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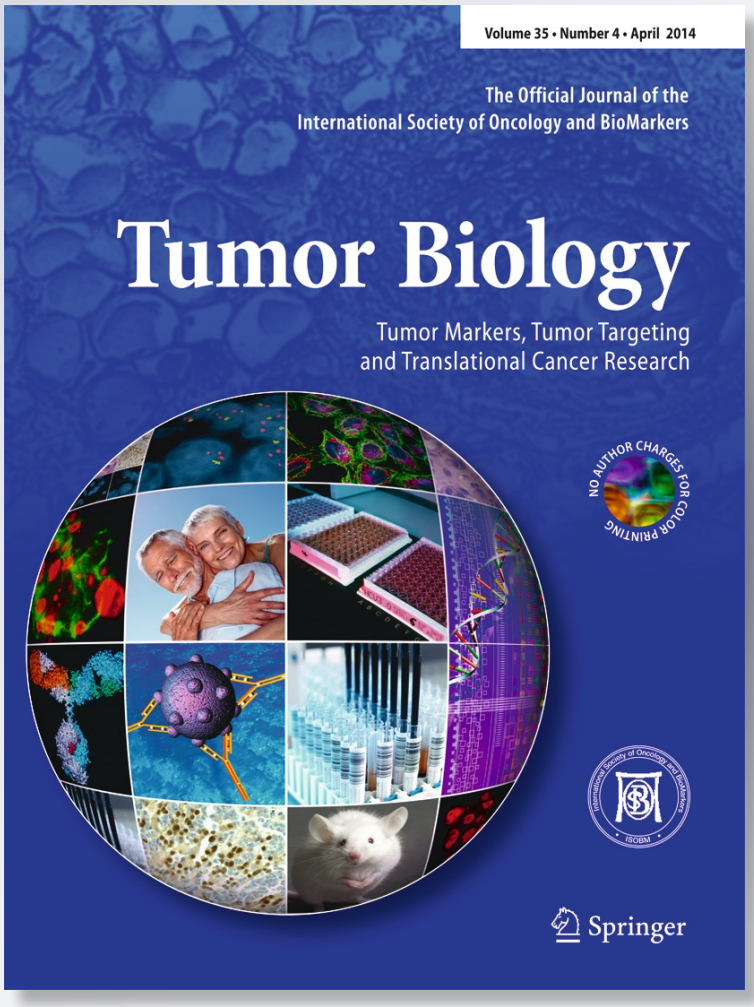

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RESEARCH ARTICLE

Staphylococcal entorotoxin B anchored exosome induces apoptosis in negative esterogen receptor breast cancer cells

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Abstract Exosomes (EXO) are acellular vehicles used for cancer immunotherapy due to their immune inducing properties. To identify whether designed structure based on tumoral EXO have a cytotoxic effect together with a potent immunological property, we synthesized a novel structure based on EXO and staphylococcal entrotoxin B (SEB), two immune inducer substances, and surveyed its cytostatic effect on the breast cancer cell line. EXO 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. We observed that EXO/SEB significantly decreased the cell proliferation and stimulated apoptosis ($P<0.001$) at all concentration after 24 h ($P<0.001$). Furthermore, EXO/SEB raised the expression rate of *bax* and *bak* ($P<0.001$) but no impact on *fas* and *bcl-xl* after 48 h. We observed reducing effect of EXO/SEB on the mRNA expression of *bcl-2*. After 24 h of exposing the cell with the EXO/SEB, a significant increase was found in the activity of caspase at the concentration of 2.5, 5 and 10 μg/100 μl for caspase-9 and at all concentrations for caspase-3 ($P<0.001$). Our designed structure, the EXO/SEB, is a novel model for apopto-immunotherapy being able to induce apoptosis in ER^- breast cancer cells.

Keywords Exosome · Staphylococcal entrotoxin B · Superantigen · Apoptosis · Breast cancer

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Introduction

Breast cancer is one of the most common causes of death in women all around the world. It is estimated more than 1.38 million new women suffered from breast cancer, have been diagnosed yearly [1]. Anti-estrogen therapy is the choice strategy for estrogen receptor positive breast cancer. However, a large number of breast cancer does not respond to this type of treatment because of lack of estrogen receptor. Furthermore, another group of breast cancer becomes insensitive to hormone therapy after first therapy and immediately promotes to grow [2]. Although Chemotherapy is an alternative strategy for treating insensitive and metastatic breast cancer but many of the treated cancers often develop a recurrence. Therefore, innovating novel cytostatic agents is an urgent requirement for attenuating the mortality rate [3]. According to approaches from recent studies, a method based on immunotherapy may be efficient to treat the breast cancer. Cancer vaccines are specific tumor antigen representors to the immune system,

