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Original Article

EL4-derived Exosomes Carry Functional TNF-related Apoptosis-inducing Ligand that are Able to Induce Apoptosis and Necrosis in the Target Cells

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Exosomes released by tumor cells play critical roles in tumor progression, immune cell suppression, and cancer metastasis. The aim of the present study was to investigate whether the exosomes released by EL4 cells carry a functional TNF-related apoptosis-inducing ligand (TRAIL) molecule. Exosomes were harvested from the supernatants of EL4 cell culture, and the shape, size, and identity of EL4-derived exosomes were evaluated by utilizing scanning electron microscopy, dynamic light scattering, and dot-blot method. The expression of mRNA and TRAIL protein in EL4 cells and EL4-exosomes were investigated using real-time PCR method and dot-blot analysis. Moreover, the effects of EL4-derived exosomes on cell death in a TRAIL-sensitive cell line (4T1) were studied by using flow cytometry (annexin V/propidium iodide (PI) staining) and fluorescent microscopy analyses (acridine orange/ethidium bromide staining). The results showed that EL4 cells continuously and without the need for stimulation, produce exosomes that carry TRAIL protein. In addition, EL4-derived exosomes were capable to induce apoptosis as well as necrosis in 4T1 cells. It was ultimately revealed that EL4 cells express TRAIL protein and release exosomes containing functional TRAIL. Moreover, the released exosomes were able to induce apoptosis and necrosis in a TRAIL-sensitive cell line. Further studies are needed to reveal the potential roles of tumor-derived exosomes in the pathogenesis of cancers.

Key words: Apoptosis, exosomes, necrosis, TNF-related apoptosis-inducing ligand, tumor

The programmed cell death or apoptosis has an indispensable role in the regulation of normal cells and cancer biology. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane, homo-trimmers apoptosis messenger protein, and belongs to the

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TNF superfamily of cytokines (1). TRAIL induces apoptosis in target cells through binding to death domain (DD)-containing receptors. In murine, a unique death- mediating TRAIL receptor 2 (TRAIL-R2) and two decoy receptors, DcTR-AILR1 and DcTRAILR2 have been identified (2).

Exosomes are lipid membrane-enclosed nano-particles released by most of the cells (3). These vesicles carry surface receptors, cytosolic proteins, RNAs, miRNAs, enzymes, and originating cells' DNA (4). TRAIL is one of the important molecules transferred by some exosomes; it is assumed that the exosomes-containing-TRAIL are able to induce apoptosis in target cells. Stenqvist et al. (2013) indicated that the human placenta releases exosomes that contain functional TRAIL, which are capable of inducing apoptosis in the activated immune cells (5). Yuan et al. (2017) showed that the exosomes derived from TRAIL-transfected-mesenchymal stem cells expressed TRAIL on their surface. These exosomes were able induce apoptosis in 11 human cancer cell lines, in a dose-dependent manner (6). In another study, Rivoltini et al. used a lentiviral vector containing the human membrane-bound TRAIL cassette to transduce K562 cells. They showed that TRAIL-armed exosomes released by transfected cells could induce apoptosis in cancer cells, and also control tumor progression *in vivo* (7).

Despite the fact that in several studies, the TRAIL-containing exosomes have been used to evaluate their effects on tumor cells, no study has investigated the expression and activity of TRAIL in exosomes that are produced by tumor cells.

The aim of the present study was to determine whether exosomes released by a tumor cell line carry functional TRAIL. For this purpose, the TRAIL-producing EL4 cell line was selected for investigation. To the best of our knowledge, this is the first report that shows EL4-exosomes carry the functional TRAIL.

Materials and methods

Cell culture

The study was conducted according to criteria set by the ethics committee of Shahid Beheshti University of Medical Sciences. EL4 is a TRAIL-resistant, leukemia/lymphoma cell line (of C57BL/6 origin), and 4T1 is a TRAIL-sensitive, mouse mammary carcinoma cell line. The above-mentioned cell lines were purchased from the Pasteur Institute of Iran. Dulbecco's modified Eagle's medium (DMEM) was used to culture 4T1 cells. For EL4 cell culture, RPMI-1640 was used instead of DMEM. The medium was supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were grown in a 5% CO_2-air atmosphere and a 37°C environment. All culture reagents were purchased from Gibco (Gibco; Thermo Fisher Scientific Inc., USA).

Exosomes isolation

To isolate 4T1- and EL4-derived exosomes, the cells were cultured in DMEM and RPMI-1640 medium, respectively. The FBS concentration was reduced gradually and was replaced with insulin-transferrin-selenium (IT'S) (Gibco; Thermo Fisher Scientific Inc., USA) as a basal-supplement. Before exosomes isolation, the final concentration of FBS and ITS in culture media was 0% and 1%, respectively. The cell culture conditioned media was collected twice every other day; then was centrifuged at 300×g for 10 min. Exosomes were separated by using EXOCIB isolation kit (CIB Biotech Co., Iran) according to the instructions of the manufacturer. Eventually, the isolated exosomes were diluted in 200 µL PBS, and were stored at -20°C until the usage. By using a bicinchoninic acid (BCA) protein assay kit (Aryatus, Mashhad, Iran), the concentration of exosomes-related proteins was measured and was considered as an equivalent to exosome concentration.

