Targeting the Proliferation Inhibition of Chronic Myeloid Leukemia Cells by Bone Marrow Derived-Mesenchymal Stem Cells via ERK Pathway as a Therapeutic Strategy

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Abstract- Bone marrow-derived mesenchymal stem cells (BMSCs) are of specific attention due to their potential clinical use in cell transplantation. These cells could secrete cytokines and growth factors upon stimulation or suppression in regenerative medicine. This study was to evaluate the influence of BMSCs on the proliferation of K562 cells as chronic myeloid leukemia (CML) cell lines through the ERK pathway. For this purpose, BMSCs were extracted from Rattus Norvegicus and were co-cultured with K562 cells. In the following, at the end of the 7th day, the K562 cell was collected and subjected to ERK protein expression measurement as well as ERK gene expression by flow-cytometry and real time-PCR, respectively. Also, the cell proliferation and PDT of K562 cells were measured in the control and experimental groups. The results were shown that BMSCs were positive for mesenchymal (CD44 and CD90) and negative for hematopoietic (CD34 and CD56) markers. In addition, it was shown that BMSCs mediated CML cell line proliferation arrest via a significant reduction of the ERK protein expression in the co-culture groups versus in the K562 cell line control group. Taken together, the data concluded that the co-culture of BMSCs with CML cell lines could secrete a substantial amount of cytokines and growth factors, thus inhibiting the proliferation of CML cell lines via the ERK signaling pathway. The identity of molecules, cytokines, and growth factors involved in the anti-proliferative effect of bone marrow-derived MSCs require further investigation, and this effect could important in the basic experimental study as a therapeutic strategy.

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Keywords: Bone marrow-derived mesenchymal stem cells (BMSCs), Chronic myeloid leukemia (CML) cell line; Co-culture; Extracellular-signal-regulated kinase (ERK) pathway

Introduction

The pioneering work of Friedenstein and co-workers in 1974 on the isolation, culture, and differentiation of bone marrow-derived cells from pigs opened a new field of stem cell identification and research (1). Subsequent investigations by other researchers introduced the mesenchymal stem cells (MSCs) terms and mentioned that MSCs were also able to self-renew and differentiate into many different cell types in the body such as adipocyte, osteocyte, chondrocyte, *etc*. (2). There are two kinds of stem cells isolated from the animals and humans: embryonic stem cells (ESCs), which are derived from the inner cell mass of the blastocyst, and non-embryonic stem cells, also known as adult stem cells that are attained from different tissues. MSCs are a kind of adult stem cells. On the other hand, cell transplantation by MSCs is one of the alternative therapeutic approaches for cancer, especially for leukemia. Today, different types of MSCs are considered for cell therapy. Bone marrow-derived stem cells, a type of distinct mesenchymal stem cells (MSCs), are sparsely distributed within bone marrow with broad prospects for the clinical applications in tissue engineering, regenerative medicine and cell transplantation (3). Bone marrow-derived MSCs are immune suppressive to lymphocyte proliferation and could inhibit tumor growth by induction of apoptosis (4). However, others have found the opposite. Hence, there

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are disagreements regarding the effect of bone marrow-derived MSCs on tumor cells. These inappropriate results with their inadequate explanations are clear indicators that the effect of MSC on tumor cells and the mechanisms involved need resolution.

Although previous studies have found that MSCs could mediate immunosuppression through secreting soluble cytokines and growth factors, cellular signaling and interaction between bone marrow cell types and molecules which can disrupt the normal hematopoiesis have yet to understand (5). K562 as chronic myeloid leukemia cells are regarded as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, and megakaryocytic lineages as defined by a surface-antigen expression. For this reason, this study was to explore the influence of MSCs on proliferation and apoptosis of chronic myeloid leukemia (CML) cell line and the underlying mechanisms. In brief, two groups of cells were used in this experimentation; control group (culture of bonemarrow-derived-MSCs alone) and experimental group (co-cultured K562 and bone marrow-derived MSCs). On the other hand, cultured K562 alone and co-cultured K562 with MSCs (10:1) were collected at day seven and subjected to ERK protein expression and cell proliferation.

