Mesenchymal Stem Cells as a Cell-Based Therapeutic Strategy Targeting the Telomerase Activity of KG1 Acute Myeloid Leukemia Cells

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Abstract- A predominant challenge in the discovery approach to curative leukemia is investigating the effect of mesenchymal stem cells (MSCs) on leukemic cells. We aimed to investigate the role of MSCs in targeting telomerase enzyme and consequently telomere length of leukemic cells. For this purpose, the KG1 cell as leukemia cell line was co-cultured with MSCs in the trans-well system. After seven days of co-culture, KG1 cells were collected, and telomerase activity, telomere length, and hTERT gene expression were analyzed by PCR-ELISA TRAP assay and real-time PCR, respectively. Also, the potentially involved ERK pathway was analyzed at gene and protein levels by real time PCR and flow cytometry, respectively. It was found that MSCs caused a significant decrease in telomerase activity, telomere length, and hTERT gene expression. The following results showed that MSCs resulted in a significant decrease in the ERK expression levels. It can be concluded that the co-culture of MSCs with KG1 cells be involved in the telomerase targeting via ERK signaling pathways. This study concluded that the co-culture of MSCs with AML leukemic cells could secrete a significant amount of cytokines that cause to inhibit the proliferation of AML cell lines via ERK signaling pathway. The recognition of cytokines as well as growth factors involved in the anti-proliferative effect of MSCs requires further investigation. This effect as a therapeutic strategy could be considered in the basic experimental studies.

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Introduction

Regarding the acute myeloid leukemia (AML), as a most common leukemia in adults, some treatments have been considered to prevent the progression of disease, including chemotherapy and radiotherapy. However, these methods are limited by the regression of metastasis and drug resistance (1). Stem cell transplantation as another treatment has attracted clinicians and researchers. MSCs are spindle-shaped cells derived from various tissue sources which have multi-lineage differentiation and self-renewal capability are the suitable choice for cell-based studies (2). Despite researches performed recently, it is yet to be known whether MSCs have tumor-suppressing or tumor-promoting effects. In one study, Manabe *et al.*, (1992) reported that MSCs prevent apoptosis of acute lymphoblastic leukemia (ALL) cells via secreting cytokines (3). In another study, Zhu *et al*., (2009) demonstrated that adipose tissue-derived MSCs inhibit AML cells proliferation through induction of cell cycle arrest (4).

On the other hand, the mutation in the telomerase complex has been involved as a risk factor for adult AML (5). As previously investigated, the telomerase enzyme is expressed in highly proliferative cells such as cancer and stem cells (6). Impaired telomerase function may lead to chromosomal instability and predisposing to malignant transformation (6). It has been reported that up to 90% of human cancers reactivate telomerase, and targeting the telomerase activity and telomere length can be considered

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as a strategy for cancer therapy (7). Disturbance of telomere homeostasis are signified in the pathogenesis of hematologic malignancies such as myeloid disorders.

Herein, we focus on the effect of adipose tissue-derived MSCs on telomerase activity as well as telomere length in KG1 cells as AML cell line. For this purpose, co-cultured Molt-4 cells with MSCs (10:1) were collected and subjected to PCR ELISA TRAP assay and real time PCR for investigating the telomerase activity and telomere length measurement. In the following, the signaling pathway involved in this process including extracellular signal-regulated kinase (ERK) was also evaluated.

Materials and Methods

Cell culture plates and materials were obtained from SPL Life Sci. and Gibco Co., respectively. Other materials which used are specified in the text.

KG1 cell line culture

The KG1 cell line was provided by Pasteur Institute. The cells were grown in RPMI 1640 complete media containing 10% FBS and 100 U/ml of penicillin/streptomycin and incubated at 37° C with 5% CO_2. To maintain a log phase of growth, cells were cultured at 15×10^4 cells/cm^2 and passaged twice weekly.

Mesenchymal stem cell culture and characterization

Adipose tissue derived-MSCs were purchased from Royan Institute and cultivated in DMEM complete medium containing 10% FBS in a 37º C incubator with 5% CO^2 until reaching to 80-90% confluency. Upon reaching this point, the cells underwent be passaging. The culture medium was refreshed twice weekly. Cells of passages 3-6 were used in the experiment. As previously performed by Fathi *et al.*, (2020), the cells were stimulated to differentiate into adipogenic, osteogenic, and chondrogenic lineage (8). Briefly, the cells were cultured in complete culture medium containing (1) adipogenic differentiation medium; (2) osteogenic differentiation medium; and (3) chondrogenic differentiation medium. At the end of the 21st day culture, the cells were fixed with 4% (v/v) paraformaldehyde for staining differentiation-specific proteins to evaluate the differentiated cells. For this purpose, Oil red O, Alizarin Red S, and toluidine blue (0.1% in distilled water) were used for adipogenesis, osteogenesis, and chondrogenesis, respectively. The cultured MSCs were also characterized by flow cytometry using a panel of MSCs markers, including CD73, CD90, CD34, and CD56. (9). For this purpose, the cells were trypsinized from a culture plate and incubated with specific antibodies anti-CD73, 90, 34, and 56. A FACScalibur flow cytometer (Becton Dickinson) was used to examine the samples, and FlowJo software (version 6.2) was used to analyze the obtained results.

