JBUON 2014; 19(2): 440-448
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Exosome/staphylococcal enterotoxin B, an anti tumor compound against pancreatic cancer

Hamideh Mahmoodzadeh Hosseini[1], Abbas Ali Imani Fooladi[2], Jafar Soleimanirad[3], Mohammad Reza Nourani[4], Mehdi Mahdavi[5]

[1]Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz; [2]Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran; [3]Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz; [4]Chemical Injury Research Center, Baqiyatallah University of Medical Sciences, Tehran; [5]Department of virology, Pasteur Institute of Iran, Tehran, Iran

Summary

Purpose: *Exosomes (EXOs) are acellular vehicles used for cancer immunotherapy due to their immune-inducing properties. We synthesized a novel structure based on EXOs and staphylococcal enterotoxin B (SEB) and surveyed its cytostatic effect on a pancreatic cell line.*

Methods: *EXOs were purified from tumor cells and SEB was anchored on it by protein transfer method. To determine the cytotoxic and apoptosis-inducing effect of this structure, treated cells with different concentrations of EXO/SEB were examined by MTT assay and Hoechst staining method. In addition, the expression rate of bcl-2, bax, bak, fas, bcl-xl and the activity of caspase-3 and caspase-9 were assessed.*

Results: *We observed that 0.5 and 2.5 μg/100μl of EXO/SEB significantly ($p<0.001$) stimulated apoptosis after 24 hrs. The concentrations of 0.5 and 2.5μg/100μl of EXO/SEB raised the expression rate of bax, bak, fas ($p<0.001$) but had no impact on bcl-2 and bcl-xl after 48 hrs. Furthermore, it was shown that 0.5, 2.5 and 5 μg/100μl of EXO/SEB only increased the activity of caspase-3 after 48 hrs ($p<0.001$).*

Conclusion: *Our designed structure, the EXO/SEB, is a novel model being able to induce apoptosis.*

Key words: *apoptosis, exosome, pancreatic cancer, staphylococcal enterotoxin B*

Introduction

Pancreatic cancer, an aggressive neoplasm with the poor prognosis, is considered as the fifth cause of cancer-related deaths worldwide. The most important clinical feature of this cancer is the local invasion and the emergence of metastasis in early stage [1]. The discovery of an effective therapy for pancreatic cancer is a controversial topic for researchers because of poor response to traditional treatments. Surgical resection, chemotherapy and radiation therapy are the current therapeutic modalities without positive impact on survival [2]. Hence, exploring a novel therapeutic strategy is necessary to improve pancreatic cancer therapy.

Immunotherapy is a new approach to improve cancer therapy and increase disease free survival. Cancer vaccines are specific tumor antigen presenters to the immune system, inducing effective antitumor immune responses [2]. One of the intriguing topics in cancer immunotherapy is the usage of EXOs as a cell-free and specific strategy for combating cancer [3]. The EXO, a 30-100 nm endosomal vesicle surrounded by a membrane, is released from various types of normal and cancer cells [4]. The EXOs reversely bud from multivesicular bodies (MVB). After fusing the MVB with the plasma membrane, EXOs are secreted into the extracellular space [5]. Tumor-derived EXOs consist of various cytosolic and membranous tumor antigens accompanied with molecules participated in antigen presentation which make them

Correspondence to: Jafar Soleimanirad, PhD. Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Valiasr, Tavaneer Ave. No 7, Tabriz, Iran. Tel: +98 411 3313053, E-mail: soleimanirj@yahoo.com
Received: 10/01/2014; Accepted: 08/02/2014

a promising candidate for cancer therapy. These kinds of EXOs are able to activate T cells by providing antigens for antigen presenting cells such as dendritic cells [6,7]. On the other hand, carrying large quantities of antigens by tumor-derived EXOs increase the possibility of anergy. Administration of adjuvants could be a solution for this problem. Furthermore, local administration of combination of EXOs with cytostatic agents could offer an effective therapy for established tumors.

Reportedly, the majority of previous studies have focused on the investigation of the immunizing properties of EXOs and their antitumor behavior [7]. A few studies surveyed EXOs impact on the fate of tumor cells. Two recent studies revealed that EXOs released from pancreatic cancer cells can trigger the mitochondrial-dependent apoptosis and increase the caspase-3 and caspase-9 activities. However, it was reported that EXOs from MIA Paca-2, a poorly differentiated pancreatic cancer cell line, are not capable to affect cell proliferation and death [8,9].

Current tumor cell death inducers are chemotherapeutic compounds which usually suppress the immune system. Superantigens as another group of T cell activators could be appropriate compounds for this purpose. Staphylococcal enterotoxin B as a potent superantigen, binds to major histocompatibility class II (MHC II) molecules on the surface of antigen-presenting cells and stimulate T cell proliferation and activation. The SEB-MHC II complex occurs outside of the antigen-binding site, then it attaches to the variable region of β chain of T cell receptor [10,11]. Several previous studies have found that SEB has potential to stimulate antitumor immune responses [12,13,14]. In addition, SEB influences the extrinsic apoptosis pathway, also termed Fas-mediated apoptotic pathway, and changes the expression of proteins involved in this pathway [15].

In the current study we firstly designed a structure based on Exos derived from tumor cells carrying the tumor antigens, and anchored them with SEB as a potent superantigen. Based on our hypothesis, this construct had two intrinsic properties: the cytotoxic and the immune stimulation ones. Then, we investigated the cytotoxic effect of this structure on original tumor pancreatic cells.

Methods

Cell culture

The MIA Paca-2, an epithelial-like pancreatic cancer cell line, was obtained from the Pasteur Institute (Tehran, Iran). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ºC in a 5% CO_2 atmosphere. All the cell culture reagents were purchased from PAA company of Holland.

