Effect of Extracellular Vesicles Derived From Peripheral Blood Mononuclear Cells on K562 Leukemia Cell Line

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Received: 21 Sep. 2022; Accepted: 14 Jul. 2023

Abstract- Recently, extracellular vesicles (EVs) are generating considerable interest in terms of their ability. EVs defined themselves as a route for intercellular communication between the origin cells and the recipient ones by transferring information. This paper investigates the influence of peripheral blood mononuclear cells derived EVs on proliferation and apoptosis of the chronic myelogenous leukemia cell line k562. Trypan blue staining was used to detect cell viability subsequently, metabolic activity was assessed by the MTT assay. Cell apoptosis and cell cycle progression were evaluated using flowcytometric assay after treatment of k562 cell line with MNC derived EVs .Our results showed that MNC-EVs have no inhibitory effect on k562 cell growth and proliferation. Our data did not reveal any significant variations in the case of enhancing k562 cell line growth following treatment with MNC derived EVs. It seems tumour-derived extracellular vesicles support tumour cells growth by communicating with each other through their extracellular vesicles.

Acta Med Iran 2023;61(8):449-454.

Keywords: Chronic myelogeneous leukaemia; k562 cell; Extracellular vesicles; Peripheral blood mononuclear cells

Introduction

Chronic myelogeneous leukaemia (CML) is a myeloproliferative neoplasm, caused by a transformation in the normal haematopoietic progenitors. In most of the patients, CML is characterized by the presence of Fusion oncogene BCR-ABL1 arising upon Philadelphia chromosome. Nowadays these patients can be treated with tyrosine kinase inhibitors as well as BMT. TKI drugs are highly effective in the chronic phase on the other hand, TKI do not demonstrate the same efficacy in the treatment of patients in accelerated phase or blast crisis. Moreover, drug resistance may also exist in patients (1).

Recently, attention has been focussed on identifying the extracellular vesicles (EVs) released from cells and their activities. EVs are a heterogeneous population, surrounded by a lipid bilayer. These particles are released from variety of cells, including epithelial, fibroblast, haematopoietic, immune, placenta and tumor tumour cells. They encompass a heterogeneous population including exosomes and microvesicles. Exosomes and MVs are nano particles with a diameter of <100 nm and 100-1000 nm respectively (2).

In addition to constitutive release of EVs by cells, their secretion is increased through activation and apoptosis. The membrane of the EVs consists of lipids, proteins and also some typical surface molecules of the cell origin. Not only the membrane molecular of EVs but also their composition depends on their cellular origin. EVs can target cells, in the relative vicinity or in the distant environment. EVs are able to trigger signalling cascades, due to the fact that these particles carry different biological materials including lipids, proteins, mRNAs and microRNAs (3,4). They can also change phenotypes (5).

Interestingly not only EVs can change cellular phenotype, but also they can suppress tumor cell growth (6,7). EVs important role in exchanging information

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relied on their ability to deliver bioactive factors and genetic materials from the origin cell to the target cell. EVs encompass several biological activities of the origin cell as well as carrying information that can reprogram target cells (8).

As normal cells have molecules and proteins to control their characteristics, growth and proliferation, this research was carried out to determine how MNC derived EVs can change k562 cell line features including apoptosis and proliferation.

Materials and Methods

The current experimental study was conducted in Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran in 2016. All the equipment was calibrated.

Human peripheral blood mononuclear cell isolation

Initially, Mononuclear cells were collected from a normal heparinized blood sample using ficoll-hypaque gradient centrifugation. Afterwards the collected cells were washed twice using phosphate buffered saline. Then the pellet was cultured in a RPMI 1640) (ATOCEL, Austria) containing 0.5% BSA (Roche, Switzerland) and 1% penicillin-streptomycin (Invitrogen, USA). Finally, the mixture was placed in a T75 flask and maintained at 37° C incubator with humidified 5% CO2 for two days.

EVs isolation

To isolate EVs, incubated MNCs in RPMI1640 were then centrifuged twice at 2500g for half an hour at 4° C. Resulted supernatant was centrifuged at 20,000 g for an hour at 4° C. The pellet was resuspended in FBS-free RPMI1640 and stored at -80° C.