inducing effective antitumor immune responses [4]. One of the intriguing topics in cancer immunotherapy is the usage of exosomes (EXO) as a cell-free and specific strategy for combating cancer [5]. The EXO, a 30- to 100-nm endosomal vesicle surrounded by a membrane, is released from various types of normal and cancerous cells [6]. The EXO reversely bud from multivesicular bodies (MVB). After fusing the MVB with the plasma membrane, EXO are secreted into the extracellular space [7]. Tumor-derived EXO consist various cytosolic and membranous tumor antigens accompanied with molecules participated in antigen presentation which make them a promising candidate for cancer therapy. These kinds of EXO are able to activate T cells through providing antigens for antigen presenting cells such as dendritic cells [8, 9]. On the other hand, carrying large quantities of antigens by tumor derived EXO increase the possibility of anergy. Administration of adjuvants could be a solution for this problem. Furthermore, local administration of combination of EXO with cytostatic agents could offer an effective medicine for establishing tumors.

Reportedly, the majority of previous studies have focused on the investigation of immunizing properties of EXO and their anti tumor behavior [reviewed in 9]. A few studies surveyed EXO's impact on the fate of tumor cells. Two recent studies revealed that EXO released from pancreatic cancer cells cause to trigger the mitochondrial dependent apoptosis and increase the caspase-3 and caspase-9 activities. However, it was reported that EXO from MIA Paca-2, a poor differentiated pancreatic cancer cell line, are not capable to affect cell proliferation and death [10, 11]. Current tumoral 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 entorotoxin B as a potent superantigen, binds to major histocampatibility class II (MHC II) molecules on the surface of antigen presenting cells and stimulate T cell proliferation and activation. The staphylococcal entrotoxin B (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 [12, 13]. Several previous studies found out that SEB has potential to stimulate antitumor immune responses [14–16]. In addition, SEB influences the extrinsic apoptosis pathway, also termed Fas-mediated apoptotic pathway, and changes the expression of proteins involved in this pathway [17].

In the current study, we purposed to construct a structure with a simultaneous characteristic of an intrinsic immune stimulator and a cytostatic compound. Thus, we firstly designed a structure based on EXO derived from tumoral cells, carrying the tumoral antigens, and anchored them with SEB as a potent superantigen. After that, we investigated the cytostatic effect of this structure on original tumoric breast cells.

Materials and methods

Cell culture

The MDA MB-231, an epithelial like breast cancer cell lines, was obtained from the Pasture 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 °C and under 5 % CO_2 atmosphere. All the cell culture reagents were purchased from PAA company of Holland.

Exosome purification

MDA MB-231cells were grown in T175 flasks (Nest, Holland). After reaching 85–90 % confluency, 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 h, the supernatant of each flask was transferred to a fresh tube and EXO was purified according to the method described by Battke et al. [18]. Firstly, the supernatant was sequentially centrifuged at $2{,}000\times g$ for 10 min and $10{,}000\times g$ for 30 min to remove dead cells and debris. 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, the ulteracentrifugation was carried out in a TL-100 rotor at $100{,}000\times g$ for an hour to pellet the ulterafiltrated concentrate. The obtained pellet was resuspended in 1 ml PBS and stored at −80 °C for later examinations. The protein concentration of purified EXO was evaluated at 280 nm using a nanodrop spectrophotometer (Thermos, USA).

Transmission electron microscopy

In order to evaluate the morphology and size of purified EXO, Transmission Electron Microscopy (TEM) was used. Five micrograms of suspended EXO in PBS was 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 Whatman filter paper NO1. After air-drying the grid, the morphology and size of EXO were viewed by TEM (LEO906, Germany) at 80 kV.

SDS-PAGE and Western blotting

Hsp-70 as one of the most common identifier markers for EXO [19] was detected using Western blotting. An equal amount of MDA MB-231 cells lysate (lysis buffer containing

10 mM triton X-100 pH 7.5 and 150 mM NaCl) and purified EXO were separated by SDS-PAGE containing 12.5 % polyacrylamide (Merck, Germany) with 0.1 % SDS (Merck, Germany). Consequently, proteins were electrophoretically transferred to a PVDF (Bio-Rrad, USA) and then blocked using 5 % skim milk solution in Tris buffered saline with 0.1 % Tween-20 (TTBS) and incubated for an overnight at 4 °C. To specifically detect Hsp-70, the membrane was incubated with mouse anti-Hsp70 primary antibody (Abcam, USA) for 2 h 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 chromagenic stain, DAB/$NiCl_2$, was utilized.