Exosome purification

MIA Paca-2 cells were grown in T175 flasks (Nest, Holland). After reaching 85-90% confluence, the attached cells were washed three times with phosphate buffered saline (PBS) for removing FBS and then the fresh medium without FBS was added to each flask. After 48 hrs, the supernatant of each flask was transferred to a fresh tube and EXOs were purified according to the method described by Battke et al. [16]. Firstly, the supernatant was sequentially centrifuged at 2000×g for 10 min and 10000×g for 30 min to remove dead cells and debries. Then it was filtered using a 0.22 μm filter (GSV Filter Technology, USA). The filtrate was concentrated using a Centricon Plus-70 centrifugal filter device (Millipore, USA). Finally, ultracentrifugation was carried out in a TL-100 rotor at 100,000×g for an h to pellet the ultrafiltered concentrate. The obtained pellet was resuspended in 1 ml PBS and stored at -80 ºC for later examinations. The protein concentration of purified EXOs was evaluated at 280 nm using a nanodrop spectrophotometer (Thermos, USA).

Transmission electron microscopy

In order to evaluate the morphology and size of purified EXOs, Transmission Electron Microscopy (TEM) was used. Five μg of suspended EXOs in PBS were fixed with an equal amount of 2.5% glutaraldehyde and transferred on to a formware/carbon coated grid (IBB, Iran). Following incubation for 20 min at room temperature , the grid was transferred to 50 μl of uranyl oxalate pH 7 (Merck, Germany) for 5 min and washed with PBS. Then, the methyl cellulose/uranyl acetate (Merck, Germany) was added to the grid and allowed to stand for 10 min on an ice container. The excess fluid was removed by Watmen filter paper No.1. After air-drying the grid, the morphology and size of EXOs were viewed by TEM (LEO906, Germany) at 80kV.

SDS-PAGE and Western blotting

Hsp-70 as one of the most common identifier markers for EXOs [17] was detected using Western blotting. An equal amount of MIA Paca-2 cells list (lysis buffer containing 10 mM triton X-100 pH 7.5 and 150 mM NaCl) and purified EXOs were separated by SDS-PAGE containing 12.5% polyacrylamide (Merck, Germany) with 0.1% SDS (Merck, Germany). Then, proteins were electrophoretically transferred to a PVDF (Biorad, USA) and then blocked using 5% skim milk solution in Tris buffered saline with 0.1% tween-20 (TTBS) and incubated overnight at 4 ºC. To specifically detect Hsp-70, the membrane was incubated with mouse anti-Hsp70

Table 1. Sequences, the annealing temperature and size products of primers utilized for the related PCR reactions

Gene	*Forward primer (5´-3´)*	*Reverse primer (5´-3´)*	*Annealing temperature (°C)*	*Product size*	*Reference*
fas	TGGAATCATCAAGGAATGCA	GCCACTGTTTCAGGATTTAAGG	54	242	21
bcl-2	CGACTTCGCCGAGATGTCCAGCCAG	ACTTGTGGCCCAGATAGGCACCCAG	56	388	22
bcl-xl	GGAGCTGGTGGTTGACTTTCT	CCGGAAGAGTTCATTCACTAC	54	379	23
bax	AGGGTTTCATCCAGGATCGAGCAG	ATCTTCTTCCAGATGGTGAGCGAG	51	490	24
bak	TCAACCGACGCTATGACT	TCTTCGTACCACAAACTGG	52	368	25
β-actin	TCATGAAGATCCTCACCGAG	TTGCCAATGGTGATGACCTG3	58	190	26

primary antibody (Abcam, USA) for 2 hrs at room temperature and then washed by TTBS three times. In the next step, the anti-mouse IgG conjugated by horseradish peroxidase (Razi, Iran) was added to the membrane and incubated for 1 h at room temperature. To visualize the interested protein, a chromogenic stain, DAB/NiCl2, was utilized.

Protein anchorage of SEB on exosomes

To anchor SEB on EXOs, the protocol described by McHugh et al. [18] was utilized. Briefly, 10 µg SEB (Sigma-Aldrich, Germany) was added to 100 µg of purified EXOs in 100 µl PBS. The mixture was shacken at 1000 rpm for 4 hrs at 37 °C and was filtered using ultrafree-0.5 biomax100k (Millipore, USA) at 3000×g for 20 min to remove the unbound SEB. The EXOs anchored by SEB were named EXO/SEB.

Proliferation assay

The effect of EXO/SEB on the proliferation of MIA-Paca-2 cells was investigated by MTT assay. Briefly, 10^4 cells were seeded on each well of a 96-well plate and incubated at the culture condition. After 24 hrs, the cells were treated with four different concentrations of the EXO/SEB including 0.5, 2.5, 5 and 10 µg/100µl culture medium. An equal amount of EXOs without modification, SEB and the mixture of SEB and EXOs, indicated as EXO, SEB and EXO+SEB, respectively, were tested as controls. In addition, the cells treated with PBS were considered as a negative control. Twenty µl MTT reagent (5 mg/ml) (Sigma-Aldrich, Germany) were added to each well and incubated for 4 hrs at 37 °C. The supernatants were replaced with 100 µL dimethyl sulphoxide (Sigma–Aldrich, Germany) and finally, the optical density of each well was measured by micro plate reader (Tecan, Switzerland) at 570 nm. All the tests were carried out in triplicate. In addition, human embryonic kidney cells (purchased from Pasteur Institute, Iran) and human lymphocytes obtained from bloodstream were tested as normal cells to investigate the cytotoxic effect of EXO/SEB on normal cells.

Apoptosis assay

The induction of apoptosis by the EXO/SEB was detected by Hoechst 33258 staining method [14]. This stain specifically binds to the A-T regions of intact DNA strands and forms a fluorescent complex. The fragmented nuclear DNA does not create fluorescent signals. As described above, cells were treated with four concentrations including 0.5, 2.5, 5 and 10 µg/100µl of the EXO/SEB. EXO, SEB, EXO+SEB and PBS were tested as controls. After 24 hrs, cells of each well were detached by trypsin/EDTA and fixed on a slide by 4% formalin solution. Cells were stained with 1% Hoechst 33258 and observed by fluorescent microscope (Micros, Austria) under the high power field (×400) of the UV beam. 1000 cells were randomly counted and the number of apoptotic cells per 1000 cells was determined. Finally, the apoptotic index of each well was calculated according to the following formula:

$$\text{Apoptotic index} = \frac{\text{The number of apoptotic cells} \times 100}{\text{Total number of counted cells}}$$

All tests were performed in triplicate.