On the other hand, the aims of this study were to: (I) evaluate the effect of bone marrow-derived MSCs on the survival and proliferation and of K562 cell line (as a chronic myeloblastic leukemia cell line) and (II) determine the cell proliferation arrest by ERK pathway.

Materials and Methods

Reagents

All chemicals, culture consumables, and media, if not otherwise specified, were purchased from Sigma-Aldrich (Invitrogen, Carlsbad, Calif., USA) and SPL Life Sciences Co., Ltd. (Gyeonggi-do, Korea), respectively. A comprehensive overview of methods that have been used in this paper was described in Figure 1.

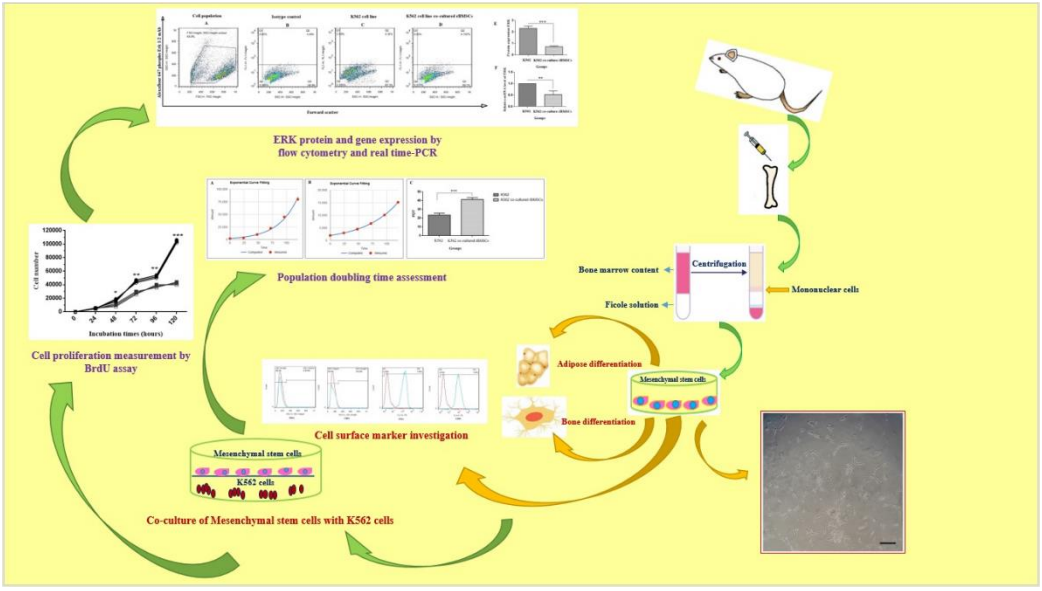

Figure 1. Flow chart of an overview of the experimental procedures that have been used in this paper

Isolation of bone marrow-derived mesenchymal stem cells (BMSCs)

In this research, before surgery of rats, ethical consent was given by the Committee on the Ethics of Tabriz University of Medical Sciences, Tabriz, Iran, in accordance with the guidelines of the Helsinki ethical code regarding experiments performed on animals. About 3 (6- to 8-week-old) male Rattus rats were purchased from animal home in Tabriz University of Medical Sciences (Tabriz, Iran), and euthanized using Xylazine (13 mg/kg)/ketamine (87 mg/kg). Bone marrow from femur and tibia was obtained by flushing with 24-gauge syringe needle containing washing buffer (phosphate-buffered saline (PBS) supplemented with 5% fetal bovine serum (FBS)) in a sterilized culture dish. Bone marrow contents were washed with washing buffer and centrifuged at 360×g (1500 rpm) for 5 min. In the following, 5 mL of diluted cell suspension was layered

over 5 ml of Ficoll-Paque (Baharafshan, Tehran, Iran) and centrifuged at 850×g (2298 rpm) for 25 minutes at 4° C. After that, middle phase containing mononuclear cell layer was transferred to a new 15 mL conical tube and was washed for two times with washing buffer. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) containing 10% FBS and 1% penicillin/streptomycin solution (complete culture medium). Cultures were maintained at subconfluent levels in a 37° C incubator with 5% CO_2 and passaged with 0.25% trypsin (Gibco, UK) and one mM ethylenediaminetetraacetic acid (EDTA; Invitrogen, UK) when required. The medium was replaced with fresh medium after two days of culture. Cells of the passage 3-6 were used throughout the present study (6).

