

Mitochondria play a vital role throughout the life cycle of the eukaryotic cells. It is involved in many metabolic tasks such as apoptosis-programmed cell death and cellular proliferation [1]. One fundamental function of mitochondria is the production of Adenosine Triphopshate (ATP) via oxidative phosphorylation. This could be disrupted when a critical threshold of mutant mitochondrial DNA (mt DNA) is met, thus inactivating the cell [2]. Therefore, the evaluation of common mtDNA deletion could be used as a marker to detect the quality of adult stem cells. Numerous laboratories have detected a specific mtDNA mutation, known as the common deletion (ΔmtDNA4977). This mutation is associated with some neoplasms [3,4] and also aging processes in some tissues and cells [5-10]. Adult cell lines may harbor mutant mtDNA that may further accumulate during cell culture, and thus have consequences for long-term viability. On the other hand, future medical applications of stem cell therapies include the use of cells originating not only from embryos but also from adult tissues [11]. Usually, it is essential to culture and populate adult stem cell line or other adult cell sources like fibroblasts before putting them to therapeutic use. Therefore, monitoring the volubility of these cells after multiple cultures is very important.

In addition to mutant mtDNA, telomere shortening is another good indicator of stem cells volubility. There are several methods for monitoring or measuring the condition of telomeres such as Terminal Restriction Fragment (TRF) analysis, Fluorescence *in situ* Hybridization (FISH), Polymerase Chain Reaction (PCR) and telomerase activity [12]. These measurements are important because extensive sub-culturing and expansion of adult stem cells is necessary for an effective therapy [13].

In this paper, adult stem cell lines were established from human third molar teeth and cell lines from human foreskin fibroblast in Yazd Research and Clinical Center for Infertility [14,15]. The goal of this study was to determine whether the human mtDNA common deletion and shortening of telomere length as two aging biomarkers were present in these cell lines during several passages.

Materials and Methods

Isolation of stem cells and cell culture

The normal and non-decayed human third molars were extracted for orthodontic treatment purposes from 20 adults (15-22 *yrs*) after receiving their informed consent at the Department of Oral and Maxillo-Facial Surgery, Dental School of Shahid Sadoughi University of Medical Sciences, Yazd, Iran [14]. Dental School Ethics Committee approved the research and all the participants submitted their signed consent letter.

The pulp tissue was gently separated from the crown and root. Tooth germs at the root-forming stage were obtained and named periapical follicles. These were removed from the root dentin with a scalpel. The tissue segments were digested in a solution of 3 *mg/ml* collagenase type I and 4 *mg/ml* dispase dissolved in DMEM for 1 *hr* in CO_2 incubator. The suspensions of cells were cultured into 25 cm^2 plastic flask with alpha modification of Eagle's medium (alpha-MEM, Gibco BRL, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL), 100 *U/ml* penicillin, 100 *mg/ml* streptomycin (Gibco BRL) and amphotericin B (BIOCHROM AG) for primary culture and then incubated at 37^oC. In this study, 2 types of derived dental stem cells, DPSCs and PAFSCs were passaged 21 times and after each passage 3 to passage 21, mtDNA deletion was investigated.

Isolation of human foreskin fibroblast

Pieces of human foreskin with the area of 1-2 cm^2 were obtained from circumcisions of 9 human neonatas. These pieces were washed with phosphate saline buffer solution (Invitrogen Corporation, Carlsbad, CA), then they were cut into 4-6 *mm* pieces, washed again, and incubated enzymatically by collagenase I and IV with a 1:1 ratio (Invitrogen Corporation, Carlsbad, CA) overnight at 37°*C*. The tissue pieces were fragmentized mechanically with insulin syringes (29 gauge), and then cell suspension was centrifuged at 1,000 *rpm* for 5 *min* and the supernatant was removed. Next, the pellet was resuspended in 10 *ml* proliferation DMEM medium supplemented with 15% FBS and 1% penicillin/streptomycin, then transferred to a 25 cm^2 tissue culture flask (Falcon) [15]. Multiple passages were performed for Human Foreskin Fibroblast (HFF) and passages 1, 5, 10, 15 and 20 were used for comparing them with two adult dental stem cell lines in the study.