Assessment of caspase-3 and caspase-9 activity

The alterations of the caspase activity following EXO/SEB treatment were evaluated via caspase-3 and caspase-9 colorimetric assay kits (Genscript, USA). According to the manufacturers' instruction, 3×10^6 treated cells were lyzed by 50µl cold lysis buffer. After 60 min, each sample was centrifuged at 10,000 rpm for 1 min at 4 °C. A clear supernatant was transferred to a clean tube on the ice and the protein concentration was measured by nanodrop spectrophotometer at 280 nm. Fifty µl of 2× reaction buffer were added to an equal volume of cell lysate containing 100-200 µg protein followed by addition of 5 µl caspase-3 substrate (DEVD-*p*NA). After 4 hrs incubation at 37 °C, the extinction value of each test was measured at 400 nm using a micro plate reader (Techan, Switzerland). The relative changes of caspase-3 activity were determined by calculating the optical density (OD) $_{\text{test}}$/OD $_{\text{negative control}}$. The relative activity of caspase-9 was measured in a similar way. All tests were performed in triplicate.

Gene expression analysis

To analyze the effect of the EXO/SEB structure on

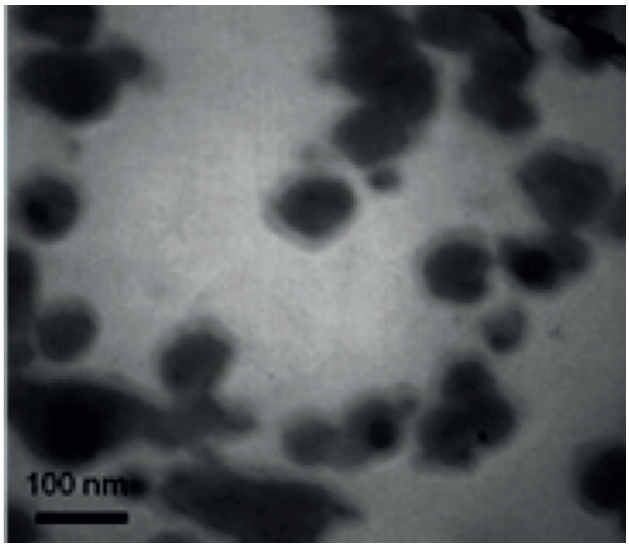

Figure 1. Transmission electron microscopy image of the purified exosomes. Negative stained isolated exosomes were observed by TEM (×60000). The exosomes' diameters are 40-150 nm.

the expression of some genes involved in apoptosis, the reverse transcriptase-polymerase chain reaction (RT-PCR) was used. After treating the cells with 0.5, 2.5, 5 and 10 µg/100µl of the EXO/SEB for 48 hrs, the total mRNA was isolated using the RNX-Plus kit (Cinnagen, Iran) according to the manufacturer's instruction. The mRNA was precipitated in isopropanol solution and finally resususpended in 20 µl DEPC-water. The concentration of each mRNA was measured by the nanodrop spectrophotometer (Thermos, USA). Bioneer kit (Takara, Japan) was used to synthesize related cDNA. In each RT reaction, 1 µg of isolated mRNA was converted to cDNA using the M-MLV RT, random hexamers and oligo dT. In the second step, the expression of the *fas, bak, bax, bcl$_2$, bcl-xl* genes was determined by PCR using specific pair primers for each gene. The expression of the *B-actin* was assessed as an internal control. The sequences, annealing temperature and the product sizes of each primer listed in Table 1. One µl of each cDNA was amplified in 20 µl of mixture reaction containing 10-fold reaction buffer, 0.2 mM of the deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, 10 pmol of each primer and 1.5 U of Taq DNA Polymerase (Cinnagen, Iran). PCR procedure was performed by thermocycler (Eppendorf, Germany) with an initial denaturation step of 6 min at 94 °C, 30 cycles of 30 sec at 94 °C, 45 sec at specified annealing temperature for each primer and 45 sec at 72 °C followed by 5 min final extension at 72 °C. The PCR products were visualized using electrophoresis on 1.5% agarose gel stained with ethidium bromide. The density of product bands was measured by the ImageJ software (National Institutes of Health, USA).

Statistics

Data obtained from all tests were assessed by the nonparametric Mann–Whitney U test using SPSS.15 software (SPSS, Chicago, IL). A p value less than 0.05 was considered statistically significant.

Results

Identification of exosomes purified from MIA Paca-2 cells

The purified EXOs were stained using uranyl acetate and observed by TEM to assess their morphology and size. As illustrated in Figure 1, the EXOs were round-shape membranous vesicles with 40-150 nm diameter. The presence of Hsp-70 as an exosomal protein marker was examined by Western blotting. We demonstrated that purified EXOs and MIA Paca-2 cell lysates had the Hsp-70 protein. A scanned image from the PVDF membrane is shown in Figure 2.

Cell proliferation analysis (MTT assay)

MIA Paca-2 cells were treated with different concentrations of the EXO/SEB for 24 and 48 hrs and cell proliferation was tested by MTT assay. After 24 hrs, all EXO/SEB concentrations except 10µg/100 µl, significantly decreased the proliferation of the cells compared to negative controls in a dose-dependent manner ($p<0.001$). Lower EXO/SEB concentration resulted in more cell proliferation reduction (Figure 3a). Moreover, 0.5 and 2.5 µg/100µl of EXO/SEB significantly attenuated the cell proliferation in comparison to an equal concentration of EXO, EXO+SEB and SEB ($p<0.01$ and $p<0.001$, respectively). Although 48-hour exposure to EXO/SEB showed a negative effect on cell proliferation, it was milder than the result of 24-hour incubation. Furthermore, no statistically significant differences in cell proliferation was observed compared to the control group (Figure 3b). Additionally, 0.5 and 2.5 µg/100µl of EXO/SEB significantly increased the cytotoxicity after 24 hrs compared to 48 hrs ($p<0.001$). The highest cytotoxic impact was observed following 0.5 and 2.5 µg/100µl of EXO/SEB treatment after 24 hrs. However, EXO/SEB had no cytotoxic effect on human embryonic kidney cells and peripheral blood lymphocytes, assessed as normal control cells (Figure 4).