Characterisation of EVs

EVs became characterized using Bradford assay, a method quantifying the protein content of these particles via NanoDrop spectrophotometer (WPA, UK). In brief, 200 µl of Bradford solution was diluted with different concentrations of bovine serum albumin (125, 250, 500, 1000 and 2000 µg/ml). Then the OD was determined at 595 nm and the standard curve was plotted using the results. Afterwards, 10 µl of obtained MNC derived EVs was added to 200 µl of Bradford solution (Sigma-Aldrich, USA. The OD was assessed at 595 nm. Finally, the protein content of EVs was determined using the standard curve.

Furthermore, to evaluate MNC derived EVs size distribution, dynamic light scattering (DLS) by a Malvern Master sizer 5111 laser diffraction system was used.

Cell culture

K562 as human chronic myeloid leukaemia cell line was obtained from Iranian Cell Bank of Pasteur Institute. A growth medium containing Roswell Park Memorial Institute 1640 medium (RPMI1640) supplemented with 10% fetal bovine serum (Gibco, USA) and 1%penicillin-streptomycin solution was used to culture the cells. The cultured cells were incubated in 37° C with 5% CO2.

Viability assay

In order to evaluate possible cytotoxic effects of the collected EVs, k562 cells were exposed to MNC derived EVs with different concentrations (0, 50, 100, 200 and 500 µg/mL) for 10 days. To evaluate cell viability rate, trypan blue (Sigma-Aldrich, USA) exclusion assay was performed. Cells were stained with 0.4% dye (in equal volume with cells). Viable cells were counted using Neubauer chamber and invert microscope.

Metabolic activity

To measure metabolic activity, MTT (Sigma-Aldrich, USA) assay was performed. Briefly, K562 cells were plated into three, 96 well plates at the density of $5x10^3$ cells/well. Cells were then exposed to five different concentration of MNC derived EVs (0, 50, 100, 200 and 500 µg/mL) for an interval of three days. Next, 100 µl of MTT (Sigma-Aldrich) Solution was added to each well and incubated for 3 to 4 hours at 37° C. After incubation period, DMSO was added. The mixture was left at RT in the dark for 15 min. Finally, to assess the absorbance for each well at 570 nm, an ELISA reader was used. By dividing the treated cells OD to the control cells OD, the metabolic activity rate of the treated cells was evaluated. Control group was defined as K562 cells culturing in an EV free RPMI 1640 medium.

Annexin/PI staining

To detect the apoptosis, following the manufacturer's protocol k562 cells exposed to MNC-EVs (0,500 µg/mL). Cells (100 cells/well) were stained with annexin V/PI (FITC annexin V apoptosis detection kit, BD bioscience). Firstly, in a RPMI1640 medium, k562 cells were exposed to MNC derived EVs for three days. After collecting cells, cold phosphate buffered saline solution was used to rinse the cell pellet for two times. The resulting pellet was then maintained in a binding buffer(1X). Subsequently, Cells were added to binding buffer (100 µL) containing FITC conjugated annexin V (5 µL) and PI (5 µL). The solution was incubated in the room temperature for an

interval of 15 minutes. Rinsed cells using binding buffer (1X), were finally analysed using Partec CY-Flow Space.

Cell cycle analysis

To analyze cell cycle status, PI staining was used to detect the DNA content of K562 cells. In brief, K562 cells at 100 cells/well were seeded in a six well plate and incubated with 500 µg/mL of MNC derived EVs for three days. At this point, Phosphate buffered saline solution was used to rinse the cells for two times. propidium iodide (eBioscience, USA) staining solution (500 µl) and RNase A (50 µl) were then added to the obtained pellet. The mixture was then kept at 37° C incubator for 15 minutes. Finally, Partec CY-Flow Space software was used to evaluate the data.

Statistical analysis

Statistical analysis was performed using the ShapiroWilk, parametric (t-test and ANOVA), and non-parametric (median) tests (P<0.05 were considered significant).

Results

Characterisation of EVs

To detect the size of EVs isolated from MNC, size distribution analysis was performed using zetasizer. Most of EVs were in the size of 324nm as shown in Figure 1.