Protein anchorage of SEB on exosomes

To anchor SEB on EXO, the protocol described by McHugh et al. [20] was utilized. Briefly, 10 μg SEB (Sigma-Aldrich, Germany) was added to100 μg of purified EXO in 100 μl PBS. The mixture was shacked at 1,000 rpm for 4 h at 37 °C. The mixture was filtrated using ultrafree-0.5 biomax100k (Millipore, USA) at 3,000×*g* for 20 min to remove the unbounded SEB. The EXO anchored by SEB were named an EXO/SEB.

Proliferation assay

The effect of EXO/SEB on the proliferation of MDA MB-231cells 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 h, the cells were treated with four different concentrations of the EXO/SEB including 0.5, 2.5, 5 and 10 μg/100 μl culture medium. The equal amount of EXO without modification, SEB and the mixture of SEB and EXO, indicated as EXO, SEB and EXO + SEB, respectively, were tested as controls. In addition, the cells treated with PBS was considered as a negative control. Next, 20 μl MTT reagent (5 mg/ml) (Sigma-Aldrich, Germany) was added to each well and incubated for 4 h 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 tests were carried out in triplicate. In addition, human embryonic kidney cells (purchased from Pasture Institute, Iran) and human white blood cells obtained from bloodstream were tested as normal cells to investigate the effect of EXO/SEB on normal cells.

Apoptosis assay

The induction of apoptosis by the EXO/SEB was detected by Hoechst 33258 staining method [16]. The Hoechst 33258 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 respectively treated with four concentrations including 0.5, 2.5, 5 and 10 μg/100 μl of the EXO/SEB. EXO, SEB, EXO + SEB and PBS were tested as controls. After 24 h, 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. Then, 1,000 cells were randomly counted and the number of apoptotic cells per 1,000 cells was determined. Finally, the apoptotic index of each well was calculated according to the following formula:

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

All tests were repeated three times.

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 manufacturer's instruction, 3×10^6 treated cells were lysed by 50 μl 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 microliters of 2× reaction buffer was added to an equal volume of cell lysate containing 100–200 μg protein followed by adding 5 μl caspase 3 substrate (DEVD-*p*NA). After 4 h incubation at 37 °C, the extinction value of each test was measured at 400 nm using micro plate reader (Techan, Switzerland). The relative changes of caspase-3 activity were determined by calculating $OD_{test}/OD_{negative\ control}$. The relative activity of caspase-9 was measured in a similar way. All tests were performed three times.

Gene expression analysis

To analyze the effect of the EXO/SEB structure on the expression of some genes involved in apoptosis, the semi-quantitative reverse transcriptase-polymerase chain reaction (semi-RT-PCR) was done. After treating the cells with 0.5, 2.5, 5 and 10 μg/100 μl of the EXO/SEB for 48 h, the total mRNA was isolated using the RNX-Plus kit (Cinnagen, Iran) according to the manufacturer's instruction. The mRNA were precipitated in isopropanol solution and finally resuspended 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, one μg of isolated mRNA was converted to cDNA using the M-MLV reverse transcriptase, random hexamers and oligo dT. In the second step, the expression of the *fas*, *bak*, *bax*, *bcl*$_2$, and *bcl*-x_l genes were 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 micrliter of each cDNA was amplified in 20 μl of mixture reaction containing 10× reaction buffer, 0.2 mM of the deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, 10 pmol of each primers 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 s at 94 °C, 45 s at specified annealing temperature for each primer and 45 s 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). To evaluate the relative expression level, the expression ratio of each gene to β-*actin* as a housekeeping gene was measured.

Statistical analysis

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

Results

Identification of exosomes purified from MDA MB-231 cells

The purified EXO were stained using uranyl acetate and observed by TEM To assess their morphology and size. As illustrated in Fig. 1, the EXO were round-shaped 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 EXO and MDA MB-231 cell lysates had the Hsp-70 protein. The scanned image from the PVDF membrane was shown in Fig. 2.

