hTERT expression extends the life-span and maintains the cardiomyogenic potential of mesenchymal stem cells in human umbilical cord blood

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Abstract: Human umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) represent a population of stem cells that are capable of differentiation into multiple lineages and are expected to serve as an excellent alternative to bone marrow-derived human mesenchymal stem cells. However, these cells exhibit senescence-associated growth arrest and phenotypic changes during long-term culture. To overcome this problem, we established UCBMSCs (hTERT-MSCs) with human telomerase reverse transcriptase (hTERT) gene. We found that the hTERT-MSCs proliferated faster than non-infected and had longer life-span. Induced hTERT-MSCs with 5-azacytidine to cardiac muscle and detected the specific marker of myocardiocyte. The hTERT-MSCs were able to form cardiomyocyte evidenced by positive staining for Connexin-43 and α-Sarcomeric actin. We concluded that the hTERT gene does not influence some type of differentiation potential of MSCs.
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Keywords: hTERT; mesenchymal stem cell; life span; cardiomyogenic potential

1. Introduction

Umbilical cord blood (UCB) is also a source of human MSCs whose cellular characteristics and multilineage differentiation capability are equivalent to those of BM-derived MSCs[Lee MW and Yang MS,2005; Lee OKand Kuo TK,2004]. UCBMSCs will be useful sources for cell transplantation, however, it is difficult to study and apply them because of their limited life span[Warren LA and Rossi DJ,2009]. Much research showed telomeres normally shorten as human cells divide. When telomeres reach a specific senescent length the cells enter senescence.

To resolve these problems, the life span of MSCs from bone marrow can be extended by retroviral transduction of hTERT gene[Basem M. Abdallah, Mandana Haack-SØensen,2005]. The researchers improved that telomerization of hMSC by hTERT over-expression maintains the stem cell phenotype of hMSC and it may be a useful tool for obtaining enough number of cells with a stable phenotype for mechanistic studies of cell differentiation and for tissue engineering protocols.

In the present study, we investigated the growth regulatory mechanism of UCBMSCs and attempted to establish UCBMSCs with hTERT (hTERT-MSCs) to overcome their limited life span. The hTERT-MSCs can serve as an alternative source of mesenchymal stem cells and may provide a unique source for cellular and gene therapy.

2. Materials and methods

2.1 Isolation and Cell Culture of UCBMSCs

Human UCB samples (>40ml/sample)obtained with the mother's consent were processed within 4h of collection. Mononuclear cells(MNCs) were isolated from UCB on a Ficoll(density,1.077/ml)gradient and were suspended in medium consisting of high glucose-DMEM, 15% fetal bovine serum, and seeded at a concentration of 1×10^7 cells/ml. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 with a change of culture medium every 7 days until the fibroblast-like cells at the base of the flask reached confluence. Once reaching confluence, the adherent cells were resuspended using 0.25% trypsin EDTA and reseeded at 1×10^6 cells/ml.

2.2 Flow cytometry (FACS) analysis

To detect cell generation cycle, confluent cells were detached with 0.25% trypsin-EDTA , washed with phosphate-buffered saline twice and stained for viability with propidium iodide for 10 min, then assayed for size and granularity based on forward and side scattering by FACS can flow cytometer linked with Mad fit LT for Mac 3.0 software (Becton-Dickinson).

2.3 Infection with Recombinant Retroviruses

The retroviral vector, pLNCX2-hTERT, was employed in these experiments. Amphotropic viral

supernatant containing hTERT was generated by packaging cell line PT67. One million primary UCBMSCs in a 10cm dish were exposed to viral supernatant containing retrovirus at an approximate multiplicity of infection of one to ensure single-copy integration, in the presence of 8μg/ml polybrene for 3 hours. After washing with phosphate-buffered saline, the transduced UCBMSCs were selected with 600mg/L neomycin.