Co-culture of KG1 cell line and MSCs

KG1 cells and MSCs were co-cultured using a trans-well system. In brief, MSCs were seeded into trans-well inserts at 1×10^5 cells/well in DMEM-low glucose complete culture medium. Following attaching the MSCs to trans-well inserts, 1×10^6 cells/well were plated in the lower chamber of the trans-well system. Therefore, two cell groups were created; KG1 cells alone as the control group, and co-cultured MSCs with KG1 cells as the experimental group. The co-cultured cells were incubated at 37º C and 5% CO_2 for seven days. Following the co-culture period, KG1 cells were harvested from control and experimental groups for subsequent experiments.

Cell proliferation assay

Cell proliferation was measured by co-culturing of KG1 cell line with MSCs at the following time points: 24, 48, 72, 96, and 120 hours. Trypan blue staining was used to determine cell proliferation after the termination of the co-culture period.

Real-time PCR

Cultured KG1 cells alone (as control) and co-cultured KG1 cells with MSCs were subjected to RNA extraction. Following this, cDNA was synthesized of total RNA (2 µg) from each group. Real-time PCR detection assay was done to determine the mRNA expression of hTERT and ERK. The mRNA expression levels were normalized to the expression level of β-actin using the $2^{-\Delta\Delta CT}$ method. The sequences of primers used in this work are shown in Table 1.

ERK protein expression by flow cytometry

Upon ending co-culture time of KG1 cell line with MSCs, Erk1/2 activation we assessed. In detail, these cells were promptly fixed by FCM fixation buffer for 30 min and then washed by the washing buffer and incubated with FCM permeabilization buffer for 5 min at RT. Staining of washed cells was carried out by Alexa Fluor 647 conjugated mouse antiphosphorylated-Erk1/2 (pT202/pY204) (612593, BD Bioscience) for 30 min. The flow cytometry samples were investigated by FACScalibur flow cytometer (Becton Dickinson).

Absolute telomere length (aTL) measurement

KG1 cells were collected as mentioned above and genomic DNA was extracted and stored at 4° C until being used for absolute measurement of the telomere length (10).

Standard curves and calculations for aTL measurement

Standard curve for telomere

By diluting known amounts of a synthesized 84-mer oligonucleotide that contains only TTAGGG, repeated 14 times, a standard curve was created. This synthetic telomere repeat sequence oligomer was identical to those reported by O'Callaghan and Fenech (2011) (10).

Standard curve for single copy gene (SCG)

As a control, SCG is used to determine the number of genome copies per sample. SCG amplification is critical for generating accurate and reliable results from the aTL assay. Based on O'Callaghan and Fenech (2011) and Fathi *et al.*, (2013) model, the genome copy number per reaction is calculated (10,11).

Assessment of telomerase activity

The total protein was lysed after the ending co-culture period. A PCR-ELISA TRAP assay was used to determine the relative telomerase activity of KG1 cells. The details of the PCR-ELISA TRAP procedure were explained by Faezizadeh *et al.*, (2012) (12).

Statistical analysis

**P*<0.05 was set statistically significant. For statistical analysis, Graph Pad Prism version 6.01 was used using t-test and Two-way ANOVA proceeded by Dennett's comparison test.

Table 1. Primer sequences used for Real time-PCR.

No.	Gene	Primer pair sequence (5'-3')	Product length (bp)
NM_001193376.1	*hTERT*	CAGCAAGTTTGGAAGAACCC GACATCCCTGCGTTCTTGG	98
NM_001040056.3	*ERK*	GGAGGACCTGAATTGTATCA CTCCACTGTGATCCGTTTAT	169
NM_017008.4	*GAPDH*	ATGACTCTA CCCACGGCAAG CTGGAGATGGTGATGGGTT	88

Results

Multi-lineage differentiation potential and cell surface markers analysis

According to the Figure 1A, MSCs are spindle-shaped cells as well as fibroblasts cells. Adipogenic, osteogenic, and chondrogenic differentiation of these cells was also investigated. Oil Red-O, alizarin red, and toluidine blue were used to stain the lipid droplets, mineralized compartments, and aggregates of aggrecan, respectively (Figures 1B, C, and D).