Apoptosis assay

To analyze the apoptotic index of the EXO/SEB, the treated cells were stained with Hoechst 33258. Figure 5 illustrates a feature of the apoptotic and non-apoptotic cells by fluorescent microscope using this method. Data obtained from

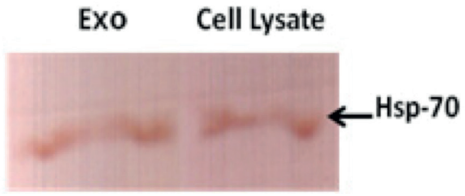

Figure 2. Western blotting of exosomal protein markers. An equal amount of lysate of MIA Paca-2 cells and their exosomes were separated by SDS-PAGE and the presence of Hsp-70 protein was identified using a specific antibody, anti-Hsp70. As shown in this figure, both EXO (an indicator for exosome) and the cell lysate contain Hsp-70.

this test showed that the EXO/SEB was able to stimulate apoptosis in a dose-dependent manner ($p<0.001$). Although the exposure to all tested EXO/SEB concentrations significantly caused apoptosis in comparison to an equal concentration of EXO, EXO+SEB, SEB and PBS ($p<0.001$), 0.5 and 2.5 μg/100μl of EXO/SEB showed the higest apoptotic index. Figure 6 displays the rate of apoptosis created by different concentrations of EXO/SEB compared to the control groups.

Gene expression

The impact of EXO/SEB on the expression of anti-apoptotic genes (*bcl-2, bcl-xl*) was quantified along with pro-apoptotic genes (*bax, bak and fas*) and was determined by RT-PCR. Because of caspase-3 activation after 48 hrs, we decided to examine the expression rate of the described genes after the same period. Our observations indicated a significant rise of bax, bak and fas gene expression at the concentration of 0.5 and 2.5 μg/100μl of EXO/SEB ($p<0.001$). However, the levels of *bcl-xl and bcl-2* expression did not show any differences between cells treated with EXO/SEB and groups exposed to EXO, EXO+SEB, SEB and PBS (Figure 7). No expression of *bcl-2* gene was seen in any group.

Caspase -3 and -9 activity assay

Specific colorimetric kits were utilized to identify whether EXO/SEB could raise the activity of caspase-3 and -9. As seen in Figure 8 (c and d), EXO/SEB had no effect on the activity of caspase-9 after 24 and 48 hrs in treated MIA Paca-2 cells. Despite the lack of activation after 24 hrs (Figure 8 a), a significant increase was observed after 48-hour exposure of the cells with 0.5, 2.5 and 5 μg/100μl concentrations of EXO/SEB ($p>0.001$) (Figure 8 b). Surprisingly,the lowest concentration

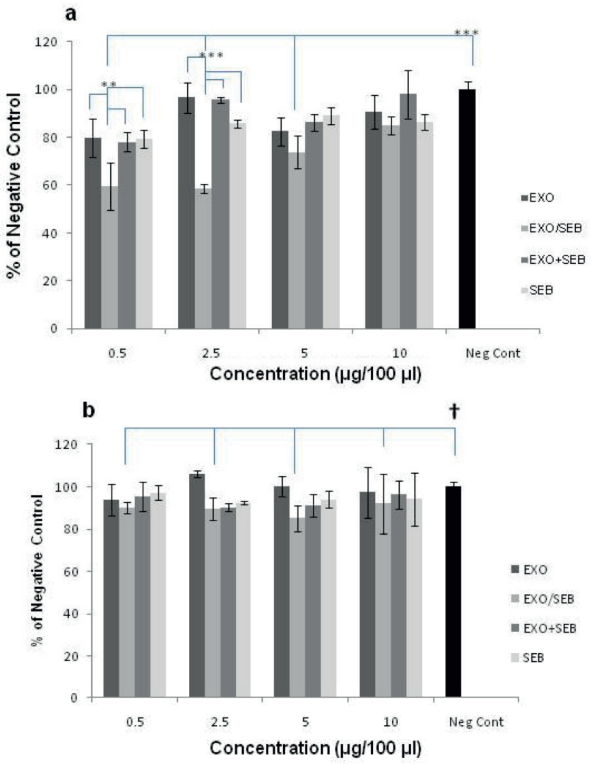

Figure 3. The cell proliferation rate of MIA Paca-2 treated with the EXO/SEB after 24 (**a**) and 48 (**b**) hours. The cells were treated with 0.5, 2.5, 5 and 10 μg/100μl of EXO/SEB for 24 and 48 hours. Also, equal amount of EXO, EXO+SEB, SEB and PBS were examined as controls ($p<0.001$). After 24 hours, significantly different proliferation rate was determined in all concentrations of the EXO/SEB, except 10 μg/100μl. After 48 hours, the EXO/SEB treatment had no effect on cell proliferation. **$p<0.01$, ***$p<0.001$, †$p>0.05$.

of EXO/SEB showed the most powerful induction for caspase-3 activity.