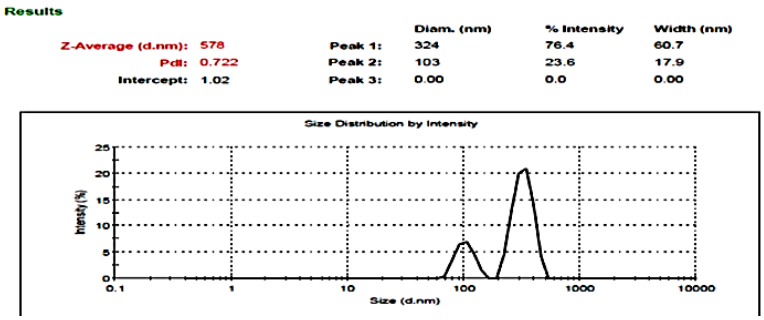

Figure 1. Determination of MNC derived EVs' size. DLS was used to evaluate EVs' size. The curves showed most of the obtained EVs were in the size of 324 nm

Cell viability

Additionally, to analyze the MNC derived EVs effect on cell viability of K562 cell line in vitro, trypan blue exclusion dye was used. Figure 2 indicates the viability of K562 cells after the exposure to different concentrations of MNC derived EVs. Overall, our results showed MNC-EVs didn't induce any alterations in the cell line viability (P>0.05).

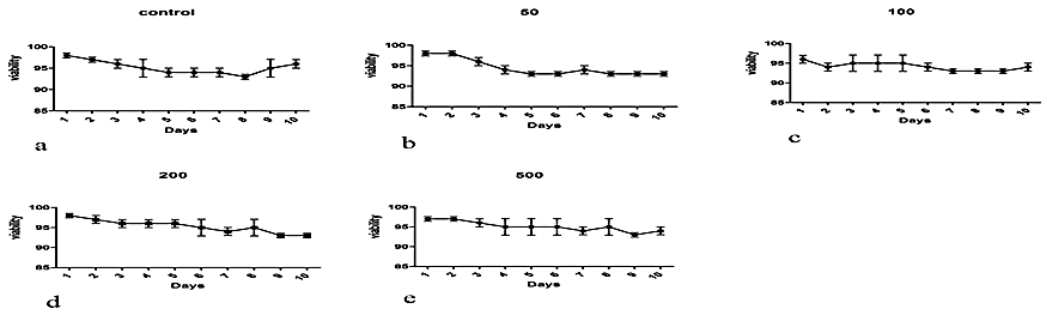

Figure 2. Effect of MNC derived EVs on k562 viability. K562 cells were exposed to MNC derived EVs in concentration of 0, 50, 100, 200 and 500 µg/ml for an interval of ten days. Cell viability was assessed using trypan blue staining. There was no significant modifications in cell viability between the treated and untreated group

Metabolic activity

MTT results exhibited no significant alterations in metabolic activity of k562 cells following treatment with EVs derived from MNCs in different concentrations (Figure 3).

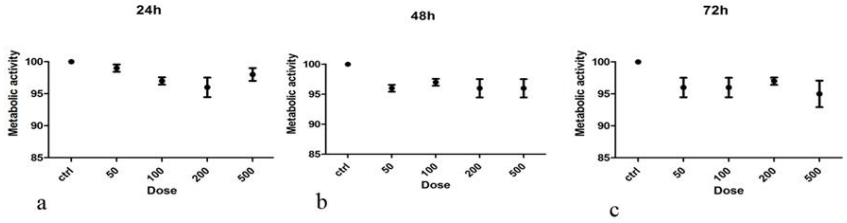

Figure 3. MNC derived EVs didn't cause any change in metabolic activity of k562 cells in vitro. After three days incubation with collected EVs in concentration of 0,50,100,200 and 500 µg no significant modification were induced in metabolic activity of k562 cells comparing to the control group. K562 cells in EV free RPMI1640 were used as control

Annexin/PI staining

To evaluate cell apoptosis, annexin V/PI staining was performed. Our data didn't show any significant changes in k562 cells' apoptosis, after three days of treatment with EVs derived from MNCs (500 µg/mL) in comparison to the control group (Figure 4).

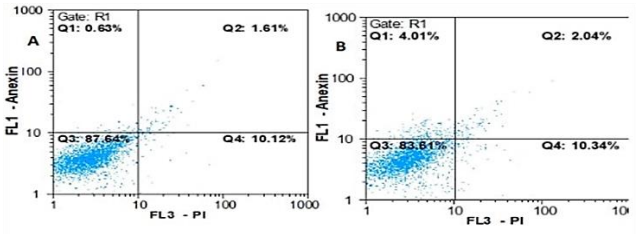

Figure 4. MNC derived EVs apoptotic rate was evaluated via annexin V/PI assay (A) k562 cells were incubated in RPMI 1640 without EVs (control group); (B) k562 cells were exposed to EVs derived from MNCs for three days (experimental group). The comparison between the two groups were not significantly different in case of apoptosis (P>0.05)

Cell cycle analysis

PI staining and flowcytometry analysis were conducted to detect the effect of MNC-EVs on cell cycle progression. No significant difference was highlighted between k562 cells treated with MNC-EVs and control (Figure 5).