Adipogenic and osteogenic differentiation of bone marrow-derived MSCs

The bone marrow-derived MSCs were grown to confluence, and adipogenic and osteogenic differentiation of cells was induced as previously described by Fathi *et al*., (2019). In brief, the cells were cultured in complete culture medium containing (1) adipogenic differentiation medium: 1 µM dexamethasone, 0.5 mM 1-methyl-3 isobutylxanthine, 10 µg/ml insulin and 200 µM indomethacin; (2) osteogenic differentiation medium: 10 nM dexamethasone, 10 mM b-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate. At the end of the 21st day, the cells were fixed with 4% paraformaldehyde for appropriate differentiation-specific staining: Oil red O for adipogenesis and Alizarin Red S for osteogenesis (7).

Characterization of bone marrow-derived MSCs

Characterization of mesenchymal stem cells (MSCs) was done by flow cytometry, as previously described by Fathi *et al*., (2017) (8). In brief, approximately 10×10^5 bone marrow-derived MSCs from the passage four cultures were collected and incubated with an appropriate amount of fluorescein isothiocyanate (FITC)-conjugated antibody CD34 and phycoerythrin (PE)-conjugated CD44, CD56 and CD90 (BD Pharmingen™, San Diego, CA, USA) (1 µg/10^6 cells) in PBS supplemented with 3-5% FBS (washing buffer) for 30 min on ice. After washing the cells, a fluorescence-activated cell sorter (FACS) instrument (Becton Dickinson Franklin Lakes, USA) was used to quantify the fluorescence intensity of bone marrow-derived MSCs and data were analyzed with FlowJo software (version 6.2) (9-11).

Cell culture of the chronic myelogenous leukemia cell line (K562)

K562 (ATCC CCL-243) as an undifferentiated erythroleukemic cell line was purchased from Institute Pasteur (Iran). Cells were culture in suspension in Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco, UK) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cultures were maintained at subconfluent levels in a 37° C incubator with 5% CO_2 and passaged with pipetting without trypsin-EDTA. K562 cell line reaches the log phase in 5-7 days.

Co-culture of bonemarrow-derived MSCs and CML-cell line (K562)

Cultured K562 cell lines were routinely counted using Trypan blue staining. The cell density was adjusted to 150×10^3/cm2 using a complete culture medium containing RPMI 1640, 10% FBS, and 1% (v/v) penicillin/streptomycin solution. Bone marrow-derived MSCs of passage three were detached using trypsin-EDTA, collected and plated into three 6-well plates at 10×10^4 cells/well in 1 ml DMEM complete culture medium solution. After 24 h, 10×10^5 cells/well in 2 mL RPMI 1640 complete culture medium solution was added respectively into two bone marrow-derived MSCs groups; control group (culture of bone marrow-derived MSCs alone) and experimental group (co-cultured K562 and bone marrow-derived MSCs). At day 7, cultured K562 alone and co-cultured K562 and MSCs (10:1) were collected and subjected to cell cycle and annexin/PI analysis. Also, at the end of the 7th day, the supernatant of two groups of cells was collected for cytokine antibody array.

Population doubling time (PDT) assessment and cell proliferation assay

To detect bone marrow-derived MSCs' effect on K562 cell line proliferation and population doubling time, K562 cell line co-cultured with bone marrow-derived MSCs for 24, 48, 72, 96, 120 hours. After termination of the co-culture period, the cells were trypsinized, washed with PBS counted, and cell proliferation and PDT were calculated by BrdU assay and trypan blue staining, respectively. Doubling time was analyzed, and curves were plotted using an online doubling time calculator at the URL http://www.doubling-time.com/compute.php.