DNA extraction and mtDNA deletion analysis

Using Bioneer Genomic DNA kit, total cellular DNA was isolated from different cell lines in passages 1, 5, 10, 15 and 20. Each experiment was repeated three times for all passages. The PCR reactions were performed in a thermal cycler (MWG biotech primus) for 35 cycles with denaturation at 94^oC for 1 *min*, primer annealing at 55^oC for 1 *min* and extension at 72^oC for 35 *s*. The deletion- prone region of mtDNA between 5461 of light strand and 15000 of heavy strand was investigated in all samples using the primers ONP 86 (5461-5480), ONP 89 (5740-5721), ONP 74 (13640-13621), ONP 25 (8161-8180) [16]. The distances between the primers were long enough to allow amplification only if a part of the DNA between respective primers was deleted. Primer pairs, ONP 86 and ONP 89, were used to amplify a normal internal mtDNA fragment (279 *bp*) in a region which is rarely afflicted by deletions, and served as a control for the preparation and PCR analysis. Primer pairs, ONP 74 and ONP 25 were used to amplify a 502 *bp* amplicon for detection of deletion. One DNA sample from a patient with Ataxia-Telangiectasia who had a common mtDNA deletion was tested as the positive control. The accuracy of tests was examined by direct sequencing of some random DNA fragments amplified by the PCR reactions.

Southern blot analysis of TRF

Telomere restriction fragment analysis was performed as described by using the Telo-TAGGG Telomere Length Assay Kit (Roche Molecular Biochemical) according to the protocol provided by 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 *hr* at 37*°C*. The DNA fragments were separated by gel electrophoresis for 2-4 *hr* at 70 *V* on a 0.8% agarose gel and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments were 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 was visualized by virtue of alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. The average TRF length could 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 calculated by the multianalizor (Bio-rad) software.

Statistics

All values are expressed as means±SD. Differences between groups were tested using paired t-test. A p-value<0.05 was considered significant.

Results

mtDNA common deletion

In this study, using a molecular approach, the frequency of the mtDNA common deletion was analyzed in adult cell lines derived from dental and foreskin tissues. The common deletion was not found in any samples (Figure 1). The results demonstrated that the mtDNA common deletion was unlikely to be responsible for aging due to multiple routine passages.

Telomere length of adult stem cells

After three experiments, the mean telomere length in the first passage was 11.86±0.55 *kb* in DPSC, 11.47±0.15 *kb* in PAFSC and 11.51±0.14 *kb* in HFF. Moreover, mean telomere length in the last passage was 11.61±0.58 *kb* in DPSC, 11.57±0.28 *kb* in PAFSC and 11.19±0.17 *kb* in HFF (Figure 2). These results show that the mean difference of telomere length between first and last passage was 0.25 *kb* in

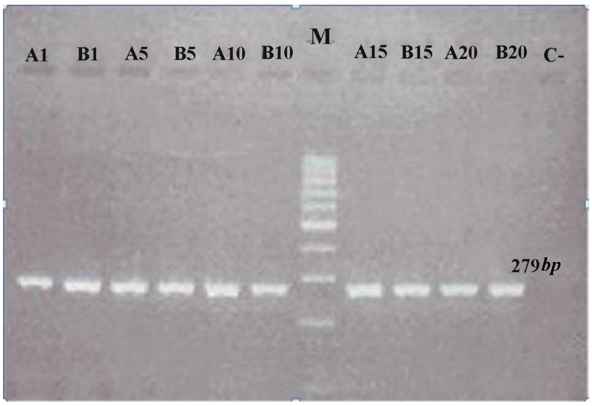

Figure 1. Gel electrophoresis to study human mitochondrial DNA (mtDNA) common deletion in adult stem cell lines (DPSCs). Marker is a 100 *bp* DNA ladder. The other lanes demonstrate 279 *bp* mtDNA amplified with primers ONP 86 and 89. No PCR amplicons were generated in any three cell lines using primers ONP 74, ONP 25, ONP 99, ONP 10 indicating no mtDNA common deletion. A1, A5, A10, A15 and A20 indicate periapical follicle stem cells (PAFSCs) after 1, 5, 10, 15 and 20 passages. B1 B5, B10, B15, B20 indicate the same results in dental pulp stem cells (DPSCs). C and M show negative control and marker, respectively