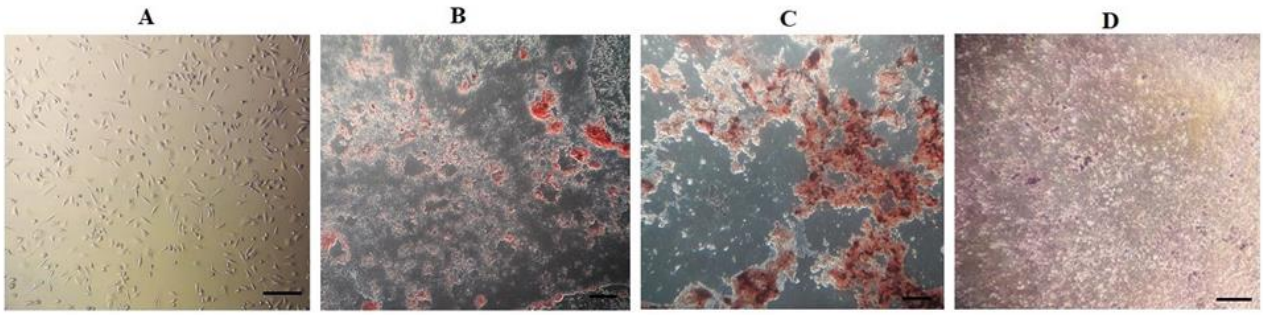

Figure 1. Morphological features of MSCs; (A) Spindle-shaped morphology of MSCs at day 7; (B) Adipogenic differentiation; (C) Osteogenic differentiation; (D) Chondrogenic differentiation.

The adipose tissue-derived-MSCs were analyzed for expression of cell surface markers. The results revealed that MSCs were positive for CD73 (96%) and CD90 (99.8%), but negative for CD34 (1.92%) and CD56 (0.72%) (Figures 2A-D).

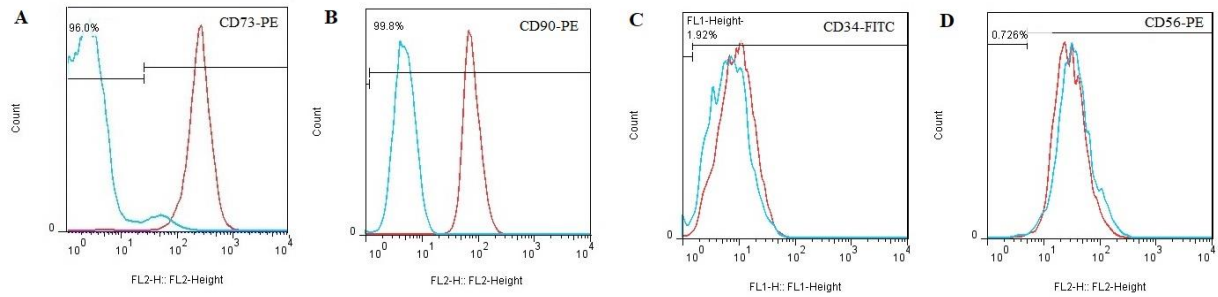

Figure 2. The expression of the cell surface markers of MSCs that analyzed by flow cytometry. The MSCs were positive for (A) CD73 (96%) and (B) CD90 (99.8%), and negative for (C) CD34 (1.92%) and (D) CD56 (0.72%).

Cell proliferation assay of KG1 following co-culture with MSCs

KG1 cells in both the control group (KG1 alone) and the experimental group (KG1+MSCs) began to proliferate at day 2 (48 hours) and no obvious difference in their proliferation speeds was observed before this time. After day 2 (days 3 and 4), cells of both groups entered the fast proliferating period, but the proliferating speed of the experimental group was significantly lower than that in the control group (Figure 3).

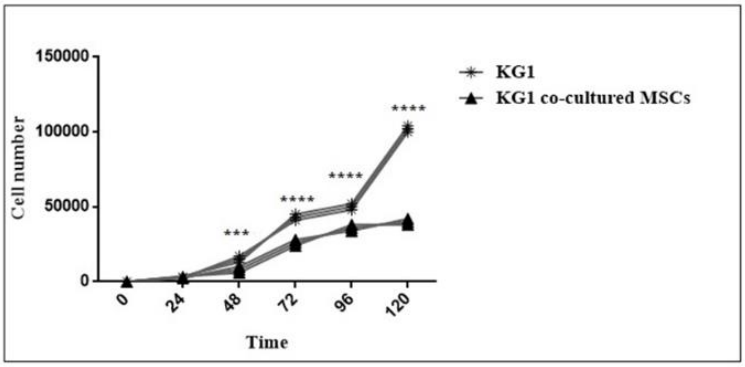

Figure 3. Growth curve of KG1 cell line following co-culture with MSCs. Cells of both groups (control and experimental) entered the fast proliferating period, but the proliferating speed of the experimental group was significantly lower than that in the control group (****P*<0.001 and (*****P*<0.0001).