Discussion

EXOs as acellular vehicles have been implicated in the design of novel vaccines for cancer therapy. In the majority of studies, the efficacy of EXOs derived from immune cells was evaluated on tumor cells. Nevertheless, few studies have been devoted to the effect of tumor-derived EXOs on the tumor cells themselves [7]. The aim of the investigators in the vast majority of previous studies was to produce a specific and efficient vaccine to improve antitumor immunotherapy [7]. In this study, we intended to design a merged structure based on EXOs that are able to activate the cytostatic signals in target cells along with the induction of particular antitumor immune responses. The EXO/SEB is made up of two parts :

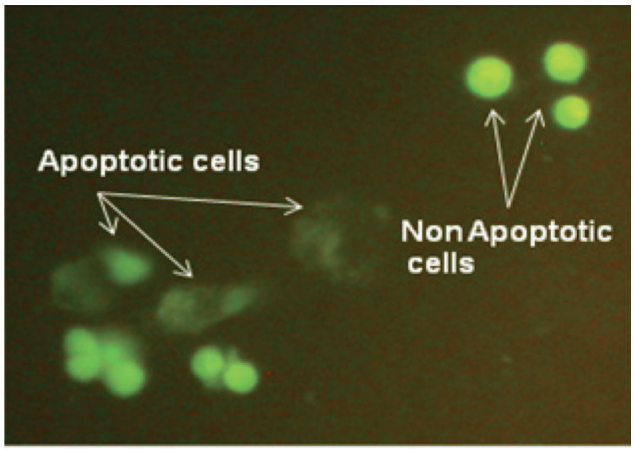

Figure 5. A Hoechst staining image. MIA Paca-2 are stained with Hoechst 33258 and observed under fluorescent microscope (×400). Cells with the degraded and fragmented chromosomes are apoptotic and demonstrate a colorless and non bright nuclei, whereas intact and non apoptotic cells have dense chromosomes and show completely stained and bright nuclei.

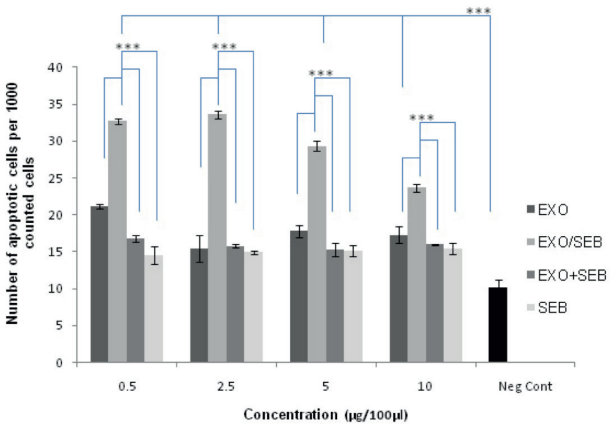

Figure 6. Analysis of apoptosis in MIA Paca-2 after treatment with four different concentrations (0.5, 2.5, 5 and 10 µg/100µl) of EXO/SEB for 24 hours. Also 0.5, 2.5, 5 and 10 µg/100µl of EXO, EXO+SEB, SEB and PBS were examined as controls. As seen in this Figure, all concentrations can significantly induce apoptosis in the MIA Paca-2 cells after 24 hours ($p<0.001$). ***$p<0.001$

