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Human embryonic stem cell-conditioned medium induces cell cycle arrest in HL60 cells by unknown soluble factor(s) in vitro

Fatemeh Adeli[1], Ameneh Alikarami[1], Shohreh Fakhari[2], Seyed Hadi Anjamrooz[1], Ali Jalili[2], Ayoob Rostamzadeh[3], Mohsen Mohammadi[4], Daem Roshani[5], Mohammad Jafar Rezaie[6]

[1] *Department of Anatomical Sciences, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[2] *Department of Immunology and Hematology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[3] *Department of Anatomy and Neuroscience, Shahrekord University of Medical Sciences, Shahrekord, Iran*
[4] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Lorestan University of Medical Sciences, Khorramabad, Iran*
[5] *Department of Biostatistics, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[6] *Department of Anatomy, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran*

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ABSTRACT

Introduction: Acute myeloid leukemia (AML) is a type of cancer that affects the blood and bone marrow, characterized by the rapid growth of abnormal white blood cells.

Aim: The aim of the present study was to evaluate the inhibitory effect of human embryonic stem cell – conditioned medium (hESC-CM) on the proliferation of human leukemic HL-60 cells.

Material and methods: We measured proliferation of myeloid leukemia cell by XTT assay. Also cell cycle and apoptosis was measured by flow cytometry after the cells stained.

Results and discussion: Our experiment indicated that the human embryonic stem cells conditioned medium has anti-proliferative effects on the progression of HL60 cell. HL60 cells were treated with conditioned medium; the results of the flow cytometry demonstrate that hESC-CM was capable of increasing apoptosis of HL60 cells and inhibit the cell cycle progression.

Conclusions: Our results show that Human embryonic stem cell conditioned medium contain factors that are able to inhibit the growth and inducing apoptosis of HL60 cells, which may represent a novel therapeutic approach for leukemia. However, further investigation is needed to identify the role of these factors.

Corresponding author: Mohammad Jafar Rezaie, Department of Anatomy, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran. Tel.: +988733664653, Fax: +988716664663.
E-mail address: rezaiemjafar@gmail.com.

1. INTRODUCTION

Acute myeloid leukemia (AML) is a type of malignancy hematopoietic cells, characterized by the rapid growth of abnormal white blood cells. AML is recognized as the ordinary form of acute leukemia among adults with higher outbreak in older patients. At present, the basic therapy for leukemia includes allogeneic hematopoietic stem cell transplantation, chemotherapy and immunotherapy.[1] However, its survival rates remain down because of inappropriate selection of recipients and transplant donors, the high relapse rate, inconvenience of chemotherapy and hard complications.[2] Hence, the exploration of new methods for AML therapy is an ever ecumenical interest. Although pluripotent stem cell is an attractive source for regenerative medicine, one of the major concerns of cell therapy is defective in vitro cell differentiation, which may form teratomas in recipient tissues.[3–5] Some studies demonstrated useful effects of paracrine factors secreted by stem cells in various diseases. The secreted factors contain secretome, exosome, etc. which can be detected in the medium. Thus, this medium is called supernatant or conditioned medium (CM). The use of CM has several excellences against the use of stem cells. For example, those produced, packaged, and transported for CM are more easily than stem cells and since the CM is acellular, there are no transplant rejection problems and high risk development of cancer.[6,7] Examinations in recent years have shown that embryonic stem cells (ESCs) make anti-tumor effects in both vitro and vivo.[8–12] Researches have revealed that the embryonic microenvironment plays a key role in blocking cancer cells proliferation.[8,13–15] We hypothesized that ESCs could secrete soluble factors that are capable of arresting cancer cells proliferation. Previous studies have demonstrated that human ES cells and embryonic zebrafish microenvironment could suppress the tumorigenicity of breast, prostate and metastatic melanoma cancer cells.[13,16] Giuffrida et al. assessed effect of hES cells conditioned medium on human epithelial, ovarian, prostate and breast cancer cells proliferation. In their study, cancer cells proliferation were reduced after exposure to hES cell-conditioned media.[17] Considering that there are slight reports in relation with antitumor effect of embryonic stem cells conditioned medium on cancer cells.

