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Leukemia- Derived Exosomes Induce Migration and Tumor Initiating in Astrocytes from Human Brain Tissue

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Abstract

Background: In children with lymphoblastic leukemia (ALL), cancerous metastasis and involvement of central nervous system (CNS) is common. Despite such commonality, there is insufficient information on the metastasis process, although the role of exosomes as extracellular vesicles on cancer growth and metastasis has been the focus of many studies. Thus, due to the significant presence of astrocytes in the central nervous system, we decided to explore the effect of ALL-derived exosomes on human astrocytes.

Methods: In this study, the exosomes, extracted from the surface fluid of Nalm6 cells culture medium after different stages of centrifugation, were identified. Trypan blue staining and scratch methods were used to evaluate the effect of exosomes on the proliferation and invasion of astrocytes. Real-time PCR also analyzed the mRNA expression of cancer-related genes.

Results: Astrocytes were treated with concentrations of ALL- derived exosomes (5, 10, 30, and 50 µg/ml) in the Trypan blue test. According to the results, 50 µg/ml exosomes led to a significant ($P<0.05$) differences in the increase of proliferation in the experiment as compared to the control. It was considered as the lowest concentration in other tests. In addition, the results of Scratch test revealed a significant ($P<0.01$) increase in migration of astrocytes after 24 h. Finally, a significant rise in the expression of MMP9 and Cox2 genes and a considerable decline in P53 in astrocytes exhibited their stimulated and anti-apoptotic phenotype under the impact of ALL- derived exosomes.

Conclusion: Exosomes can promote tumor behavior by increasing the expression of tumor-related genes in astrocytes. Therefore, it is necessary to understand the complex factors governing metastasis of leukemia in the CNS for diagnostic and clinical applications.

Key Words: Astrocyte, Exosome, Leukemia, Tumorigenesis.

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1- INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide. One of the main challenges to clinical diagnosis and treatment of ALL is that it affects central nervous system (CNS)(1), so that about 15-20% of children with ALL experience a recurrence of the disease. Accordingly, CNS is one of the sites frequently affected by ALL(2). Research shows that 1 in 5 children relapse, among which 3 to 5% of cases are detected in the early diagnosis and 30 to 40% during the disease recurrence(1). Patients with brain metastases go through severe complications and fatal prognosis. Our knowledge of how cancer cells metastasize to the brain tissue is limited, so finding answers to questions in this field could promise more targeted and specific treatments in future (3).

Astrocytes are one of the most abundant cells in the CNS, which play a major role in metastatic cascade of the brain. Also, in malignancies, they transform into astrocytoma, which is one of the most common forms of glioma(4). For this reason, active astrocytes have always been of great interest to researchers in relation to nerve damages and tumor progression(5). In this study, we also targeted human astrocytes.

Another point is that cancer cells, compared to normal cells, secrete a greater deal of extracellular vesicles, including exosomes. Recently, exosomes with a dimeter of 40 - 150 nm and a double-layer phospholipid membrane have received growing attention in many diagnostic and therapeutic studies(6). Therefore, in the present study, we looked into the effects of exosomes derived from leukemia cells on the biology of CNS astrocytes and evaluated their role in metastasis.

Regarding the metastasis tendency of some cancers in organs such as the brain and their tumorigenesis in brain tissues, a number of specific genes have been identified(4). One of the factors considered by researchers in relation to cancer and tumorigenesis is the presence of enzymes that destroy extracellular matrix including matrix metalloproteinase (MMPs) and their role in attack (7). Several studies have reported high levels of MMPs, including MMP-9, in astrocytic tumors (7). Glioblastoma is one of the most fatal and aggressive forms of astrocytic tumors with poor prognosis, where a reduction in the tumor suppressor (p53 gene) results in the proliferation and an uncontrolled cell cycle (8). Previous research has shown that p53 expression is inhibited in astrocytes as a result of cancer malignancy (9). Previous studies have also shown that COX-2 gene expression improves in glioma tissue (10). Therefore, in this study, we evaluated the expression of target genes associated with cancer, such as MMP9, P53 and Cox2 in astrocytes treated with exosomes derived from leukemia.