Flow cytometry analysis of ERK protein expression

To analyze activated Erk1/2, in the following co-culture of K562 cell line with bone marrow-derived MSCs, K562 cells were harvested, washed twice by

washing buffer (PBS supplemented by 5% FBS). These cells were immediately fixed using FCM fixation buffer (sc-3622, Santa Cruz, CA) for 30 min, washed by washing buffer, and permeabilized by FCM permeabilization buffer (sc-3623, Santa Cruz, CA) for 5 min at RT. Washed cells were immediately stained using Alexa Fluor 647 conjugated mouse antiphosphorylated-Erk1/2 (pT202/pY204) (612593, BD Bioscience)

for 30 min, the phospho-flow analysis was performed using a BD FACS Calibur flow-cytometer system (BD Biosciences, Heidelberg, Germany). Normal mouse IgG1 Alexa Fluor 647 was used as isotype control (sc-24636, Santa Cruz, CA) (10).

Statistical analysis

The results were analyzed using the software program Graph Pad Prism version 6.01. We used one-way, and two-way ANOVA followed by Dunnett's post hoc test to determine the significant difference among groups. Also, statistical significance was determined at $P<0.05$.

Results

In vitro, two lineage differentiation potential and PDT assessment of bone marrow-derived MSCs

As shown in Figure 2A-C, bone marrow-derived MSCs are spindle-shaped cells that have the capacity to adhere to culture plastic flasks as well as fibroblasts cells. Also, adipogenic and osteogenic differentiation of these cells was evident in Oil Red-O and alizarin red staining, respectively. In brief, at the end of adipogenesis, Oil Red-O was used to staining the lipid droplets. Also, redness of the nodules indicated the presence of mineralized compartments as a result of the osteogenic treatment following alizarin red staining (Figure 2D and E).

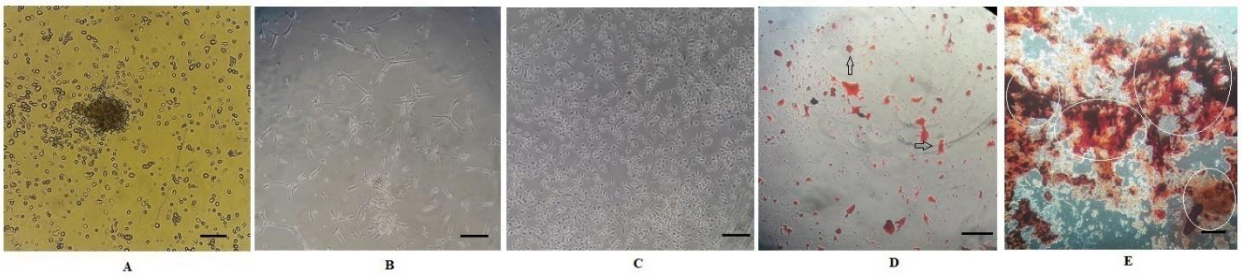

Figure 2. Morphological features of bone marrow-derived MSCs. (A) Spindle-shaped morphology of bone marrow cells that appear at day 1; (B) The number and size of colony appears to gradually increase on days 3–7; (C) More confluent bone marrow-derived MSCs at 14 days of culturing cells; (D) Generation of lipid vacuoles after adipogenesis; (E) Osteogenic differentiation and alizarin red staining of mineralized cell aggregates

Flow cytometry characterization of bone marrow-derived MSCs

The cells (bone marrow-derived MSCs) were analyzed for the expression of a panel of cell surface markers, as shown in Figure 3. The results showed that bone marrow-derived MSCs were positive for mesenchymal markers CD90 (100%) and CD44 (100%), but negative for hematopoietic markers CD34 (0.67%) and CD56 (0.62%).