2. AIM

The aim of the study was to evaluate the inhibitory effect of human embryonic stem cell-conditioned medium (hESC-CM) on the proliferation of human leukemic HL-60 cells.

3. MATERIAL AND METHODS

3.1. Cells and reagents

The HL-60 cell obtained from the Iranian national cell bank (Pasteur Institute, Tehran, Iran). hESC and MEF cell was purchased from ROYAN Institute (Tehran, Iran). RPMI 1640 medium (Bio Idea Co., Tehran, Iran) that was used for positive control. DMEM/F12, knockout serum, non-essential amino acids (NEAAs), L-glutamine, penicillin/streptomycin (Pen/Strep), insulin, transferrin, selenium (ITS), collagenase IV solution, 0.05% Trypsin/EDTA solution and PBS were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Basic fibroblast growth factor (bFGF) was purchased from ROYAN Institute (Tehran, Iran). Furthermore, dimethyl sulphoxide (DMSO), rock inhibitor (Y 27-632) (1 μM), beta-mercaptoethanol (β-ME), $CaCl_2$, mitomycin C powder and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moreover, colorimetric cell viability kit with the tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was purchased from Promokine (Heidelberg, Germany). Propidium iodide (PI), RNase type A and annexin V-FITC were purchased from eBiosciense (San Diego, CA).

3.2. Cell culture

HL-60 cells were suspended cell types. HL-60 cell were incubated in RPMI 1640 media supplemented with 10% FBS and 100 l Penestrapat 37°C in an atmosphere of 5% CO_2.

3.3. Stem cell culture

This cell has been of the type adhesion. hESC cell were cultured in 90 mm dishes coated with MEF cell that had been mitotically inactivated with mitomycin. We used hESCs passages 21. These cell were maintained in DMEM-F12, supplemented with 20% Knockout serum, 0.1Mm non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/μg/mL Penstrep, 5 mg/μg/mL insulin, transferrin, selenium 12 ng/mL basic fibroblast growth factor and then placed the cells in the incubator at 37°C in an atmosphere of 5% CO_2.

3.4. Preparation condition medium

Control conditioned medium were collected by incubating 10 mL of hESC medium on dish covered with MEF cells for 24 h and 48 h. hESC conditioned medium was obtained by incubation of stem cells on a dish covered with MEF cells with 10 mL stem cell media for 24 h or 48 h. Stem cells should be examined for stem cell markers such as SSEA1, TRA-1-60, TRA-1-81 to undifferentiate during the time intended. Feeders were plated at 600 000 cells per plate and hESCs were plated at 1000 000 cells per plate. Then conditioned medium was harvested from the dishes with the initial centrifuge at 3000 rpm for 5 minutes, and then it was passed through a 0.2 μm syringe filter to remove cellular debris.

3.5. Cell proliferation assay

Cell growth and cell viability was assessed using the Colorimetric Cell Vability Kit (WST-8) based on the reduction of tetrazolium salt to soluble formazan compounds by mitochondrial enzymes. Cells (5000 cells/well) with growth medium were seeded in a 96-well plate and cells then in-

cubated with different conditioned media for each experiment. At the end of the incubation time-periods (24 h ,48 h) 10 l CCK8 solution was added to each well to be analyzed and cells were incubated for 4 h at 37°C. After an incubation period of 4 h the absorbance of the samples was measured using an ELISA reader at 450 nm.

3.6. Apoptosis assays

The cell apoptosis was assessed by dual staining with annexin V-FITC and PI. Cells were plated at a density of 200 000–250 000 cells/well in 2 mL of growth medium or CM in 6-well plates. HL-60 was treated with conditioned media: day 2 MEF-CM as control and day 2 hESC-CM for 48 h. HL-60 cells were centrifuged and washed in PBS then they were resuspended in 100 μL binding buffer and were stained with 5 μL of annexin V-FITC and 10 μL of PI for 15 minutes in the dark and analyzed by flow cytometry.

3.7. Cell cycle analysis

Cells were plated at a density of 106 cells in 6 mL of growth medium or CM in flask then cultured for 48 h. After harvesting, washing and resuspending in 1 mL PBS, then 2 mL FCM buffer and resuspended in 500λ sodium citrate solution containing 3 μL of RNase and 15 μL of PI. Cells were analyzed by flow cytometry.