2.4 RT-PCR analysis of hTERT gene transcripts expressed

Total RNA was isolated from three cell groups hTERT-MSCs, USBMSCs (negative control)and K562 cells(positive control) using the Trizon Total RNA Isolation System according to the manufacturer's instructions. The integrity and purity of total RNA was verified by gel-electrophoresis on 0.8% agarose. For reverse transcript, one hundred nanograms of total RNA was reverse transcribed and amplified with the use of the AMV First Strand cDNA Synthesis Kit. A equal volume of each sample was amplified by Polymerase Chain Reaction by using the following primers:the primers specific for retrovirally encoded hTERT were 5′TCTGGATTTGCAGGTGAACAG3′ and 5′GTAGGTGACACGGTGTCGAG′3, with a product of 310bp. The primers of β-actin were 5′GGC ATG GGT CAG AAG GAT TCC ′3 and 5′ATG TCA CGC ACG ATT TCC CGC′3, with a product of 500bp. The PCR reaction was performed at 94°C for 5 minutes, followed by amplification of 35 cycles consisting of 94°C for 30 seconds, 65°C for 50 seconds, and 72°C for 60 seconds.

2.5 Analysis of telomerase activity

hTERT-MSCs , USBMSCs (negative control)and K562 cells(positive control) were cultured in standard growth medium to 80% confluence. Telomerase activity in each sample was detected by using the Telo TAGGG Telomerase PCR ELISA kit according to the manufacturer's instruction.

2.6 proliferation rate (MTT assay)

The comparison of proliferation rate between the UCBMSCs and the hTERT-MSCs was performed by MTT assay. Cells were trypsinized and seeded in 96-well plates at a density of about 4×10^4/ml. Then cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. The measurement was done at the same time everyday. The cells were incubated with DMEM-FBS containing 0.5mg/ml MTT for 4h at 37°C. The medium was discarded and 200μl dimethyl sulfoxide was added to the wells. After 10 min of incubation at room temperature, the colored formazan salt was measured at 492nm using a spectrophotometer.

2.7 Evaluation of the cardiomyogenic potential of hTERT-MSCs

To induce cardiomyocyte differentiation. hTERT-MSCs and long-term culture, UCBMSCs were seeded at a density of 10^5 cells/ml in 6-well plates (for cytochemical staining) and grown for 24h in standard growth medium. At 80 – 90% cell confluence, the medium was supplemented with 10μmol/L 5-azacytidine and this medium was replaced after 24h. Then cultures were maintained with a change of culture medium every 3 days for at least two weeks.

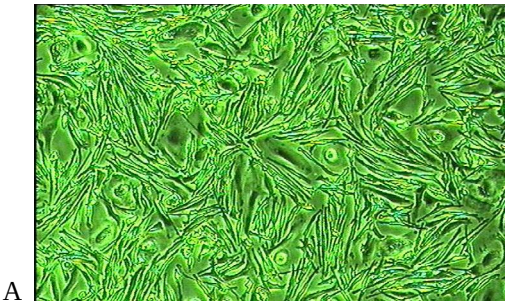
After 14 days in culture, the hTERT-MSCs and long-term culture UCBMSCs on chambered slides were fixed with 4% paraformaldehyde diluted in PBS for 20 min at 4°C and permeabilized in 0.03%TritonX-100 for 10 min , then washed with PBS and blocked in 10% sheep serum for 15min. Primary antibodies were incubated overnight at 4°C, including Anti-human α-Sarcomeric actin(mouse monoclonal antibody, 1:100) and Connexin-43(rabbit polyclonal antibody, 1:100. Secondary antibodies, anti-mouse IgG for α-Sarcomeric actin and anti-rabbit IgG for Connexin-43 were incubated on slides for 30 min at 37°C. After intensive washing with PBS, the slides were incubated in streptavidin conjugated to horseradish peroxidase in Tris-HCL buffer for 30 min. Prepared substrate-chromogen solution using DAB chromogen tablets was applied for 30s and the slides were counterstained in Meyer's hematoxylin for 2 min . Coverslips were mounted on slides with propyl gallate. Then all slides were examined under microscope.