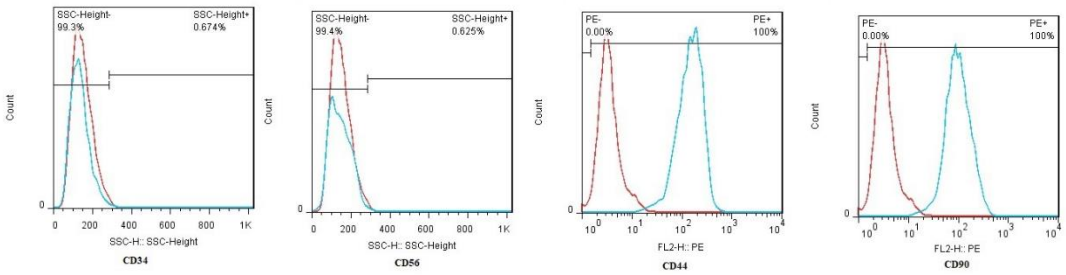

Figure 3. Phenotypical characterization of the cell surface markers of bone marrow-derived MSCs by flow cytometry. Each antibody was tested individually, and the isotopes controls were used as the negative control in this experiment; the bone marrow-derived MSCs were negative for CD34 (0.67%) and (C) CD56 (0.62%) and positive for CD44 (100%) and CD90 (100%). For CD34 and CD56, the isotype control was mouse IgG1, and for CD44 and CD90, the isotype control was mouse IgG2b. Also, isotype control is seen with red dots

Bone marrow-derived MSCs increased PDT OF K562 cell

The population was doubling time for the K562 cell line as the control group (K562 alone), and co-cultured cells (K562+BMSCs) as the experimental group was 24 and 41 hours, respectively. These results showed that cell doubling time was significantly increased in the experimental group (co-cultured cells) as compared to the control group by more than 1.7 (Figure 4A-C).

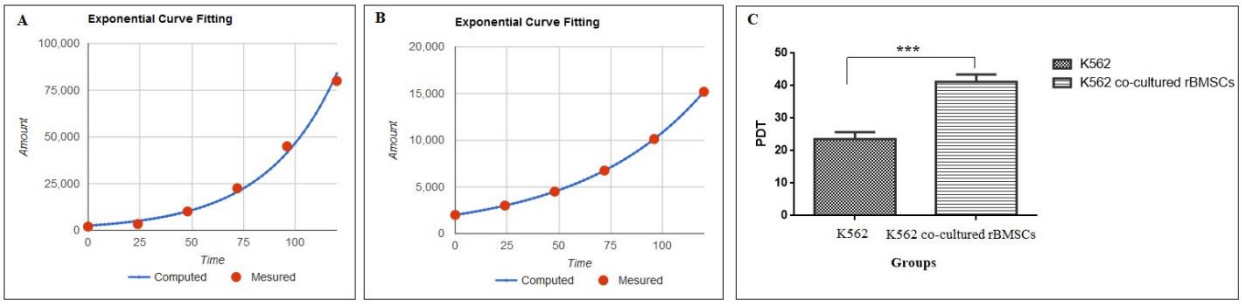

Figure 4. Population doubling time (PDT) of K562 cells in control (A) and experimental (B) groups. These results showed that cell doubling time was significantly increased in the experimental group (co-cultured cells) as compared to the control group by more than 1.7 (C). Values are mean±SD from independent experiments; (****P*<0.001)

Cell proliferation assay of K562 following co-culture with bone marrow-derived MSCs

K562 cells in both the control group (K562 alone) and the experimental group (K562+BMSCs) began to proliferate at day two, 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 5).

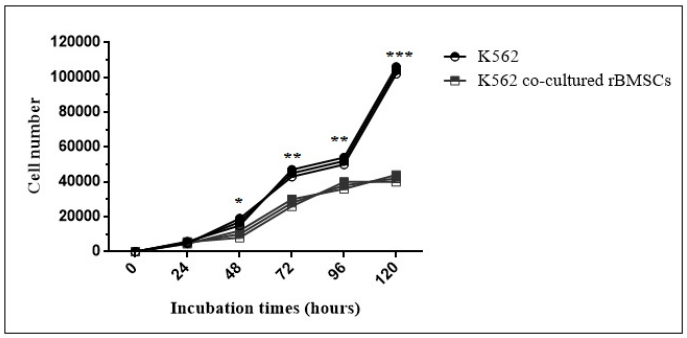

Figure 5. Growth curve of K562 cell line following co-culture with bone marrow-derived MSCs. Harvested cells were evaluated with BrdU assay. Cells of both groups (control and experimental) entered the fast proliferating period, but the proliferating speed of the experimental group was significantly lower than that in the control group. Values are mean ± SD from independent experiments; ((**P*<0.05; ***P*<0.01 and (****P*<0.001).