3.8. Statistical analysis

Results are reported as mean ± standard error of the mean of three to six replicates per group. Data were analyzed using oneway ANOVA and Tukey test using SPSS (V.20, USA). A statistically significant difference was accepted when the *P* value was lower than 0.05.

4. RESULTS

4.1. Effects of hESC-CM on HL60 cell proliferation

The present study initially investigated anti-cancer effect of human embryonic stem cells condition medium on the proliferation of HL60 cells in vitro. We examined the population progression of HL60 cells exposed to 1 and 2 day hESC CM by the cell counting kit-8 assay or haemocytometer counting (Figure 1). hESC-CM exhibited significant decrease in cell proliferation rate in HL60 in the experimental group after 48 h. CM was collected from MEF alone as main control and RPMI medium + 10% serum and embryonic stem cell medium were used as additional controls. There was no difference between cells exposed with the control groups after 48 h (Figure 1a). Incubation of the HL60 cell with one day hESC-CM suppressed cell proliferation significantly, after 48 h of culture. Utilization of two day CM caused stronger suppression compared to one day CM (Figure 1b). Results revealed significant difference in percent cell viability between treatment and control groups in the direct cell counts (Figure 1c).

4.2. Effect of dilutions of hESC CM 48h on HL60 cancer cell proliferation

Furthermore, in the next experiments the anti-proliferative effect of CM on HL60 cell was evaluated by incubation with dilution of CM 1 : 1 with fresh medium. Cell propagation should not be suppressed if the effect was due to cell hunger. Data cell counting kit-8 assay indicated significant inhibitory effect of two day hESC-CM 50% on HL60 cell proliferation after 48 h incubation (Figure 2).