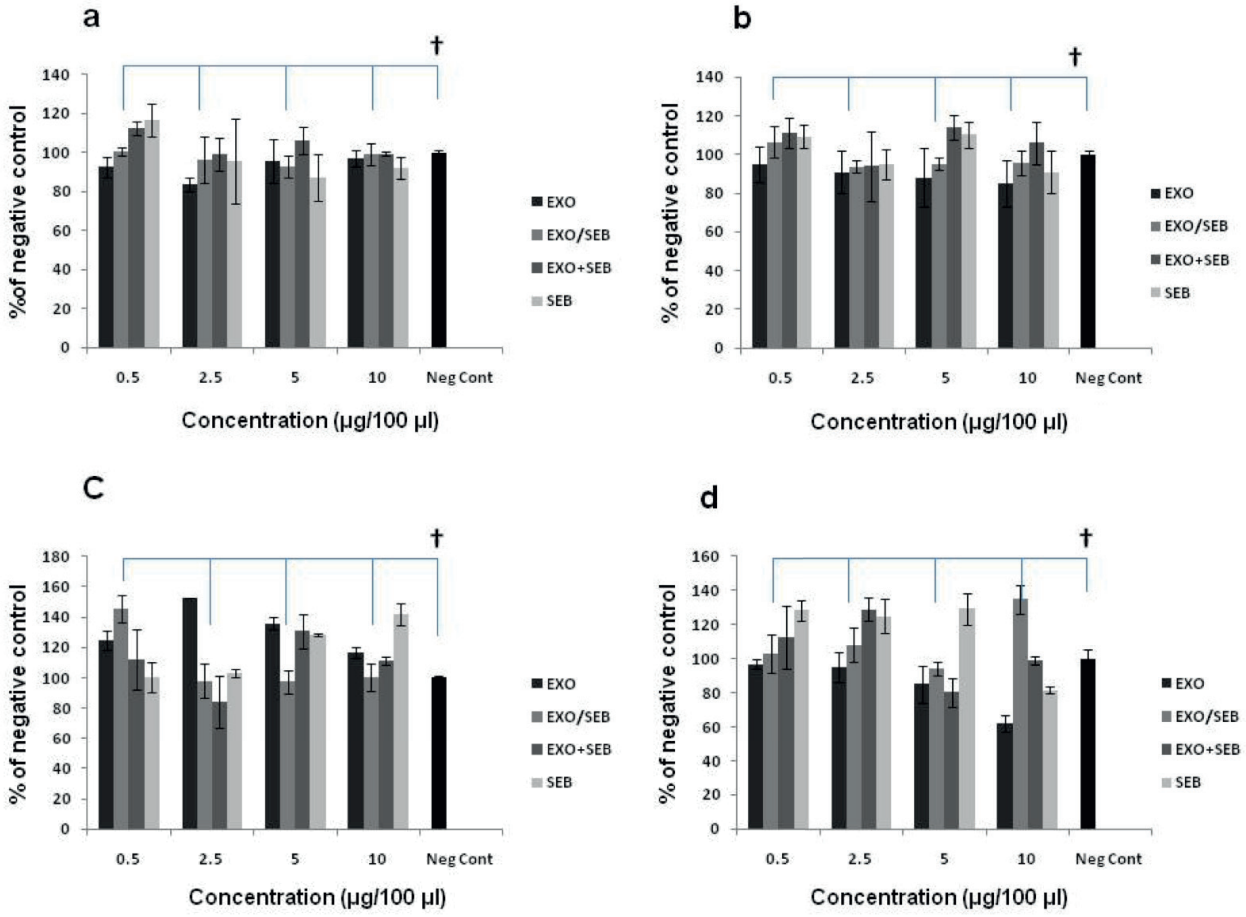

Figure 4. The cell proliferation rate of human kindley embryonic cells and peripheral human white blood cells treated with EXO/SEB after 24 (**a** and **c**, respectively) and 48 (**b** and **d**, respectively) hours. The cells were treated with 0.5, 2.5, 5 and 10 µg/100µl of EXO/SEB for 24 and 48 hours. Also, equal amounts of EXO, EXO+SEB, SEB and PBS were examined as controls. After 24 and 48 hours, EXO/SEB had no cytotoxic effect on both kinds of cells. †$p>0.05$.

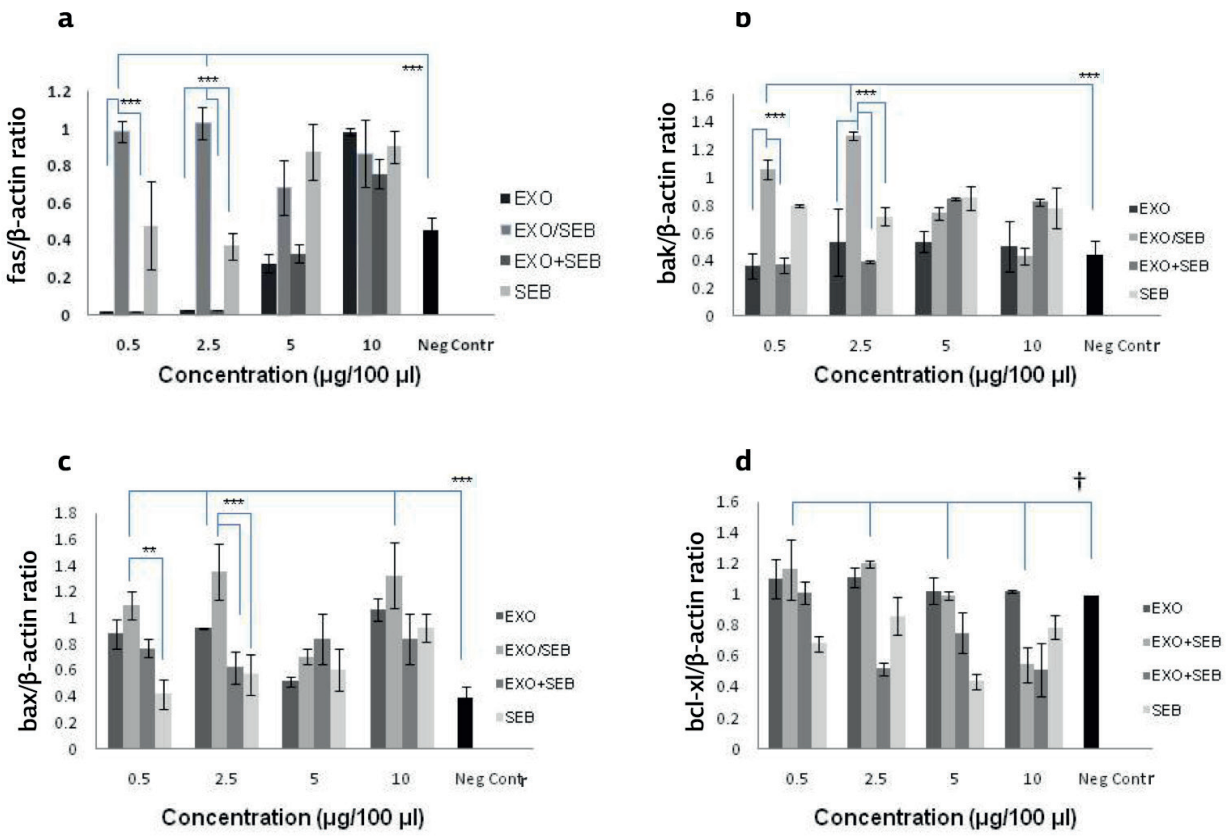

Figure 7. Effect of EXO/SEB on the expression of *fas, bcl-xl, bax* and *bak* in MIA Paca-2. The mRNA levels of the mentioned genes were relatively quantified by RT-PCR in the MIA Paca-2 cell treated with the four different concentrations of EXO/SEB (0.5, 2.5, 5 and 10 μg/100μl) after 48 hours. *β-actin* was examined as a housekeeping gene and consequently the results were shown as a ratio of desired gene expression/ *β-actin* expression. EXO/SEB increased the expression of *fas* **(a)** and *bak* **(b)** at the concentration of 0.5 and 2.5 μg/100μl ($p<0.001$). **c)**: The expression of *bax* was raised after treating cells with 0.5, 2.5 and 5 μg/100μl of EXO/SEB ($p<0.001$). **d)**: EXO/SEB showed no effect on the expression of *bcl-xl* gene. **$p<0.01$, ***$p<0.001$, †$p>0.05$.