3 Results

3.1 The proliferative ability of MSCs

Concomitant with the growing, UCBMSCs became broad and flat and ceased to proliferate. It's life span didn't exceed 40 days while hTERT-MSCs did not show the senescence phenomena (>90days).

Cell cycle analysis of UBCMSCs was performed. Results showed that during proliferation they were all normal diploid, suggesting that their karyotype remained stable during proliferation. 85.1% MSCs were in the quiescent period (G0-G1 phase), and 14.9% UBCMSCs were in the proliferating stage (S+G2+M phase), demonstrating that only a small fraction UBCMSCs are of potent proliferative ability.



(×40)

Fig.1. Photomicrographs showing hTERT-MSCs. A. Confluent hTERT-MSCs B. Cell cycle by flow cytometry analysis.

3.2 Detection of hTERT mRNA in hTERT-MSCs and UCBMSCs

We confirmed the integration and mRNA expression of the exogenous hTERT in hTERT-MSCs by RT-PCR. As shown in Fig.2, 310bp fragment was observed in the hTERT-MSCs and K562 cellsbut not in the UBCMSCs, which demonstated the integration and mRNA expression of the exogenous hTERT gene in hTERT-MSCs.

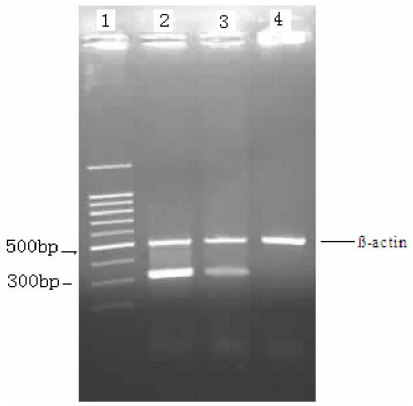


Fig.2.RT-PCR analysis of hTERT gene transcripts . 1 represents Marker, 2 represents K562 cells(as positive control) , 3 represents the hTERT-MSCs, 4 represents the UBCMSCs.

3.3 Detection of telomerase activity in transfected and untransfected MSCs

The absorbance of negative controls in which telomerase was in by heat treatment was then subtracted to remove PCR artifacts. As shown in Fig.3, As samples are regarded as telomerase-positive if the difference in absorbance(A450nm-A690nm) is >0.2 A450nm-A690nm units, we concluded that the UBCMSCs remained telomerase-negative but the hTERT-MSCs showed robust telomerase activity.

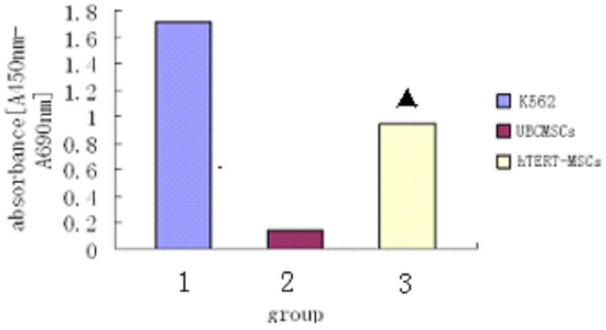


Fig. 3 the telomerase activity in transfected and untransfected MSCs, PD＝15。

3.4 Growth kinetics

Growth kinetics of hTERT-MSCs were measured by MTT assay and compared with those of UCBMSCs. hTERT-MSCs growth curves showed an initial lag phase of 1-2 days. This was followed by a log phase in which the hTERT-MSCs divided at exponential rates for 3-5 days. With the increase of passage, the hTERT-MSCs growth rates were slower and the number of cells generated by the end of 7 days in culture was reduced. UCBMSCs showed the same results. hTERT-MSCs cultures grew at faster rates as compared to UCBMSCs cultures.