Bone marrow-derived MSCs reduced K562 cell viability via the ERK pathway

The predominant effect of bone marrow-derived MSCs on the ERK pathway was detected via gene and protein expression of ERK by real quantitative time-PCR and flow cytometry, respectively. As shown in Figure 6A-D, it was established that bone marrow-derived MSCs attributed to a significant decrease in ERK protein expression in co-cultured cells (K562+BMSCs) as compared to control groups (K562 alone), in which seven days after co-culture the percentage of ERK protein in control cells and co-cultured cells as scored 2.32% and 0.72%, respectively (Figure 6D and E) (****P*<0.001). Also, the expression of the ERK mRNA level was significantly decreased as compared with the control group by more than two-fold (Figure 6F) (***P*<0.01).

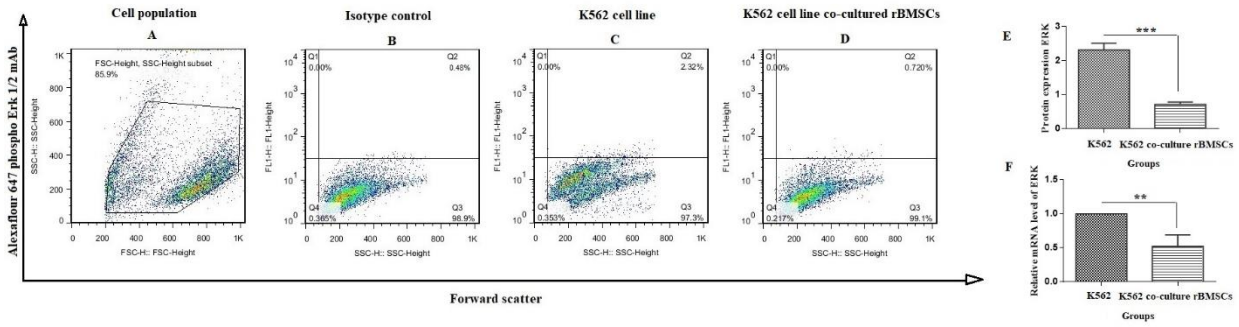

Figure 6. ERK protein expression of K562 cell line following co-culture with bone marrow-derived MSCs. The ERK protein expression was assessed by flow cytometry. In this figure, A is selected cell population, B is isotype control, C is K562 cells alone, and D is K562 cells co-cultured with bone marrow-derived MSCs. Values are mean ± SD from independent experiments; (***P*<0.01, ****P*<0.001).

Discussion

Chronic myeloid leukemia (CML) is a serious hematopoietic stem cell, and myeloproliferative disorder, characterized (in most patients) by a reciprocal t (9;22) (q34; q11) chromosomal translocation, which create the Philadelphia (Ph) chromosome and leads to the generates the BCR-ABL fusion gene (11). In other words, in the Philadelphia chromosome, the BCR gene on chromosome 22 is fused with the ABL gene on chromosome 9 and the resulting fusion gene BCR-ABL. The fusion gene (BCR-ABL) encodes a constitutively active cytoplasmic tyrosine kinase responsible for inducing and maintaining leukemic transformation (12). Thus, inhibition of tyrosine kinase by tyrosine kinase inhibitors (TKIs) has become the mainstay of chemotherapy for CML. A recent document suggests some additional changes may be required for the development of CML (13). In other words, BCR-ABL confers a proliferative advantage to hematopoietic cells by activating several signaling pathways such as mitogen-activated activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) (14-16). In addition to molecular changes, hematoproliferative disorder, like CML, gradually target the bone marrow niche, converting it to a neoplastic niche, which is a specific microenvironment for the proliferation of malignant cells. Bone marrow niche is a three-dimensional microenvironment structure consisting of hematopoietic and non-hematopoietic stem cells (HSCs) such as mesenchymal stem cells (MSCs), endothelial cells, adipocytes, fibroblasts, as well as stromal and non-stromal cells like osteoblasts and osteoclasts (15). The important duties of this niche are maintaining self-renewal, pluripotency, and proliferation of stem cells as well as the determination of the fate of these cells (16). Although the molecular mechanisms that underlie BCR-ABL-mediated transformation have been extensively studied, cellular signaling and interaction between bone marrow niche cell types and molecules that can disrupt the normal hematopoiesis are incompletely understood.