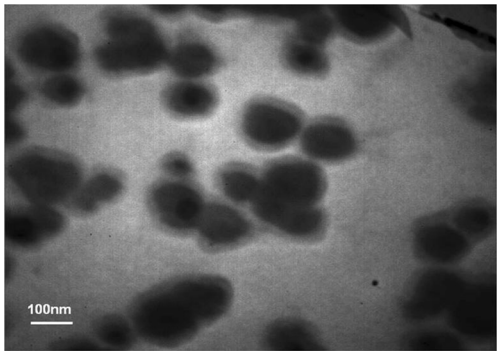

Fig. 1 Transmission electron microscopy image of the purified exosomes. Negative stained isolated exosomes was observed by TEM (×60,000). The range of exosomes' diameter is 40–150 nm

Cell proliferation analysis (MTT assay)

MDA MB-231 cells were treated with different concentrations of the EXO/SEB for 24 and 48 h and cell proliferation was assessed by MTT assay. After 24 h, all concentrations of EXO/SEB significantly attenuated the proliferation of treated cells compared to negative controls ($P<0.001$). The highest inhibitory effect was seen after exposing the MDA MB-231 cells to 10 μg/100 μl of EXO/SEB (Fig. 3a) that it significantly was more cytotoxic than the EXO/SEB at both 0.5 and 2.5 μg/100 μl ($P<0.01$). Although 48-h exposure to EXO/SEB showed a negative effect on the cell proliferation, it is milder than the result of 24 h incubation. Furthermore, no statistically significant difference in cell proliferation was observed compared to the control group (Fig. 3b). Except for a concentration of 0.5 μg/100 μl, no significant difference was seen between the inhibitory effect of EXO and EXO/SEB after 24 h (Fig. 3a). However, no concentration of SEB had an inhibitory impact on cell proliferation after 24 h. However, EXO/SEB did not impact on the proliferation of human embryonic kidney cells and peripheral blood lymphocytes assessed as normal control cells (Fig. 4).

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	Product size (bp)	Reference
fas	TGGAATCATCAAGGAATGCA	GCCACTGTTTCAGGATTTAAGG	54	242	[26]
bcl-2	CGACTTCGCCGAGATGTCCAGC CAG	ACTTGTGGCCCAGATAGGCACC CAG	56	388	[27]
bcl-xl	GGAGCTGGTGGTTGACTTTCT	CCGGAAGAGTTCATTCACTAC	54	379	[28]
bax	AGGGTTTCATCCAGGATCGAGCAG	ATCTTCTTCCAGATGGTGAGCGAG	51	490	[29]
bak	TCAACCGACGCTATGACT	TCTTCGTACCACAAACTGG	52	368	[30]
β-*actin*	TCATGAAGATCCTCACCGAG	TTGCCAATGGTGATGACCTG3	58	190	[31]

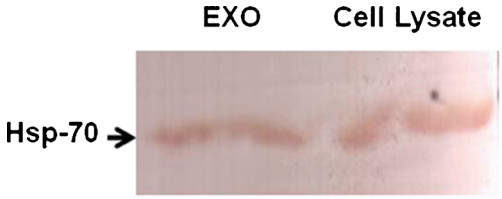

Fig. 2 Western blotting of exosomal protein markers. An equal amount of lysate of MDA MB-231 cells and their exosomes were separated by SDS-PAGE and the presence of Hsp-70 protein was identified using a specific antibody, anti-Hsp70. As shown in this figure, both EXO (an indicator for exosome) and the cell lysate contain Hsp-70

Apoptosis assay

To analyze the apoptotic index of the EXO/SEB, the treated cells described before 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 this test showed that the EXO/SEB was able to stimulate apoptosis ($P<0.001$). However, the exposure to all tested EXO/SEB concentrations significantly caused apoptosis rising in comparison to an equal concentration of EXO, EXO + SEB, SEB and PBS ($P<0.001$), 5 and 10 μg/100 μl of EXO/SEB showed the most apoptotic index. In addition, we observed significantly higher inducing of apoptotic index by EXO/SEB at the concentration of 10 μg/100 μl compared to three rest concentration of EXO/SEB ($P<0.001$). We showed that all concentrations of EXO, EXO + SEB and SEB except 2.5, 5 and 10 μg/100 μl of SEB significantly induced apoptosis compared to the negative control ($P<0.001$). Figure 6 demonstrated the rate of apoptosis created by different concentration 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 relatively quantified along with pro-apoptotic genes (*bax*, *bak* and *fas*) was determined by semi-RT-PCR. Our observations indicated a significant rise of *bax* gene expression at all tested concentrations and *bak* at the concentration of 2.5, 5 and 10 μg/100 μl of EXO/SEB ($P<0.001$). However, levels of *fas* remarkably reduced in the cell treated with EXO and EXO/SEB at all concentrations studied in this study ($P<0.001$). What is more, the mRNA level of *bcl-2* significantly reduced at all concentrations ($P<0.001$). On the other hand, *bcl-xl* expression did not point any differences between cells treated with EXO/SEB and groups exposed to EXO, EXO + SEB, SEB and PBS (Fig. 7).