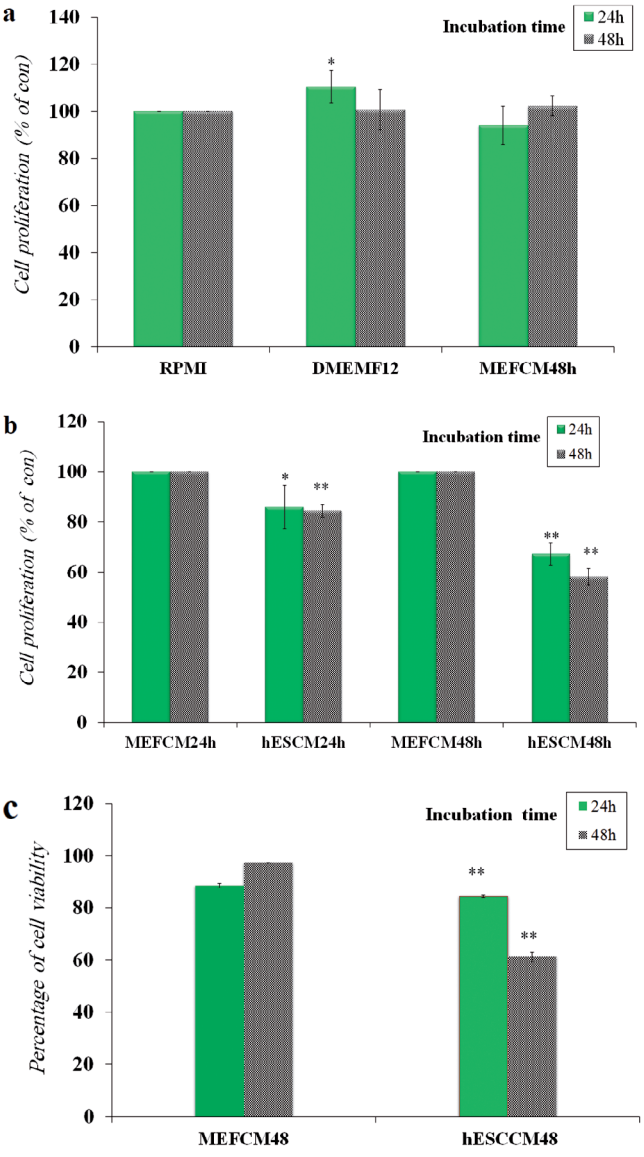

Figure 1. The effect of hESC-CM on the proliferation of HL60 cancer cells. HL60 cells were treated with MEF--CM, hESC-CM, RPMI and ES media and incubated for 24–48 h. (a) HL60 cell proliferation did not change significantly between the control groups. (b) The number of HL60 cell was less significantin the treated groups than the control groups.(c) Percent cell viability decreased significantly in the treatment group. Values are presented as the mean ± SD of six replicates (* *P* < 0.05 , ** *P* < 0.001 vs. control).

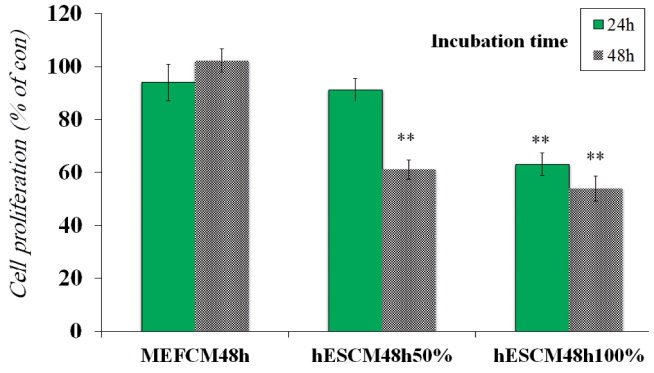

Figure 2. The effect of dilutions of 2 day hESC-CM on the proliferation of HL60 cancer cells. HL60 cells were exposed to hESC-CM 50% and 100% dilution for 48 h. Treatment of hESC-CM led to a significant reduction in the cell number versus control. Values are presented as the mean ± SD of six replicates ($P < 0.001$ vs. control).**

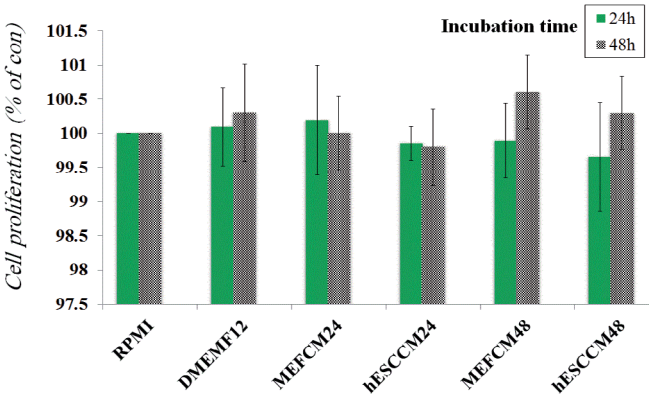

Figure 3. The effect of two day hESC-CM on the proliferation of MNC. MNC were treated with two day hESC--CM for 24h, 48 h. MNC cell proliferation did not change significantly after treatment with hESC-CM. Values are presented as the mean ± SD of six replicates.

4.3. hESC-CM did not affect the population proliferation of MNC

To demonstrate the therapeutic effect, we investigated ability of CM to suppress cell proliferation of normal mononuclear cell (MNC). Cell proliferation was evaluated by XTT assay. The results showed one, two days hESC-CM did not change significantly on MNC proliferation than the control groups. The findings confirmed that there was no inhibitory effect of CM on normal cells and there is safer clinical application (Figure 3).

4.4. Induction of apoptosis of cancer cells by hESC-CM

As mentioned above two day hESC-CM had the strongest effect on cancer cell growth, and then we investigated whether hESC-CM induced cell apoptosis. HL60 cell were treated with two day hESC-CM or MEF-CM for 48 h. Cells were stained with annexin and PI and were analyzed by flowcytometry. Cells that were negative for annexin and PI are live cells (Q1), cells positive for annexin and negative for PI, cells underwent early apoptosis (Q2), while cells positive for annexin and PI, cells underwent late apoptotic (Q3), (necrotic cells = Q4). We observed increased percentage of early and late apoptotic cells in the test group more than the control group (Figure 4).