tumor cells-derived EXO and anchored SEB as a superantigen. The combination of SEB and EXOs was performed through the glycosyl phosphatidyl inositol anchorage using the protein transfer method [18]. The presence of high levels of lipid raft domain, rich in sphingomyelin and cholesterol on the surface of EXOs [9] provides functional regions to contribute to the augmentation of apoptotic signals. Furthermore, SEB can induce apoptosis via the FAS/FASL system [15]. Our findings supported the cytostatic properties of the EXO/SEB. Results from MTT assay confirmed the cytotoxic impact of EXO/SEB on the MIA Paca-2 cells. Despite the outcomes reported by Ristorcelli and colleagues [9] that showed no antiproliferative effect of tumor-derived EXOs on poorly differentiated cancer cell lines such as MIA Paca-2 because of the high level of hes-1 [8], our results illustrated significant antiproliferative properties of EXOs derived from MIA Paca-2 cells at both concentrations of 0.5 and 2.5 μg/100μl. Moreover, the EXO/SEB was able to inhibit the proliferation of MIA Paca-2 cells in a reverse dose-dependent manner. The more the EXO/SEB concentration was increased, the lower the cell proliferation was inhibited. Since significant differences were seen between the exposure to the EXO/SEB at the concentrations of 0.5 and 2.5 μg/100μl and an equal concentration of SEB, EXO and EXO+SEB groups, it could be suggested that EXO/SEB has achieved new functional properties. Of note, antiproliferative and cytotoxic effects of EXO/SEB on the two normal cell lines (embryonic kidney and peripheral blood lymphocytes) were observed, which demonstrates that EXOs derived from each tumor cell line may influence only them. Moreover, results from Hoechst staining identified that treatment with EXO/SEB led to activation of apoptotic signals and consequently cell death. The increased expression of antiapoptotic genes including *bax, bak* and *fas* in cells treated with the EXO/SEB causes enhancement of apoptosis. On the other hand, lack of alterations in the expression of *bcl-xl* gene and loss of *bcl-2* expression in the MIA Paca-2 cells as antiapoptotic effectors can accelerate apoptosis [19]. Inducing the expression of an-

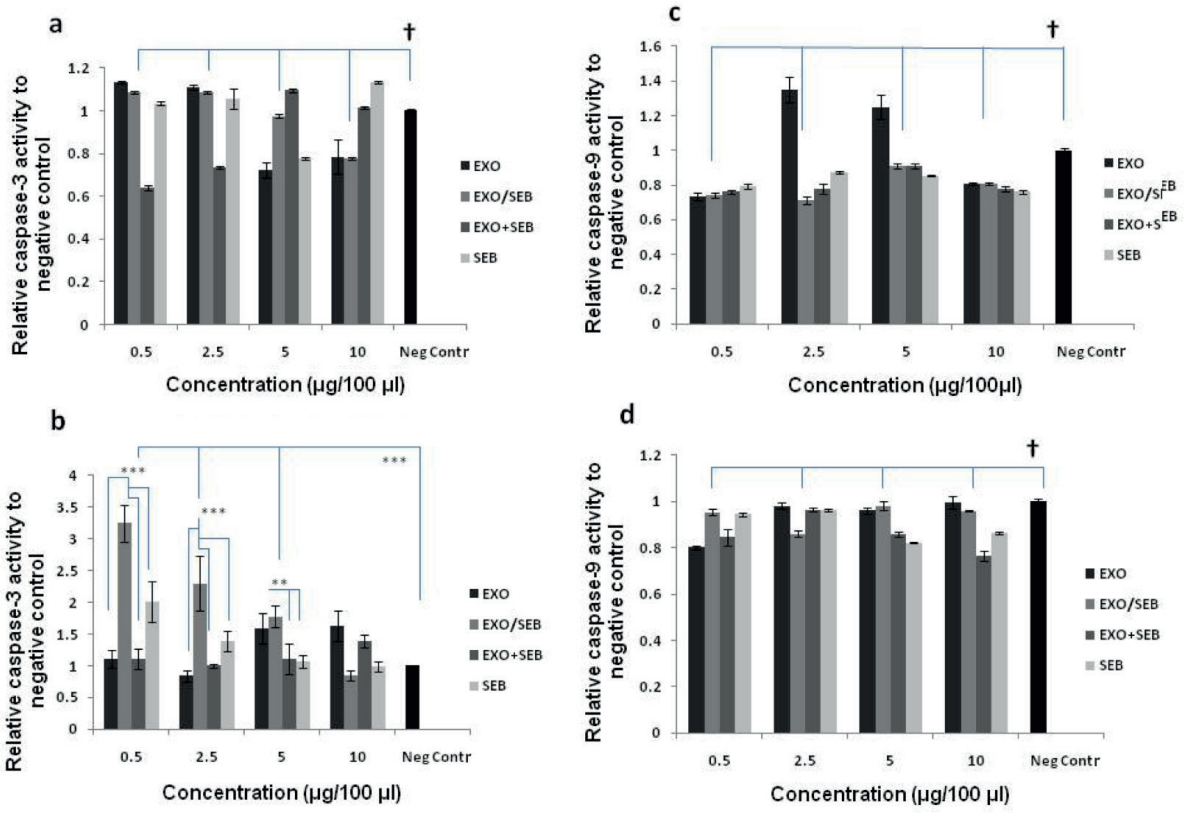

Figure 8. Effect of EXO/SEB on the induction of caspase-3 and caspase-9 activity in MIA Paca-2 cells. Caspase activity was quantified by the colorimetric method in the MIA Paca-2 cells following treatment with the four different concentrations of EXO/SEB (0.5, 2.5, 5 and 10 µg/100µl) after 24 and 48 hours. Our product had no impact on the activity of caspase-3 after 24 hours (**a**) and caspase-9 after 24 and 48 hours (**c**, **d**). EXO/SEB increased the activity of caspase-3 after 48 hours (**b**) at the concentration of 0.5, 2.5 and 5 µg/100µl ($p<0.001$). **$p<0.01$, ***$p<0.001$, †$p>0.05$.

tiapoptotic genes emerged at the concentrations of 0.5 and 2.5 µg/100µl.

In addition, we observed an increase in the activity of caspase-3, after 48 hrs but no changes were seen in the activity of caspase-9, which might be due to inability of EXO/SEB to activate caspase-9 or it required more time to be activated.