Caspase-3 and caspase-9 activity assay

The specific colorimetric kits were utilized to identify whether EXO/SEB could raise the activity of caspase-3 and caspase-9. After a 24-h exposure to EXO/SEB, a significant increase was found in the activity of caspase at the concentration of 2.5, 5 and 10 μg/100 μl for caspase-9 (Fig. 8a) and at all concentrations for caspase-3 (Fig. 8c) ($P<0.001$). The influence of EXO/SEB on the activity of caspase-9 is a dose dependent that more effect was induced after treating by 10 μg/100 μl of EXO/SEB ($P<0.001$). Unlike to caspase-9, increasing changes in the activity of caspase-3 was independent from raising the EXO/SEB level. As shown in Fig. 8b and d, Exo/SEB had no effect on the activity of caspase-3 and -9 after 48 h in treated MDA MB-231.

Discussion

In spite of maintaining the homeostasis in biological condition, apoptosis is inefficient in tumoral tissues, thereby leading to the development of tumorigenicity; therefore, introducing a novel apoptotic inducer is critical for cancer therapy [21].

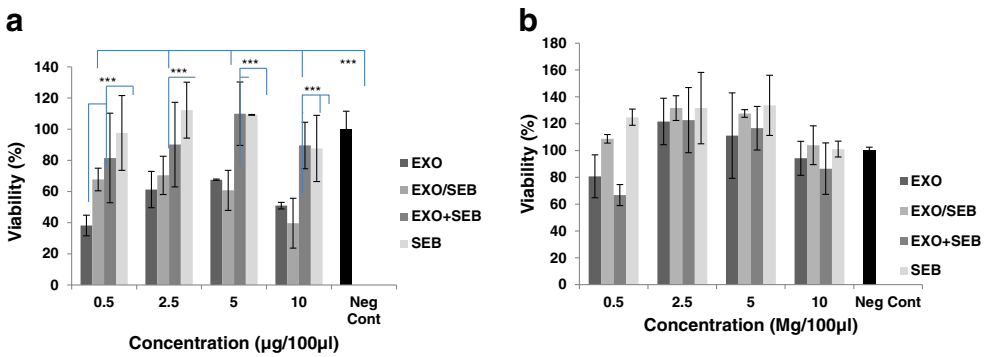

Fig. 3 The cell proliferation rate of MDA MB-231 treated with the EXO/SEB after **a** 24-h and **b** 48-h exposure. The cells were treated with 0.5, 2.5, 5 and 10 μg/100 μl of EXO/SEB for 24 and 48 h. Also equal amount of EXO, EXO + SEB, SEB and PBS were examined as controls. After 24 h, significant different proliferation rate was determined in all concentrations of the EXO/SEB. After 48 h, the EXO/SEB treated had no effect on the cell proliferation