4.5. hESC-CM treatment induces G1 phase cell cycle arrest

Evaluation of the cell cycle phase showed that HL60 cells treated with two day hESC-CM had a marked increase in the percentages of cells at the G1 phase compared to controls after 48 h with a significant reduction in both the G2 and S phase. We found that CM increased the percentage for cells of G1 from 25.37% in control to 36.04 % (for hESC CM) and

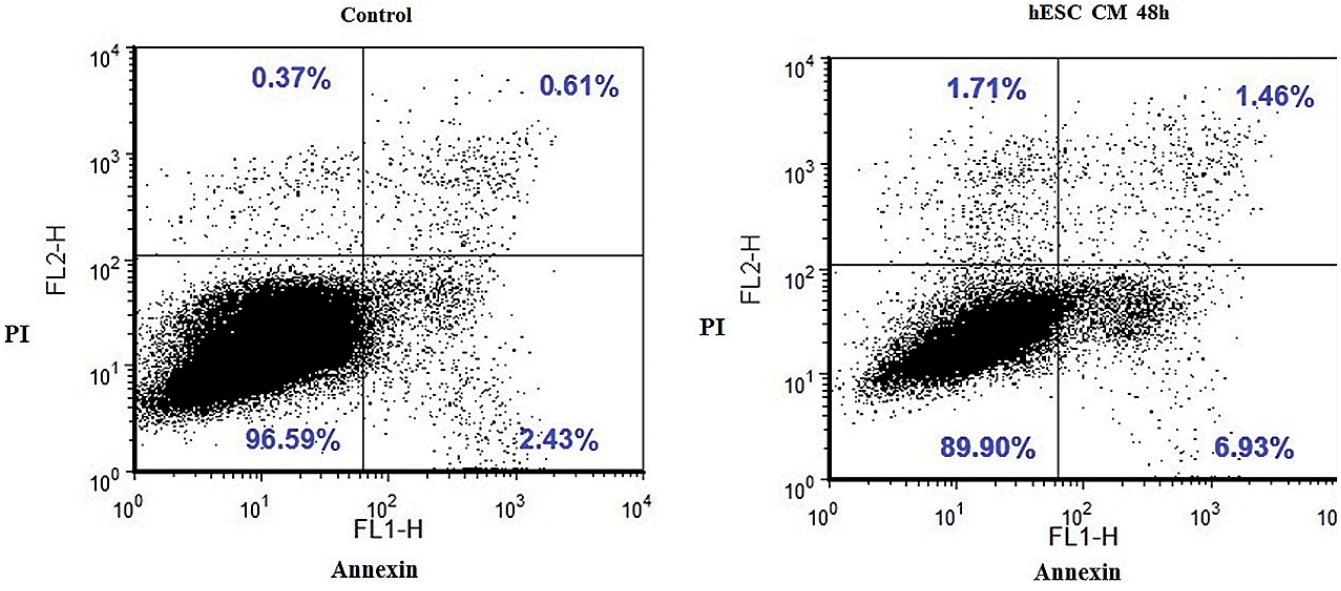

Figure 4. Annexin V-PI apoptosis assay. HL60 cell treated with two day hESC-CM and cells were stained and then analyzed by flow cytometry. HL60 cells were treated with two day hESC-CM significant induces apoptosis than the control group. Values are presented as the mean ± SD of three replicates ($P < 0.05$ vs. control).