As earlier described in the introduction section of this manuscript, the subject of this study was to explore the influence of MSCs on proliferation and apoptosis of chronic myeloid leukemia (CML) cell line and the underlying cellular mechanisms. Based on the findings, it is suggested that the bone marrow-derived MSCs are capable of inhibiting the proliferation of K562 as a chronic myeloid leukemia cell line via the ERK pathway. In brief, Following the seven days of the co-culture of K562 leukemic cells with MSCs, the proliferation of leukemic cells was significantly inhibited (Figure 5). In addition, it seems that inhibiting proliferation was associated with a significant decrease in gene and protein expression of ERK (Figure 6). Previous studies have shown that MSCs derived from different sources such as umbilical cord blood via soluble factors and cytokines are capable of suppressing immune reactions both *in vitro* and *in vivo* (17). Among the mentioned factors, PGE2 (prostaglandin E2), TGF-β (transforming growth factor-β), IL-6 (interleukin-6), and IL-8 (interleukin-8) are possible candidates (18-20). The underlying mechanism involved in the interaction of MSCs with tumor cells are still just beginning to unravel, but at least it can be stated that some of the effects may be due to the same attributes. The extracellular signal-regulated kinase (ERK) pathway, also called the mitogen-activated protein kinase (MAPK) pathway, has a major role in the control of diverse cellular processes such as survival, growth, proliferation, motility, differentiation, *etc.* (21-23). Accordingly, aberrant activation of this signaling pathway would result in the induction of many types of tumors. Therefore,

interest in the ERK signaling pathway as attractive targets for cancer chemotherapy and cell transplantation has great attention in the last few years (22). Previous studies have been shown that MEK/ERK pathway plays an important role in the malignant transformation of leukemia, especially CML. It was shown that chemotherapeutic drugs cause to dramatically decrease the protein and mRNA expression of components of the ERK/MAPK signal pathway (23).

We hypothesized that the inhibition of K562 cell proliferation by bone marrow-derived MSCs were governed by the ERK signaling pathway. Zhang had also investigated the effect of human bone marrow-derived MSCs on chronic myeloid leukemia-mononuclear cell (CML-MNC) growth curve. It was found that INF-α was the most abundant protein expressed in co-culture media that have cause to significantly inhibited the proliferation of CML-MNC (3). In another study, Kogler *et al*., (2005) were reported that human umbilical cord blood-derived MSCs (hUCB-MSCs) could secrete cytokines and growth factors, as well as some pro-angiogenic factors, such as VEGF and angiopoietin-1 that interact in co-culture system (24). Unlike other studies, we did not investigate any cytokines and growth factors, which involved in cell-cell interaction (25). Interestingly, we are the first to report that bone marrow-derived MSCs induced inhibition of the leukemic tumor cell lines was ERK-dependent. In the experimental group (co-cultured K562 and bone marrow-derived MSCs), the expression of ERK protein were significantly decreased by more than 3.2-fold, respectively, as compared with the K562 cell line control.

In conclusion, the results of this study showed that bone marrow-derived MSCs cause significant induction on apoptosis of K562 cells as CML cell lines via the ERK pathway. Taken together, the data concluded that the co-culture of bone marrow-derived MSCs with CML cell line maybe secrete a substantial amount of cytokines and growth factors, thus to inhibit the proliferation and promote the apoptosis of CML cell lines via ERK signaling pathway. The identity of molecules and factors involved in the anti-proliferative effect of bone marrow-derived MSCs requires further investigation.

Acknowledgments

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