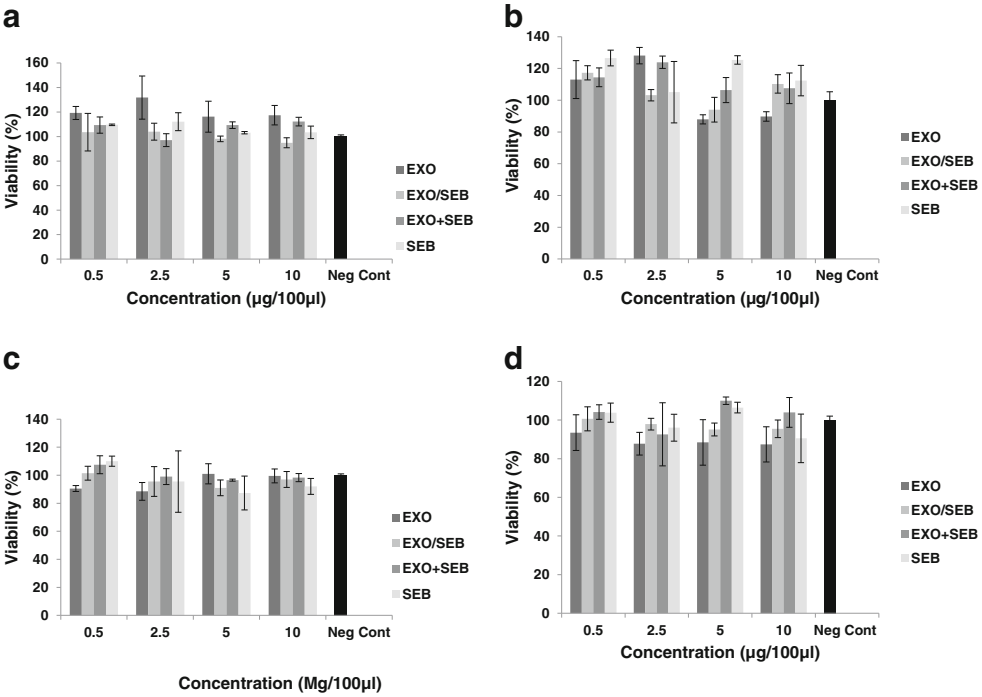

Fig. 4 The cell proliferation rate of human embryonic cells and peripheral human white blood cells treated with EXO/SEB after 24 h (**a** and **c**, respectively) and 48 h (**b** and **d**, respectively). The cells were treated with 0.5, 2.5, 5 and 10 µg/100 µl of EXO/SEB for 24 and 48 h. Also equal amount of EXO, EXO + SEB, SEB and PBS were examined as controls. After 24 and 48 h, EXO/SEB had no effect on the proliferation of both kinds of cells

Nowadays, EXO as acellular vehicles have been used to design novel vaccines for cancer therapy. In the majority of studies, the efficacy of EXO derived from immune cells was evaluated on the fate of tumoral cells. Nevertheless, few studies have been devoted to the effect of tumor-derived EXO on the tumor cells themselves [9]. The aim of investigators in the vast majority of previous studies was to produce a specific and efficient vaccine to improve anti tumoral immunotherapy [9]. In this study, our purpose was to design a merged structure based on EXO that is 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; tumor cells derived EXO and anchored SEB as a superantigen. The combination of SEB and EXO were performed through the glycosyl phosphatidyl inositol anchorage using protein transfer method [20]. The presence of high levels of lipid raft domain, rich in sphyngomyelin and cholesterol, on the surface of EXO [11] provides functional regions to contribute to the augmentation of apoptotic signals. Furthermore, SEB can induce apoptosis via the FAS/FASL system [17]. Our findings clearly supported the cytostatic properties of the EXO/SEB in ER$^-$ breast cancer cell line, MDA MB-231. Results from MTT assay showed the cytotoxic impact of EXO/SEB on the MDA MB-231cells. Furthermore, no cytotoxic and anti-proliferative effect of EXO/SEB

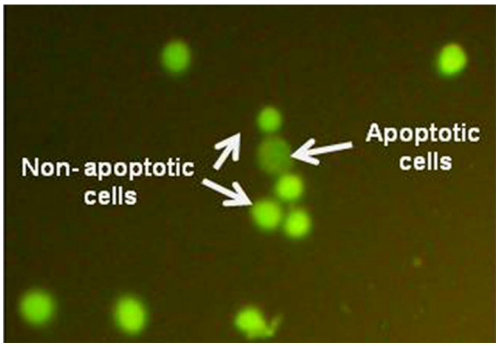

Fig. 5 A Hoechst staining image. MDA MB-231 are stained with Hoechst 33258 and observed under a fluorescent microscope (×400). Cells with the degraded and fragmented chromosomes are apoptotic and demonstrate a colorless and non-bright nuclei, whereas intact and non-apoptotic cells have a dense chromosome and shows a completely stained and bright nuclei

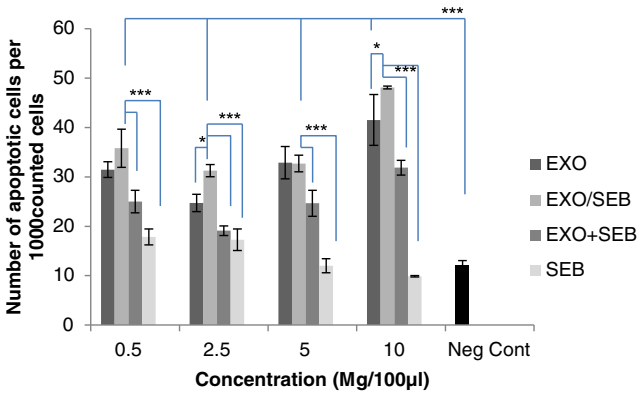

Fig. 6 Analysis of apoptosis in MDA MB-231 after treatment with four discrepant concentrations (0.5, 2.5, 5 and 10 µg/100 µl) of EXO/SEB for 24 h. Also, 0.5, 2.5, 5 and 10 µg/100 µl of EXO, EXO + SEB, SEB and PBS were examined as controls. As seen in this graph, all concentrations can significantly induce the apoptosis in the MDA MB-231 cells after 24 h ($P<0.001$)