2- MATERIALS AND METHODS

2-1. Cell culture, isolation and characterization of exosomes

When Nalm6 (Pasteur Institute, Iran) cell density reached 80% in the RPMI 1640 (Bio-Idea, Iran) culture medium, the cell supernatant was centrifuged in accordance with predefined protocols over different periods after 48 h culturing (FBS-free), and the resulting pellet exosomes were dissolved in PBS. The protein content was measured by BCA Protein Assay Kit (Kiazist Life Sciences, Iran), the protein size by dynamic light scattering (DLS), and the protein morphology by field emission scanning electron microscopy (FESEM) and atomic force microscopy AFM (11).

2-2. Isolation, culture and analysis of astrocytes

Astrocytes were isolated from other primary cells of human fetal brain tissue (obtained from *Ommol Banin Hospital,*

Mashhad, Iran) using trypsinage method and being cultured in the complete cell culture medium of DMEMF12 (Bio-Idea, Iran) (12). Astrocytes were also confirmed by immunocytochemistry (ICC) using the glial fibrillary acidic protein (GFAP)-specific antibody based on predefined protocols (13).

2-3. Astrocytic survival analysis (Trypan blue staining)

For cell viability assay, about 3×10^3 cells were cultured in 12-well plates, and after 24 h, the cells were treated with various concentrations of exosomes (5, 10, 30 and 50 µg / ml). The control group did not receive any treatment and another sham group was treated with PBS. Finally, after 48 hours, the cells were Trypsinized and centrifuged; 10 µg/ml of the obtained cell sediment was pipetting with 0.4% Tripan Blue dye and cell counting was performed using a hemocytometer with an inverted microscope. The blue-stained and colorless cells were considered as dead and living, respectively. Then, using the following formula, the percentage of living cells in each concentration was calculated (14).

Biological capacity of cells = (total number of cells / numbers of living cells) ×100

2-4. Tumor cell invasion analysis (Scratch)

For cell migration assay, approximately 1×10^4 cells were cultured in 6-well plates and treated with exosomes (50 µg /ml) after 24 h. In the next 24 h, a scratch was made at the bottom of the plate by a micropipette tip. Finally, the cells were photographed with an inverted microscope after 24 h and analyzed by Image J software (15).

2-5. Quantitative real-time PCR

To evaluate the expression of MMP9, P53 and Cox2 genes, 1×10^6 cells were cultured in each 6-well plate vial, and then treated with exosomes (50 µg /ml) after 24 h. Afterwards, cell RNA was extracted based on RNeasy Mini Kit protocols (Parstoos, Iran) and the total RNA concentration was determined by NanoDrap method (Epoch, BioTek, Winooski, VT, United States). In the next step, they were reverse-transcribed to cDNA using the Quantitect Reverse Transcription Kit (Parstoos, Iran) and thermocycler (Perkin Elmer Applied Biosystems, Boston, MA). The real-time PCR was performed using SYBR Green (Ampliqon) and specific primers as well as βActin as the internal control by considering 3 reiterations for each gene in a Bio Rad (Bio Rad, US). Finally, DDCt method was utilized to determine the relative changes in gene expression between control and treated samples and the final values were obtained by calculating the fold change.

2-6. Statistical analysis

The data analysis was conducted using SPSS 20 software and one-way ANOVA as well as Tukey test. The mean differences were considered significant at the level of $P < 0.05$

This research is a part of the PhD dissertation that was approved on 11/24/1397 in the ethics committee of the Islamic Azad University of Mashhad with the code of ethics IR.IAU.MSHD.REC.1397.092.

3- RESULTS

3-1. DLS, AFM and FESEM of ALL-derived exosomes analysis

The average size of exosome nanoparticles was 43 nm and they had a spherical morphology (**Fig. 1**).

3-2. GFAP positive cells

According to the results of immunocytochemistry, more than 90% of GFAP cells were positive (fig 2).