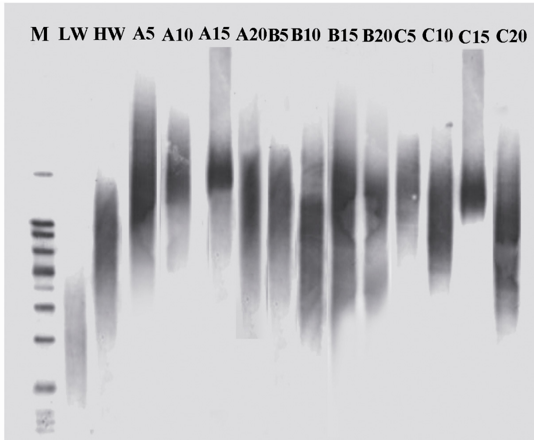

Figure 2. Southern blotting shows telomere length. A5, A10, A15 and A20 indicate telomere length in PAFSCs after 5, 10, 15 and 20 passages. B5, B10, B15, B20 and C5, C10, C15, C20 indicate the same results in DPSCs and HFF, respectively. M is molecular weight marker. LW and HW indicate low and high molecular weight of control DNA, respectively

Table 2. Primer used for common deletion detection

Primer	Primer nucleotide	Primer sequencing
ONP-86F	5480-5461	3′-CCCTTACCACGCTACTCCTA-5′
ONP-89R	5740-5721	3′-GGCGGGAGAAGTAGATTGAA-5′
ONP-74R	13640-13621	3′-GGTTGACCTGTTAGGGTGAG-5′
ONP-25F	8161-8180	3′-CTACGGTCAATGCTCTGAAA-5′

DPSC, 0.1 *kb* in PAFSC and 0.32 *kb* in HFF which indicate insignificant changes with p-values greater than 0.05 (Table 1). In addition, morphological investigation by microscope and cell proliferation rate assay for achieving cell density did not show appreciable changes during passages (Table 2).

Discussion

Investigating mtDNA common mutation and telomere length assay as aging biomarkers of adult stem cells is the subject of many recent studies. For example, several studies were done in this regard on Mesenchymal Stem Cell (MSC) lines derived from human bone marrow [12-14, 17] or animal [9] and also on oocytes, embryo or cumulus cells [18,19]. In this study, efforts were made to examine mtDNA common mutation and telomere length assay together in adult human dental stem cells and adult fibroblast line which were produced in our stem cell laboratory during multiple passages.

Adult stem cells including MSCs and Hematopoetic Stem Cells (HSCs) are generally known to have a limited life span [20]. Human Mesenchymal Stem Cells (hMSCs), because

Table 1. Characteristics of dental stem cell lines and human foreskin fibroblasts

Characteristics	Dental Follicle Stem Cell (DPSC)	Periapical Follicle Stem Cells (PAFSC)	Human Foreskin Fibroblast (HFF)
Mean telomere length in the first passage (*kb*)	11.86±0.55 *	11.47±0.15 †	11.51±0.14 ¥
Mean telomere length in the last passage (*kb*)	11.61±0.58 *	0.28†±11.57	11.91±0.17 ¥
Mean difference of telomere length between first and last passage (*kb*)	0.25	0.1	0.32
Needed time for flask confluence at first passage (day)	0.8±3	0.8±3	0.8±3
Needed time for flask confluence at last passage (day)	0.5±5	0.5±5	0.5±5

Comparisons of groups *†¥ with mean telomere length in the last passage were not statistically significant. P>0.05

of their self renewal ability, multipotentiality, and immunosuppressive capacity are considered to have high clinical potentials. These potentials could be different depending on tissue origin and some embryonic stem cell like phenotypes such as expression of embryonic markers [12,21].