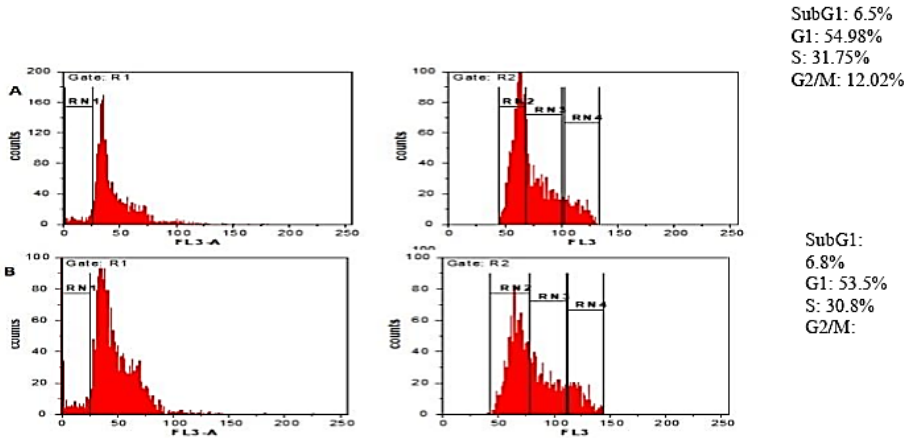

Figure 5. Cell cycle progression in EV exposed k562 cells and control group. (A)Cell cycle assessment was performed following the three days treatment of k562 cells with EVs obtained from MNCs (500 µg/ml). The result then compared with the untreated control group (B). MNC-EVs were not able to significantly induce any progression in different phases of the cell cycle

Discussion

Extracellular vesicles representing a powerful mediator for intercellular communication and may rearrange the recipient cells via proteins and nucleic acids transfer. They can transfer membrane components such as nucleic acids and proteins between different cells in both paracrine and endocrine manner (9,10).

EVs act as vectors for intracellular exchange of biological information. Emerging evidence suggests that EVs derived from different normal cell types such as prostate (7), and hepatocyte (11), affect the growth of tumour cells. The aforementioned studies showed that leukocyte-derived extracellular vesicles act in several biological process such as tumour metastasis, cell survival, fibrinolysis matrix remodelling and so on (12). In addition, several studies have shown the anti-tumour effect of EVs derived from different cells (7,13). However, other research has reported a promoting effect on tumour growth (14,15).

Controversy arises from the fact that extracellular vesicles can transfer different biological molecules such as tumour-promoting molecules and tumour suppressors (16).

In the current investigation, isolation of mononuclear cells' EVs from healthy individuals was conducted. More importantly, we Evaluated EVs potential influence on growth, apoptosis and cell cycle of k562 CML cell line. Cell viability and metabolic activity were evaluated with trypan blue exclusion dye and MTT assay, respectively. As reported earlier in the results, there were not any significant modifications in terms of cell viability and metabolic activity between treated and control group. We further analyzed apoptosis and cell cycle via flow cytometry. Our observations revealed that MNC-EVs have no significant effect on k562 cell apoptosis and cell cycle phases.

Tumour cells shed extracellular vesicles into the extracellular microenvironment. Released extracellular vesicles change the extracellular microenvironment condition, they can affect other cells. As crosstalk mediators, EVs carry the proteins, nucleic acids and also oncogenic products to the neighbouring cells. Tumour-derived extracellular vesicles promote tumours; tumour cells appear to maintain viability via shedding EVs which can affect other tumour cells (17,18).

In conclusion, the effect of MNC-EVS on k562 cells was evaluated in this study. Our observations demonstrated that MNC-EVs have no effect on viability of k562 cells. Progressive or inhibitory effect of extracellular vesicles on tumour cells depends on EVs' content as they can be tumour-promoting molecules and tumour suppressors. Furthermore, tumour cells secrete extracellular vesicles containing tumour-promoting molecules which can progress to tumours.

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