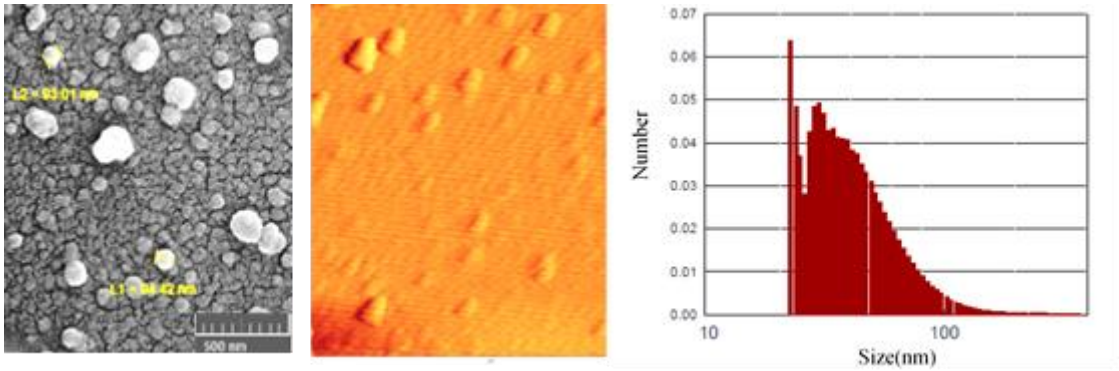

Fig. 1: Morphology and size distribution of exosomes (a and b) detected by FESEM and AFM, (c) the mean size is indicated by DLS (mean ± SD).

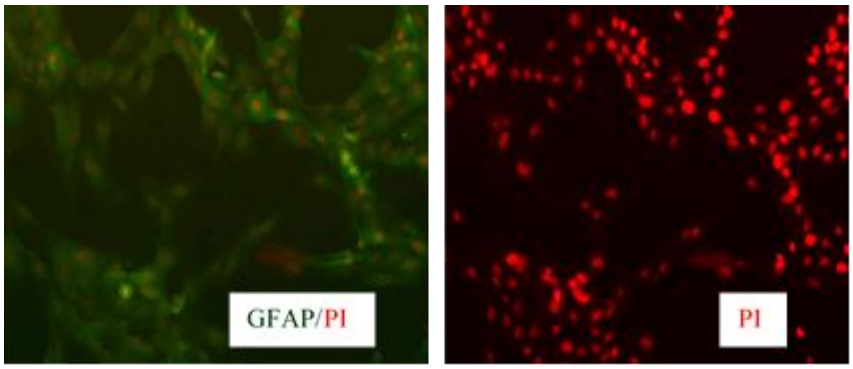

Fig. 2: Confirming astrocytes by ICC method; they were confirmed by the Olympus fluorescent microscope ×100).

3-3. ALL- derived exosomes induced survival ability to support enhancing tumorigenic behavior in human astrocytes

According to the results of the Trypan blue exclusion test, astrocytes treated with exosomes (50µg/ml) were significantly different (P <0.05) from those in the control group after 48 h. However, at lower concentrations of exosomes (30 and 10, 5 µg/ml), no significant increase was observed in the proliferation of these cells (the results are based on 3 reiterations). Therefore, the minimum significant concentration (50µg/ml) was considered for subsequent tests (fig 3).

3-4. ALL-derived exosomes induced migration in astrocytes culture

The results of the Scratch test unveiled a significant difference in the migration of astrocytes treated with exosomes (50 µg/ml) after 24 h as compared to the control group (P< 0.05) (**Fig. 4**).

3-5. ALL-derived exosomes induce MMP9 and Cox2 expression and decrease P53 of human astrocytes

According to the results of the real-time PCR test, the expression of P53 gene in astrocytes treated with exosomes (50 µg /ml) decreased significantly when compared to the control group (P <0.05*). The expression of MMP9 and Cox2 also rose significantly in comparison to the control group (**Fig. 5**).

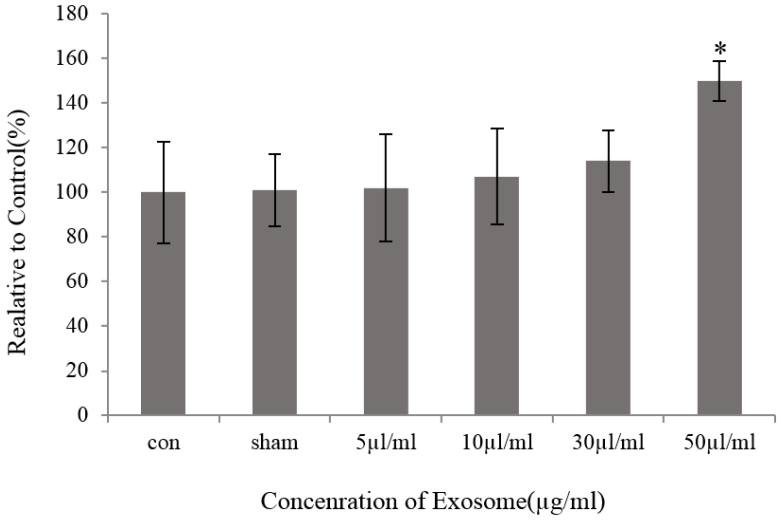

Fig. 3: Astrocytes viability by Trypan blue exclusion test after 48-h treatment with exosomes (5, 10, 30 and 50 µg/ml) (P <0.05*).