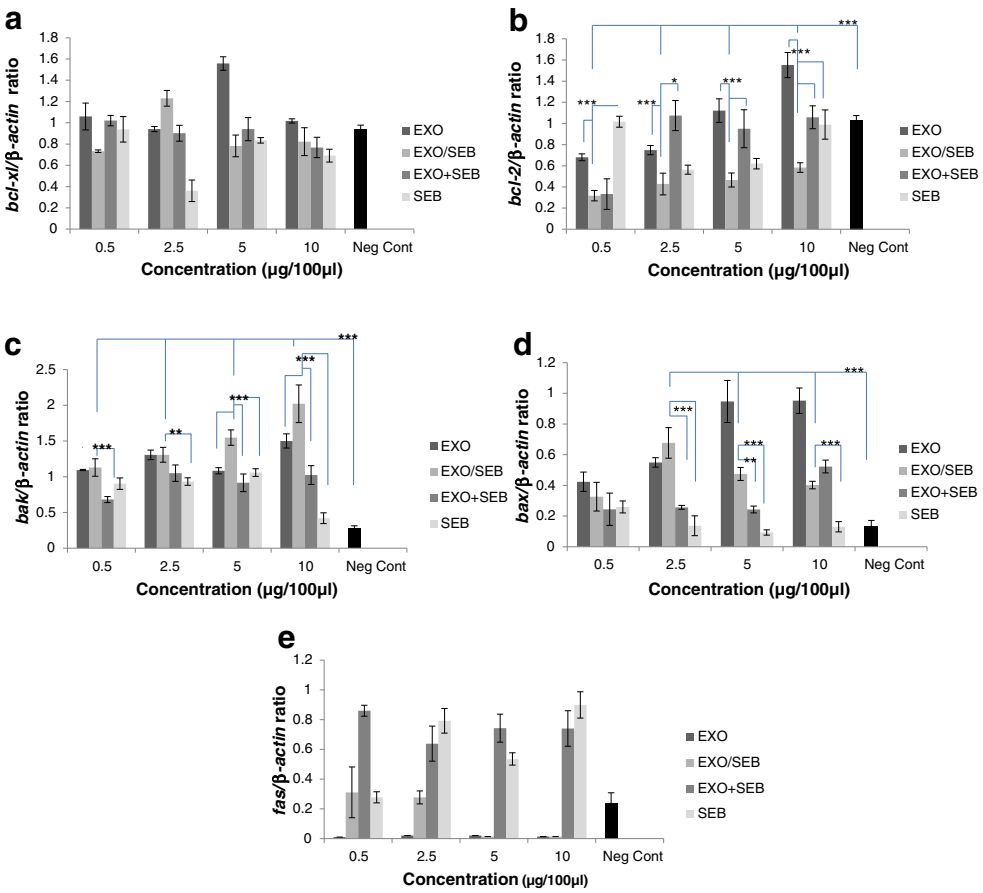

Fig. 7 Effect of EXO/SEB on the expression of *fas*, *bcl-xl*, *bax*, *bcl-2* and *bak* in MDA MB-231. The mRNA levels of the mentioned gene was relatively quantified by RT-PCR in the MDA MB-231 cell treated with the four different concentrations of EXO/SEB (0.5, 2.5, 5 and 10 μg/100 μl) after 48 h. β-*actin* was examined as a housekeeping gene and consequently the results showed as a ratio of desired gene expression/β-*actin* expression. EXO/SEB showed no effect on the expression of *bcl-xl* gene (**a**), but it can reduce the mRNA expression of *bcl-2* at all tested concentrations (**b**) ($P<0.001$). In addition, EXO/SEB increased the expression of *bak* at the all tested concentrations (**c**) ($P<0.001$) and *bax* at the concentrations of 2.5, 5 and 10 μg/100 μl (**d**) ($P<0.001$). The expression of *fas* severely attenuates after treating cells with EXO/SEB (**e**)

on two described normal cells demonstrated that EXO may influence only on the origin tumor cells. Moreover, findings from Hoechst staining demonstrated h apoptotic cells higher with raising the concentration of EXO/SEB. Outcomes reported by Ristorcelli and colleagues [11] showed anti-proliferative effect of tumor derived EXO on some pancreatic cancer cell lines because of reducing the level of hes-1 and stimulating the PTEN and GSK-3β activation [10]. Regarding the previous described results, we observed that EXO/SEB was able to induce cytostatic events via apoptosis in insensitive human ER^- breast cell line. Since a significant difference was seen between the exposure to the EXO/SEB at the studied concentrations and an equal concentration of SEB, EXO and EXO + SEB groups, it could be suggested that EXO/SEB had achieved new functional properties.

The increased expression of anti-apoptotic 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 as anti-apoptotic effectors can accelerate apoptosis [22].