Ristrocelli et al. [9] suggested EXOs could induce the mitochondria-dependent apoptotic pathway which is inconsistent with some parts of our observations. High level of bax leads to inactivation of the protective function of bcl-2. Furthermore, bax stimulates releasing of cytochrome-c from mitochondria and finally results in apoptosis [20].

Overall, our designed structure, the EXO/SEB, is a novel model for apopto-immunotherapy, being able to induce apoptosis in addition to specific immune responses. The presence of EXO and its lipid rafts in this structure provides the possibility of binding to tumor cells. SEB and the described lipid rafts trigger the apoptotic signal both via extrinsic and mitochondria-dependent pathways. Besides, the attendance of tumor antigens accompanied with superantigen leads to enhancement of specific antitumor immune responses. Another advantage of this structure is its impact on poorly differentiated cancer cell lines that are insensitive to EXOs solely. Of course, further studies are required to support our results.

Acknowledgements

This study was part of the dissertation of Hamideh Mahmoodzadeh Hosseinini, submitted to Tabriz University of Medical Sciences in partial fulfillment of the requirements for the PhD in pharmaceutical biotechnology (No.78). This work was supported by the grant from the Iranian National Sciences Foundation (INSF) and Tabriz University of Medical Sciences. We thank Dr. Elnaz Mehdizadeh Aghdam (Tabriz University of Medical Sciences, Iran) for her editorial work in the preparation of this manuscript.

References

1. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 2011;61:212-236.
2. Koido S, Homma S, Takahara A et al. Current immunotherapeutic approaches in pancreatic cancer. Clin Dev Immunol 2011;2011:267539.
3. Rieger J, Freichels H, Imberty A et al. Polyester nanoparticles presenting mannose residues: toward the development of new vaccine delivery systems combining biodegradability and targeting properties. Biomacromolecules 2009;10:651-657.
4. Hosseini HM, Fooladi AA, Nourani MR, Ghanezadeh F. Role of exosome in infectious disease. Inflamm Allergy Drug Targets 2013; 12: 29-37.
5. Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic 2008; 9:871-881.
6. Cho JA, Yeo DJ, Son HY et al. Exosomes: a new delivery system for tumor antigens in cancer immunotherapy. Int J Cancer 2005;114:613-622.
7. Tan A, De La Peña H, Seifalian AM. The application of exosomes as a nanoscale cancer vaccine. Int J Nanomed 2010;5:889-900.
8. Ristorcelli E, Beraud E, Mathieu S, Lombardo D, Verine A. Essential role of Notch signaling in apoptosis of human pancreatic tumor cells mediated by exosomal nanoparticles. Int J Cancer 2009;125:1016-1026.
9. Ristorcelli E, Beraud E, Verrando P et al. Human tumor nanoparticles induce apoptosis of pancreatic cancer cells. FASEB J 2008;22:3358-3369.
10. Choi YW, Kotzin B, Herron L, Callahan J, Marrack P Kappler J. Interaction of Staphylococcus aureus toxin" superantigens" with human T cells. Proc Natl Acad Sciences 1989;86:8941-8945.
11. Kappler J, Kotzin B, Herron L et al. V beta-specific stimulation of human T cells by staphylococcal toxins. Science 1989;244:811-813.
12. Imani Fooladi AA, Sattari M, Hassan ZM, Mahdavi M, Azizi T, Horii A. In vivo induction of necrosis in mice fibrosarcoma via intravenous injection of type B staphylococcal enterotoxin. Biotechnol Lett 2008;30:2053-2059.
13. Imani Fooladi AA, Sattari M, Nourani MR. Synergistic effects between staphylococcal enterotoxin type B and monophosphoryl lipid A against mouse fibrosarcoma. JBUON 2010;15: 340-347.
14. Fooladi AAI, Sattari M, Nourani MR. Study of T-cell stimulation and cytokine release induced by staphylococcal enterotoxin type B and monophosphoryl lipid A. Arch Med Sci 2009;3:335-341.
15. Higgs BW, Dileo J, Chang WE et al. Modeling the effects of a staphylococcal enterotoxin B (SEB) on the apoptosis pathway. BMC Microbiol 2006;6:48-60.
16. Battke C, Ruiss R, Welsch U et al. Tumour exosomes inhibit binding of tumour-reactive antibodies to tumour cells and reduce ADCC. Cancer Immunol Immunother 2011;60:639-648.
17. Exosome protein and RNA database. ExoCarta 2010 [cited 2010]. Available from: http://exocarta. ludwig. edu.au.
18. McHugh RS, Nagarajan S, Wang YC, Sell KW, Selvaraj P. Protein transfer of glycosyl-phosphatidylinositol-B7-1 into tumor cell membranes: a novel approach to tumor immunotherapy. Cancer Res 1999;59:2433-2437.
19. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609-619.
20. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005;258:479-517.
21. Ferenbach DA, Haydon GH, Rae F, Malcomson RD, Harrison DJ. Alteration in mRNA levels of Fas splice variants in hepatitis C-infected liver. J Pathol 1997;183:299-304.
22. Marone M, Mozzetti S, De Ritis D, Pierelli L, Scambia G. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biol Proced Online 2001;3:19-25.
23. Chen JH, Cao JL, Chu YL, Wang ZL, Yang ZT, Wang HL. T-2 toxin-induced apoptosis involving Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 signaling pathways in human chondrocytes. J Zhejiang Univ Sci B 2008; 9:455-463.
24. Kim H, Chung H, Kim HJ et al. Id-1 regulates Bcl-2 and Bax expression through p53 and NF-kappaB in MCF-7 breast cancer cells. Breast Cancer Res Treat 2008;112:287-296.
25. Banisor I, Kalman B. Bcl-2 and its homologues in the brain of patients with multiple sclerosis. Mult Scler 2004;10:176-181.
26. Khaheshi I, Keshavarz S, Imani Fooladi AA et al. Loss of expression of TGF-βs and their receptors in chronic skin lesions induced by sulfur mustard as compared with chronic contact dermatitis patients. BMC Dermatol 2011;11:2-10.