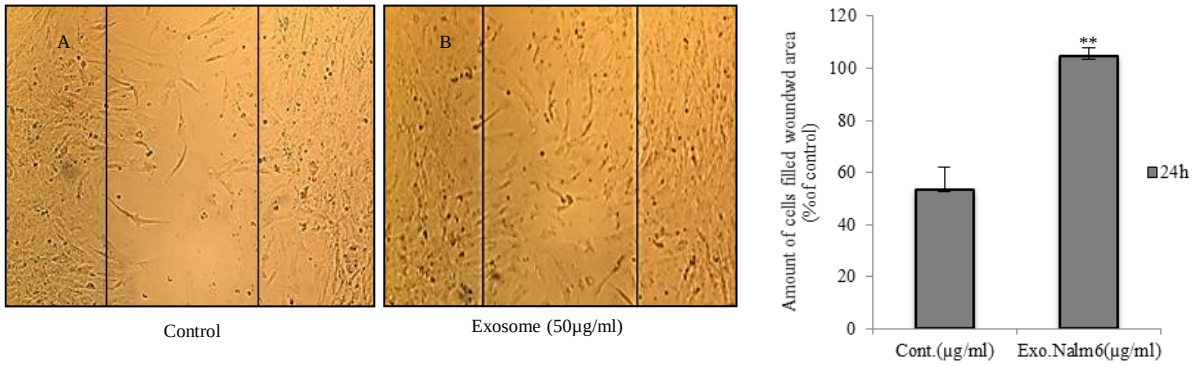

Fig. 4: Exosomes increase migration in astrocytes in comparison to the control. The image on the right displays the Excel diagram of migration (P<0.05*) and the image on the left shows cells in the control and exosome (50 µg/ml) treated group by the inverted microscope (×100).

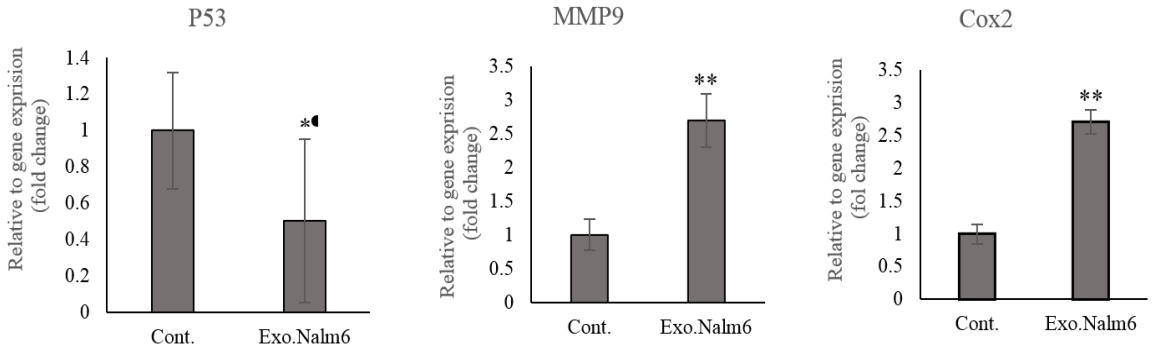

Fig. 5: Analysis of gene expression in real-time PCR showed that astrocytes increased significantly in the expression of MMP9 and Cox2 genes due to the effect of exosomes (50 µg/ml). However, the expression of P53 dwindled significantly as compared to the control group in astrocytes treated with exosomes (* P <0.05, and ** P <0.01 vs. control group).

4- DISCUSSION

We provided evidence that ALL-derived exosomes may accelerate tumor growth and viability in CNS by increasing the expression of cancer-related genes in astrocytes. For the prevention, diagnosis and treatment of cancer, it is necessary to identify important genes involved in cancer, and understand their relationship with the onset and progression of cancer (16). One reason for ineffectiveness of treatment in children with ALL is the recurrence of leukemia in the CNS. Unfortunately, the existing detection methods are not sufficiently sensitive to determine the risk caused by the invasion of cancer cells to the CNS(17).