Our results revealed that EXO/SEB augmented the mRNA level of *bak* and *bax*, the anti apoptotic genes involved in mitochondrial dependent pathways. On the other hand, it is able to attenuate the expression of *bcl-2* a pro apoptotic gene. Although we demonstrated no influence on the mRNA level of *bcl-xl*, but increase the amount of *bax* leads to increase the ratio of *bax*/*bcl-xl*, which ultimately triggers cell apoptosis. Despite treating the cells with SEB alone and the mixture of SEB and EXO, which increases the expression of *fas*, EXO/SEB and EXO have an inhibitory role in the expression of this gene. These results suggested that the nature of EXO/SEB and consequently, its features were a quiet different from SEB.

In recent years, it has been reported the direct correlation of down regulation of caspase-3, an important member of the caspase cascade, and breast carcinogenesis [23]. In this study, we observed a significant increase in the activity of caspase-3 and -9 after 24 h but no changes were seen after 48 h. Ristrocelli et al. [11] suggested EXO 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. Further, bax stimulates releasing of cytochrome *c* from mitochondria and finally results in apoptosis [24]. Furthermore, results from the caspase activity analysis confirmed our observation from Hoechst staining. Various death signals are commonly able to activate the caspase-3 that it consequently leads the cleavage of numerous significant proteins, which they are correspondence of DNA fragmentation and cellular apoptotic changes [25]. The present study pointed that mitochondrial

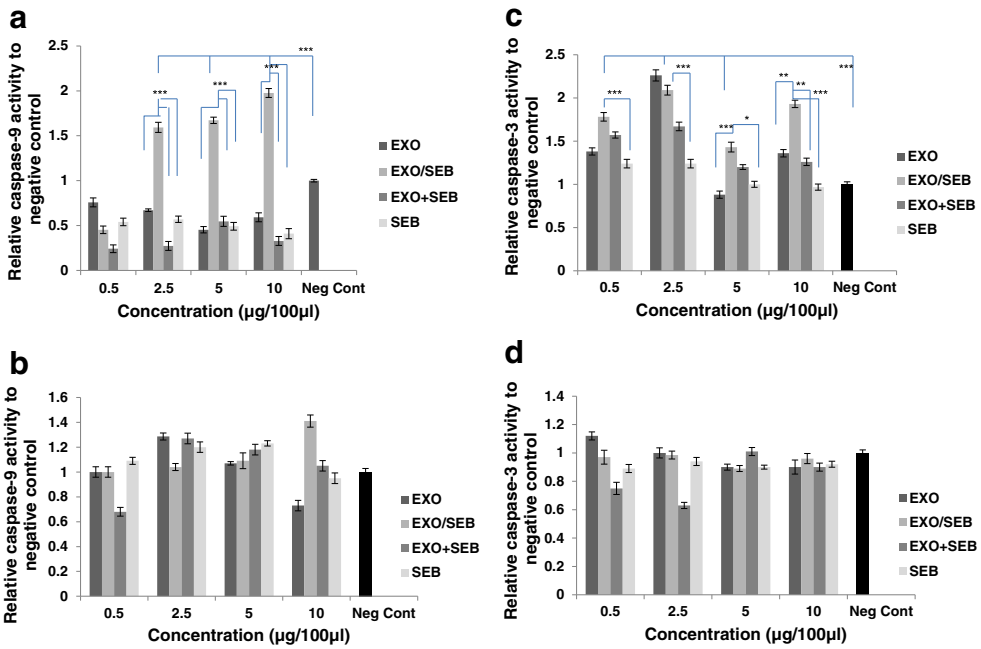

Fig. 8 Effect of EXO/SEB on inducing the activity of caspase-3 and caspase-9 in MDA MB-231. Caspase activity was quantified by the colorimetric method in the MDA MB-231cell following the treatment with the four different concentrations of EXO/SEB (0.5, 2.5, 5 and 10 µg/100 µl) after 24 and 48 h. After 24 h, EXO/SEB significantly increased the activity of caspase-9 at the concentration of 0.5, 2.5 and 5 µg/100 µl ($P < 0.001$) (**a**) and caspase-3 at all concentrations ($P < 0.001$) (**c**). Our product had no impact on the activity of caspase-3 and caspase-9 after 48 h (**b**, **d**)

dependent pathway is a main pathway involved in apoptosis stimulated by EXO/SEB.

To our knowledge, the current study is the first one to state that EXO/SEB induces cell cytotoxicity in human ER$^-$ breast cancer cell line. 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. Besides, the attendance of tumoral antigens accompanied with superantigen leads to enhancement of specific anti-tumor immune response. Another advantage of this structure is its impact on human ER$^-$ breast cancer cell lines that are insensitive to hormone therapy. Of course, further studies on the molecular and cellular mechanisms are required to support our results.

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Conflicts of interest The authors confirm that this article content has no conflict of interest.

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