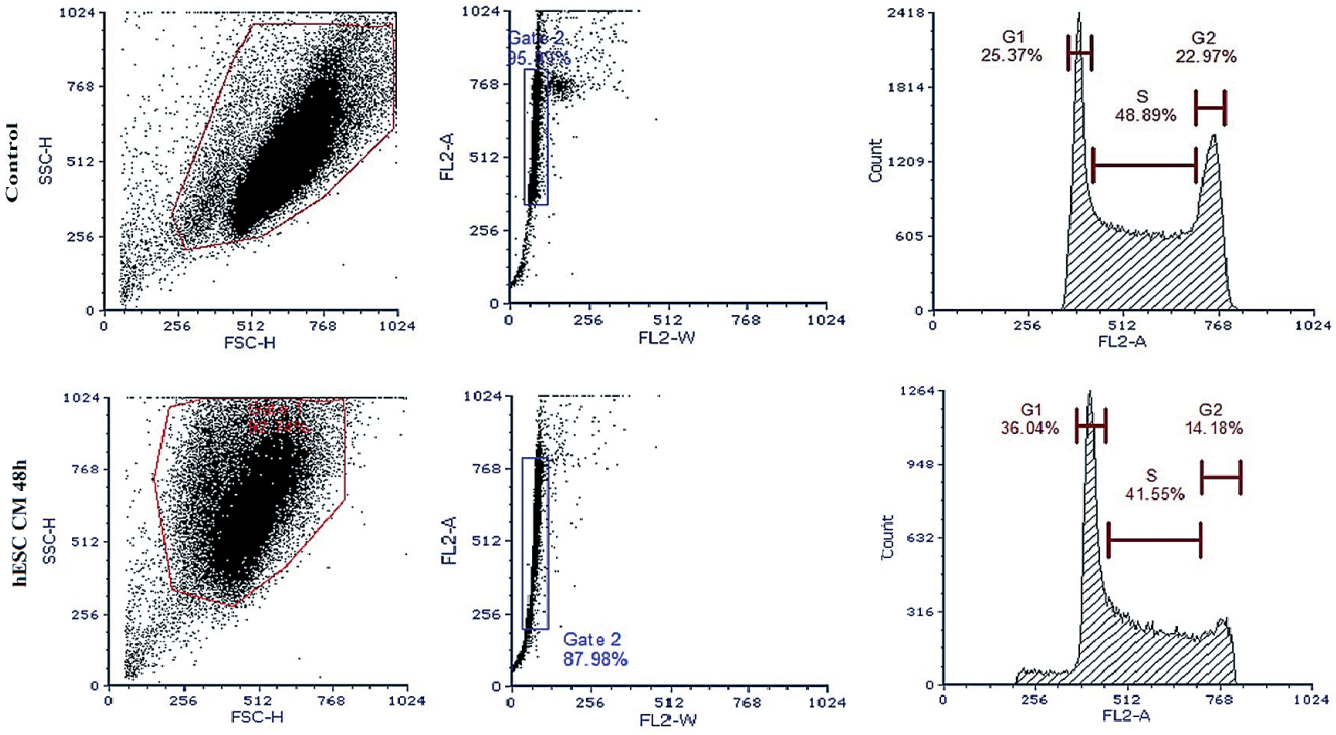

Figure 5. Cell cycle phase analysis of HL60 cells. HL60 cells incubated with two day hESC-CM for 48 h then analyzed by flow cytometry. Two day hESC-CM significant induces cell cycle arrest in HL60 cells at G1 phase with a concomitant decrease of cell number in S, G2 phase than the control group. Values are presented as the mean ± SD of three replicates ($P < 0.05$ vs. control).

decreased cell number in S phase from 48.89% to 41.55% and in G2 from 22.97% to 14.18%, indicating that hESC-CM may induce G1 growth arrest in HL60 cells (Figure 5).