MSCs changed the gene and protein expression of ERK

The gene and protein expressions of ERK was investigated by Real-time PCR and flow cytometry, respectively. As shown in Figure 4, the gene and protein expression of ERK were significantly decreased 0.6- and 5.1-fold, respectively (***P*<0.01 and ****P*<0.001).

MSCs decreased the telomerase activity, aTL, and *hTERT* gene expression of KG1 cells

Following the co-culture period, both groups of cells (KG1 cell alone and KG1 cells co-cultured with MSCs) were collected, and the telomerase activity and aTL were measured using PCR-ELISA TRAP assay and real-time PCR, respectively. As shown in Figure 5, the telomerase activity and *hTERT* gene expression have been decreased by 24% and 0.52 fold in the experimental group compared to the control group (Figure 4A) (**P*<0.05 and ***P*<0.01). Also, aTL significantly decreased in the experimental group (9.13 Kbps) compared with the control group (44.91 Kbp) (****P*<0.001).

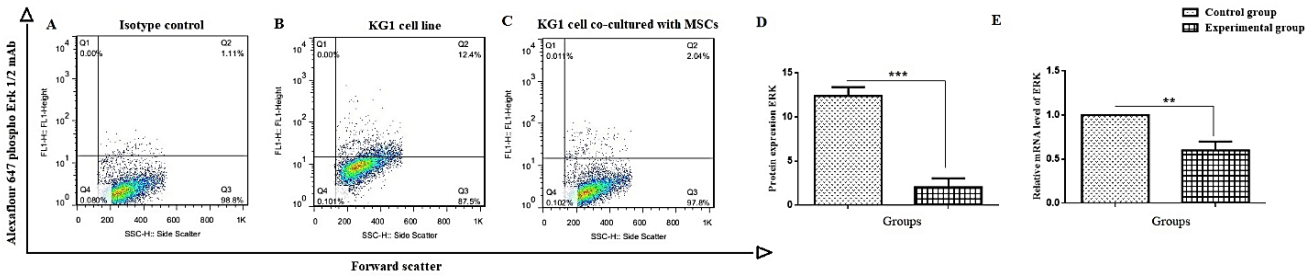

Figure 4. ERK protein expression of KG1 cell line following co-culture with MSCs. The ERK protein expression was assessed by flow cytometry. In this figure, A is isotype control, B is KG1 cells alone, C is KG1 cells co-cultured with MSCs, D is the graph of ERK protein expression, and E is the graph of the mRNA expression level of ERK (***P*<0.01, ****P*<0.001).

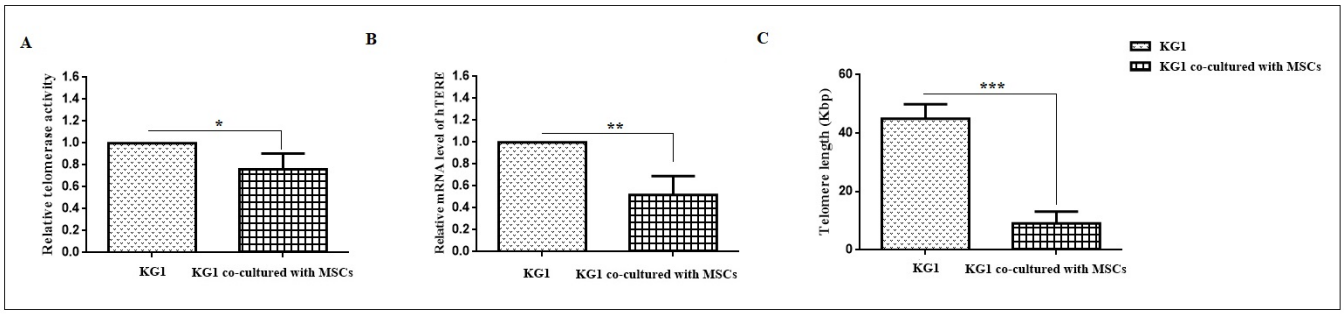

Figure 5. (A) Relative telomerase activity and (B) hTERT gene expression levels. MSCs were significantly decreased the telomerase activity (**P*<0.05) and hTERT gene expression of the KG1 cell line (***P*<0.01). (C) aTL measurement; MSCs were significantly decreased the aTL (****P*<0.001) of the KG1 cell line.