On the other hand, with the spread of metastasis in the CNS, astrocytes, as the main type of host cell, react by changing their phenotype(3). Astrocytes can be tumorigenic. For example, glioma often occurs due to Improper signaling in the genes of cell growth regulator genes(5). In this study, we focused on astrocytes, presenting them as one of the primary cells for the treatment of CNS diseases.

A notable point about exosomes, as small vesicles secreted from various cells and found in body fluids, is that they produce reproductive differentiation or apoptosis effects on recipient cells depending on their content (mRNA, miRNA, proteins) Therefore, given their role in pathological processes, they can be used for identification, diagnosis and treatment of cancer patients(18).

Previous studies on the metastasis of leukemia to the nervous system have used the pre-B ALL cell line, as this type of leukemia often metastasizes to the CNS (19). In this study, we used Nalm-6 to extract exosomes. For instance, as reported in previous studies, about 40 days after intravenous injection of Nalm-6 cells, all mice displayed signs of CNS involvement (19).

According to previous studies, the expression of some proteins, including matrix metalloproteinase 9 (MMP-9), increases after stimulation in astrocytes during metastasis. Therefore, as described in previous research, the high expression of MMP9 in astrocytes is due to the metastasis of cancer cells MDA-MB-435 and MDA-MB231 to the brain(16, 20). In the present study, the expression of this gene rose significantly in astrocytes treated with exosomes.

MMP molecules, including MMP9, play a key role in invasion, angiogenesis, and migration of GBM, and there is ample evidence that indicate their role in tumor growth, invasion, and inhibition of apoptosis (7). Therefore, from a theoretical perspective, determining the expression level of MMP9 is pivotal for diagnosing tumor progression (21). Astrocytes contribute to cell invasion by facilitating the process of ECM regeneration through expressing MMPs, including MMP9(9). Cell migration is one of the main obstacles to the treatment of patients (9). In the present study, the results of Scratch test manifested the significant migration of astrocytes treated with exosomes as opposed to the control group. It suggests that the effect of exosomes on astrocytes may sparkle their aggressive behavior.

Previous studies have shown that glioblastoma (GBM) stem cells block the expression of mRNA p53 as a tumor suppressor gene (a nuclear protein encoder involved in cell cycle regulation and cell proliferation) in surrounding astrocytes (22). In the present study, a significant reduction in the expression of mRNA P53 demonstrated the escalated tumor behavior of astrocytes in the face of exosomes derived from leukemia.

In previous reports, the regulations of MMPs expression under P53 wild−type and mutant expression were different (7). In ovarian cancer, for instance, the

expression of MMP2 and p53 facilitated cell invasion and metastasis. Therefore, the simultaneous demonstrations and biological interaction between P53 and MMP genes in tumorigenesis is possible (7). In the present study, the expression of MMP9 and P53 genes in astrocytes treated with leukemia exosomes was examined as molecules involved in pathogenesis pathways in CNS.

Contrary to the above results, it has been shown that with the growth and spread of metastases, RAS leads to the production of tumor neurogenic niches through various mechanisms such as secreting molecules and increasing Cox2 expression in metastatic brain cancer cells (3). In addition, there are ample evidence that Cox2 plays a crucial role in the proliferation and invasion of glioma, acting as a mediator for growth and survival of cancers (10). As noted in previous research, increasing the expression of Cox2 protein in the U87MG cell line affects the transcription of genes such as P53(23). In this study, we observed a significant increase in the expression of mRNA Cox2 in astrocytes treated with exosomes. Analyzing the expression of target genes in this study after the exposing astrocytes to exosomes derived from leukemia probably exhibits tumor behavior, survival and invasion.

5- CONCLUSION

In short, we studied the effect of exosomes derived from leukemia on the CNS through human astrocyte cells to investigate the expression of some cancer-related genes. The results can have broad implications for oncologists for early diagnosis and development of effective treatment methods. It is therefore recommended to compare the results of this study with the reproductive characteristics of astrocytic tumors and evaluate their invasive potential by examining genetic molecules involved in tumor signaling. It can be performed by immunohistochemistry at in vivo level and Western blotting at the protein level. The results can help obtain more accurate results on how blood cancer metastasizes to the CNS.

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7- ETHICAL APPROVAL

All procedures performed in this study were in accordance to the ethical standards of the ethical committee in Islamic Azad University-Mashhad Branch with IR.IAU.MSHD.REC.1397.092 approval ID.

8- CONFLICT OF INTEREST

The authors declare no conflict of interests

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