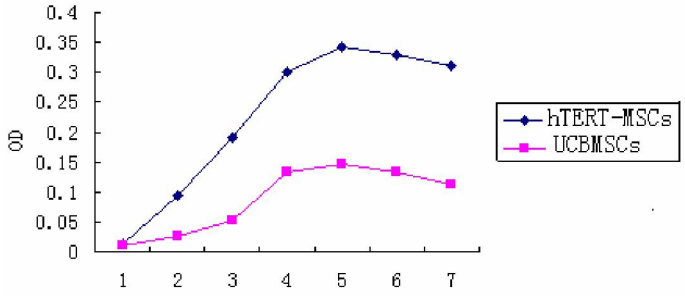


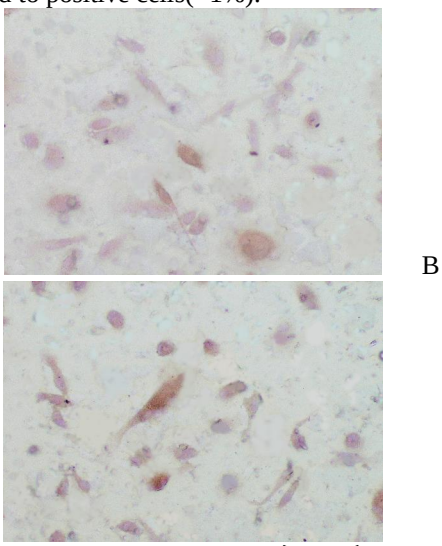
Fig.3. Growth curves of UCBMSCs and hTERT-MSCs.

3.5 hTERT-MSCs maintained differentiation capacity in vitro compared with the long-term culture of UCBMSCs

We performed studies of hTERT-MSCs (90days) and long-term (30days) culture UCBMSCs

for differentiation into the cardiomyocyte by 5-azacytidine. As shown in Fig. 4, hTERT-MSCs were able to form cardiomyocyte evidenced by positive staining for Connexin-43 (8%)and α-Sarcomeric actin(10%). But few long-term cultured UCBMSCs can be induced to positive cells(<1%).

A B



α-Sarcomeric actin （×100）
Connexin-43 （×100）

Fig.4 Characteristic antibody of cardiac muscle immuncytochemistry was showed in A. Some cytoplast was stained by Connexin-43 B. Some cytoplast was stained by α-Sarcomeric actin.

4. Discussions

The use of UCBMSCs requires their in vitro expansion and the capability to preserve their differentiation potential. To achieve this goal, we attempted to prolong the life span of UCB-derived cells even to endow them with immortality. In this study, we demonstrates that hTERT-MSCs proliferated faster than non-infected and had longer life-span.

Transfection of hTERT gene was a safe and effective way to prolong cell life span. Most somatic cells from humans and other mammals lack telomerase activity and undergo senescence after only a limited number of replications. The effect of ectopic expression of hTERT on abolishing the senescence-associated growth arrest and extending the proliferative life-span has been demonstrated in several somatic cell types including fibroblasts, osteoblasts, endothelial cells, epithelial cells, and liver cells[Tsuruga Y and Kiyono T,2008]. Morales's study[Morales CP and Holt SE,1999]showed that although ectopic expression of telomerase in human fibroblasts is sufficient for immortalization, it does not result in changes typically associated with malignant transformation.

Following the proliferation of UBCMSCs, the cells will end in replicative senescence and lose their ability of differentiation. However, in addition to effects on cell proliferation, there is an increasing recognition that telomerase activity may also contribute to the biological functions of the cells. We tested the cardiomyogenic potential of the hTERT-MSCs. Approximately 10% of the hTERT-MSCs were successfully transdifferentiated into cardiomyocytes while the long-term culture UCBMSCs had only <1%. hTERT-MSCs can be a promising cellular source for cardiac stem cell-based therapy[Nishiyama N and Miyoshi S,2007].

The maintenance of the differentiation functions of cells has also been observed in other cell types. hTERT-over expression in endothelial cells led to an improved neovascularization after in vivo implantation, compared with telomerase-negative cells[Murasawa S and Llevadot J,2002]. Also, telomerized human fetal hepatocytes exhibited an extended life span and maintenance of their liver-specific characteristics[Kim YS and Yoon SJ,2008].We have found that hTERT-MSCs responded adequately to in vitro differentiation signals in contrast to the impaired responsiveness observed in senescent cells.

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