Discussion

The therapeutic targeting of AML cells may become a conceivable approach to treat leukemia malignancy. Recent experimental studies suggest the inhibitory effects of MSCs on leukemic cells. In one study by Liang *et al*., (2008), it was shown that bone marrow-derived-MSCs lead to cell cycle arrest of AML cell lines via the induction of specific gene expression (13). In the following, Zhu *et al*., (2009) reported the inhibitory effect of adipose tissue-derived-MSCs on AML cell line proliferation through secreting cytokine such as DKK1 (4). The inhibitory effect of MSCs on leukemic cell growth via activation of the P38 MAPK signaling pathway was also demonstrated by Tian *et al*., (2010) (14). It assumed that the role of MSCs on cancer cells is through releasing the soluble mediators, cytokines, and growth factors (15,16). In our study, the inhibitory effects of MSCs mediated via the decreasing the telomerase activity of KG1 cells as a leukemia cell line was investigated. In this study, MSCs cause to decrease the telomerase activity, TL, and *hTERT*, the catalytic subunit of human telomerase gene expression. Effects of MSCs on AML cell lines have also been investigated by other studies, but none of these studies focused on the telomerase and telomere length of cancer leukemic cells. Thus, our present study expands the knowledge via its focus on telomerase activity targeting. Upregulation of telomerase activity and following that telomere lengthening is a hallmark of cancer cells, making telomerase an attractive target for cancer therapeutics (17). Telomere lengthening is exclusively important for pluripotency and multipotency characteristics of hematopoietic stem cells as well as cell and tissue regeneration (17). In more detail, the disturbance of telomere homeostasis has been implicated in the pathogenesis of hematologic disorders such as AML (18). As previously reported by Brümmendorf and Balabanov (2006), it seems telomere length to be markedly reduced in patients with myeloid neoplasms (19). In leukemic patients with abnormal cytogenetics, the telomere is significantly shorter than those with no cytogenetic abnormality (20). The results of our study are

contradictory with the results from the previous studies. We showed that MSCs could significantly decrease the telomerase activity and TL of the KG1 cell line. To identify possible signaling pathways involved in KG1 leukemia cells-MSCs interactions during the co-culturing, we performed an expression profile of several components involved in the signaling using western blot and flow cytometry analyses.

The ERK pathway, has a major role in the control of diverse cellular processes such as survival, growth, proliferation, motility, differentiation, etc. (21,22). Accordingly, aberrant activation of this pathway would result in the induction of many types of tumors. Therefore, interest in the ERK pathway as attractive targets for cancer chemotherapy and cell transplantation has great attention in the last few years (23). Previous investigations have been shown that MEK/ERK pathway plays a key role in the malignant transformation of leukemia. It was shown that chemotherapeutic drugs cause to dramatically decrease the protein expression of components of the ERK/MAPK signal pathway.

We hypothesized that the inhibition of KG1 cell proliferation by MSCs was governed by ERK signaling pathway. In the experimental group, the gene and protein expression of ERK was significantly decreased by more than 0.6- and 5.1-fold, respectively. Zhang (2009) had also reported the effect of MSCs on leukemia-mononuclear cell growth curve. It was found that INF-α as an important cytokine was the most abundant protein expressed in the co-culture media that have cause to markedly inhibited the proliferation of leukemia-mononuclear cell (24). Kogler *et al*., (2005) reported that MSCs could secrete cytokines and some pro-angiogenic factors, such as VEGF and angiopoietin-1 that interact in co-culture system (25). Unlike another studies, we did not investigate any cytokines and growth factors, which involved in KG1cell-MSCs cell interaction. Interestingly, we are the first to report that MSCs cause to reduction of telomerase activity, telomere length and *hTERT* gene expression of the KG1 leukemic tumour cell lines through ERK pathway.

In conclusion the current research indicated that MSCs cause to significant reduction in telomerase activity, aTL and *hTERT* gene expressin of KG1 cells as AML cell line via ERK pathway. This study concluded that the co-culture of MSCs with AML leukemic cells maybe secrete an amount of cytokines as well as growth factors that cause to inhibit the proliferation of AML cell lines via ERK signaling pathway.

Ethics Approval

The ethical consent was given by the Committee on the Ethics of Tabriz University of Medical Sciences, Tabriz, Iran (Ethics No.IR.TBZMED.VCR.REC.1398.056).

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