5. DISCUSSION

Cancer is an aberration of the normal cell expansion and can be regulated by signals present in the embryonic microenvironment. Previous studies demonstrated that the transfusion of embryonic carcinoma cells into a blastocyst is able to adjust cancer cell. The fate of the cancer cells inject into embryo are induced to die and to arrest growth or they are towards to differentiating pathways.[18] The potential use of hESCs as a cell appealing source for stem cell therapy is rapidly developing based on continuous proliferation of their capacity and can also be induced to differentiate.[5,16,19] ESCs could produce clear anticancer effects. ESCs could secrete paracrine and autocrine factors that are capable of regulating cellular behavior such as slow cancer cells proliferation.[10,20,21] The aim of using secretions to stem cells is to discover the factors within it and inhibit tumor cell feature and/or reprogrammed to a nonaggressive phenotype.[14,15,22] It has recently been observed that there are factors in a hESC-conditioned matrix that potentially makes the reprogramming of cancer cells.[21] Studies reveal that ESC-CM may be a proper alternative to stem cell transplantation whereas it does not have the potential risk of developing cancer, but the concentration of soluble factors in CM and components of the culture medium are improper for therapeutic use.[6] In the last decade, a novel research has been initiated with a focus on the anticancer effects of stem cells condition medium on tumor growth.[23] The exact mechanism of how SC-CM or their extracts inhibit the progression of malignant cells is not clear but several studies demonstrated that the anticancer effects can be mediated by a variety of mechanisms rather than a single mechanism different for non-solid and solid cancers.[24] These mechanisms are perhaps to be mediated secrete factors that inhibit the proliferation of the cancer cells.[24] Studies are in development to recognize the unique agents present in the CM that are responsible for the anticancer effects on types of cancers.[16,24,25] Stem cells inhibit tumor growth via three pathways: the reduction of proliferation, induction of apoptosis and the excitation of cell cycle arrest.[23] hESC-CM have been shown to demonstrate anti-proliferation properties. The present study confirmed that CM from hESC share anticancer characteristics in HL60 cells and significantly inhibited the proliferation of HL60 cells. Kim clarified the use of a mES-conditioned matrigel to prevent growth of human melanoma cells. They detected an gremlin in the ES cell microenvironment responsible for inhibiting cancer cell proliferation.[20] Giuffrida et al. assessed the effect of hESC-CM on human epithelial cancer cell proliferation. They studied cancer cell proliferation reduction then exposure to hESC-CM as contrasted to the control.[16] In this study it was shown that induction of apoptosis by CM is one of the mechanisms that reduce proliferation of HL60 cells. Studies have reported that conditioned medium derived from human Wharton's jelly stem cells increase autophagy and apoptosis

of cancer cells.[26] Postovit demonstrates that presentation of cancer cells to the ES cell-conditioned matrigel could prevent Nodal gene expression in cancer cells leading to apoptosis.[27] It is recently assumed that in CM such as chemotherapy drugs releasing hydrogen peroxide (H_2O_2), the exposure of malignant cells to high H_2O_2 levels after 48 h incubation causes their death.[24] Another anti-proliferation mechanism of the CM is cell-cycle arrest. Regulation of the cell cycle is necessary to retain harmony between differentiation and proliferation in cells. Checkpoints are regulating cell cycle arrest or progression. Cancer occurs when these checkpoints flunk and damage cells survive. Blocking cancer cells in the G1 phase or returning the cell cycle checkpoints will arrest the growth of the cancer.[28] In the present study, we found that there was an enhancement percentage of the HL60 cancer cell in the G1 phase when the cells were treated with two day hESC-CM. This enhancement was accompanied whit reduction of cells in the S and G2 phases compared to cells cultured in control group. Hence we hypothesized that CM factors arrest the propagation of cancer cells through the cell cycle. Results demonstrated that the observed repressive effects were due to inhibitory factors secreted by hESCs. Lin demonstrated that conditioned medium drived human umbilical cord Wharton's jelly stem cells (hWJSC-CM) possess tumoricidal effects and significant increases in the percentages of lymphoma cells at the G1 phase and increased apoptosis in cell compared to controls after 48 h of exposure to the hWJSC-CM.[24] Cavallari demonstrated that the embryonic soluble factors are able to decrease cancer cell proliferation by arresting their cell cycle.[29] The data showed that human fetal MSC-CM can inhibit HCC cell proliferation and induction of cell cycle arrest.[16] The results of this study confirmed that both concentrations of conditioned medium produced significant reductions of cell proliferation and similar to other studies demonstrated that evacuation of nutrients in hESC-CM do not cause the inhibition of cancer cell proliferation.[16] Also, the findings indicated that medium condition derived from adipose tissue significantly reduced the propagation of B16 melanoma cells in a time dependent manner.[30] In addition to these in vitro evidences, our results showed that hESC-CM did not induce decrease of proliferation and apoptosis of normal MNC, indicating that hESC-CM may be promising candidates for future therapeutic strategies.

6. CONCLUSIONS

In conclusion, our finding show that hESC-CM contain factors that are able to inhibit the growth and induce of apoptosis of HL60 cells, which may represent a novel therapeutic approach for leukemia. However, further investigation is needed to identify the role of these factors.

Conflict of interest

None declared.

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