Some studies introduced adult stem cell lines with detectable levels of telomerase that can extend to a large population at multiple passages *in vitro* [22]. Moreover, the mechanism of senescence for these cell lines is poorly understood. Replicative senescence proceeded and it was shown that stress-induced premature senescence, unlike replicative senescence, is largely independent of the telomere length or the number of cell divisions [20]. Chen *et al* indicated that Amniotic Fluid (AF) stem cells show senescence and longevity changes unrelated to telomere length. Therefore, AF derived cells are most likely to undergo stress induced premature senescence and not normal replicative senescence in culture [23].

In adults, telomerase is only expressed in germ cells, stem cells and some progenitor cells [24] while presence of telomerase activity in MSCs is still controversial according to different studies [25-32].

It is reported that the longest telomeres are generally marking the most primitive adult stem cell compartments and the shortest telomeres are related to more differentiated compartments within a given tissue [25]. Similar results were reported in lingual epithelium [26]. In order to better explain the role of telomeres in the aging of MSCs, initial size of telomeres and donor age dependency were examined. Interestingly, the data from different groups do not correlate with each other [28-30]. However, findings related to the gradual deterioration of telomere ends with successive cell divisions are consistent across all studies [12].

In our previous work on PAFSCs, DPSCs and HFF, high proliferative ability of these cell lines was shown by the expression of some embryonic/germ immonohistochemical or gene markers which indicate that their self renewal capacities may increase without occurrence of aging markers [14,15]. Overall, some investigators used cancer stem cells instead of adult stem cells [33]. Studying this population is easier for monitoring their behavior in response to different stimuli or therapeutic treatments [34]. However, in this study, generated stem cells plus HFF cell line were used which are the main strengths of this research.

In the present study, an insignificant change of telomere length was observed between first and last investigated passages in all three cell lines. In addition, morphological investigation by microscope and cell proliferation rate assay for achieving cell density did not show appreciable changes during passages.

In addition to the telomere length, the accumulation of common deletion as well as other mtDNA deletions and point mutations are thought to contribute to normal cell aging [27]. In the past decade, different mutations of mtDNA have been reported to frequently occur and accumulate with age in muscle and other human tissues. Among them, the 4,977 *bp* and 7,436 *bp* deletions and the A3243G and A8344G point mutations are the most frequent mutations [35]. In fact, the accumulation of mtDNA deletions and Single Nucleotide Variants (SNVs) is a well-accepted facet of the biology of aging. Recently, it was shown that a complex manner is involved in alteration of the mitochondrial genome with age [36]. It was shown that mitochondria are translocated in the oocyte during fertilization to cluster around the pronuclei and remain in a perinuclear pattern during embryo development. This clustering appears to be essential for normal embryonic development. Because embryonic stem cells are derived from fertilized oocytes, and eventually can differentiate into adult stem cells, it was hypothesized that mitochondrial perinuclear clustering persists through preimplantation embryo development into the stem cells, and that this localization is indicative of stem cell pluripotency [7,19].

The study did not reveal a specific association between common 4977 *bp* mtDNA deletion and multiple passages of adult stem cells and fibroblasts that were used as a common cell line. Similar to this study, several other studies using different tissue/cell lines had already come to the same conclusion [3].

Conclusion

With these results, it seems that common mtDNA deletion and telomere length are not related with aging in 20 first passages in adult stem cell lines and HFF cell line. Therefore, these adult stem cells may be suitable for clinical application even after 20 passages. This article suggests further investigation on different adult cell lines with more passages *in vitro* for mtDNA deletion and telomere length assay. This will expand our data regarding the effect of multiple passages and cellular senescence of stem cell lines on gene expression changes in adult stem cells.

Conflict of Interest

There were no conflicts of interest to be stated.

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