Scanning electron microscopy (SEM)

Scanning electron microscopy (Digital SEM,

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KYKY, EM3200, China) was used to analyze the size and shape of isolated exosomes. In order to analyze, the exosomes were fixed with 2.5% glutaraldehyde (Sigma–Aldrich, Germany) in PBS for 15 min, followed by twice washing with PBS, and gold-palladium sputtering.

Dynamic light scattering (DLS)

By using a dynamic light scattering (DLS) instrument (Malvern Instruments, UK), the distribution of the exosomes particle size was evaluated. Exosomes were diluted 1:1000 with PBS for analysis.

Dot-blot analysis

In order to evaluate CD63 expression as an exosomal marker and also TRAIL protein the dot-blot analysis was used (8). At first, EL4 lysate was prepared using lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM PMSF, 50 mM Tris pH 7.5) (9). The lysates were centrifuged (10,000 g, 10 min, 4 °C) after 30 min incubation. Then, 10 µg exosomes and EL4 lysate were blotted on the nitrocellulose membrane. The procedure was followed by blocking the nitrocellulose membrane using 5% skim milk. The detection of mouse CD63 and TRAIL antigens in the exosomal lysate was performed by adding 10 µL of 100 µg/mL CD63 antibody (Rat anti-mouse κ Isotype, BioLegend, USA) and anti-TRAIL antibody (Rat anti-mouse, R&D Systems, USA). Afterward, the membrane was incubated for 1 h at room temperature; then the membrane was washed using Tris-buffered saline containing 0.1% Tween-20. The membrane was incubated after adding a horseradish peroxidase-conjugated secondary antibody (Goat anti-rat R&D Systems, USA). Subsequent to immunoblotting, the substrate was added, and the emitted light was recorded using an enhanced chemiluminescence (ECL) Western blotting system (Cyto matin gene, Iran). The neutrophil lysate was used as a positive control for analysis of the presence of the CD63 molecule.

Quantitative real- time polymerase chain reaction (qRT-PCR)

In order to analyze the presence of *TRAIL* mRNA in EL4 lysate, qRT−PCR assay was performed. Total RNA was extracted using the QIAamp Qiagen kit (Qiagen, Germany), according to the instruction of the manufacturer. The extracted RNA was converted to cDNA by using a PrimeScript™ RT cDNA synthesis kit (Takara, Japan) according to the manufacturer's instructions. Subsequently, PCR amplification was performed by using SYBR Green Master Mix (Amplicon, Denmark) and Rotor gene thermocycler (Corbett Rotor-Gene, Australia). The following primers sequences were used to amplify the *TRAIL* gene. Forward: 5'CCCTGCTTGCAGGTTAAGAG3', reverse: 5'GGCCTAAGGTCTTTCCATCC3'. The experiment was performed in duplicate.

Apoptosis and necrosis assay

In order to investigate the apoptosis rate in 4T1 cells, two assays including fluorescent microscopy using acridine orange/ethidium bromide (AO/EB) staining and, flow cytometry assay using annexin V/ PI staining was performed. Primarily, the 4T1 cells (2×10^5 cells/well) were co-incubated for 24 h with EL4-exosomes or 4T1-exosomes, at 37 °C. For apoptosis assay by flow cytometry, the cells were rinsed by 0.25% trypsin-EDTA solution until the detachment of cells occurred. The procedure was followed by centrifugation at 300 ×g for 5 min. Immediately, the cells at a concentration of 2×10^5 cells/mL were suspended in 1× binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). The cells were incubated for 15 min at room temperature by fluorescein isothiocyanate labeled anti-annexin V antibody (Thermo Fisher Scientific, USA). After washing with binding buffer, the cells were re-suspended in 200 µL binding buffer. Then, the PI staining solution (5 µL, eBioscience, USA) was added. In the following, within 1 h, the cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, USA). The data were analyzed

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using FlowJo software (FlowJo). For apoptosis assay by a fluorescent microscope, fluorescent staining solution (1 µL) containing 100 µg/mL acridine orange and 100 µg/ml ethidium bromide (AO/EB, Merck, Germany) were added to the culture medium (10). Immediately, the incorporation of ethidium bromide into the dead cells' DNA was examined using a fluorescent microscope (Nikon, Japan).

Statistical analysis

For each analysis, three biological and two technical replicates were done. SPSS version 22 (SPSS) was used for statistical analyses. Mann-Whitney U Test was used to compare the means of nonparametric data. P values less than 0.05 were considered as significant. The data are shown as the mean ± S.E.M (standard error of the mean). The graphs were produced by GraphPad Prism software version 6.0 (GraphPad Prism, USA).

Results

Exosomes released by tumor cell lines showed typical features

The exosomes which were released by the EL4 and 4T1 cells were isolated from the serum-free culture supernatants. The evaluation of the size and shape of the isolated exosomes were done by using SEM (Figure 1a). The obtained images showed three-dimensional spherical vesicles, that most of them were less than 100 nm in size. Moreover, the exosomes size distribution was identified by the DLS technique (Figure 1b). The obtained data indicated that the mean size of the exosomes was 50 nm, which was in the expected size distribution range i.e. 30 to 100 nm. In addition, to verify the identity of exosomes, the presence of CD63 as one of the exosomal marker was shown using the dot-blot method (Figure 1c). Indeed, the obtained data indicated that the isolated exosomes have a typical size and morphology.

EL4 cells produce TRAIL- expressing exosomes

Initially, the expression of *TRAIL* in the EL4 cell line was shown by RT-qPCR analyses (Figure 2a). Additionally, the expression of TRAIL in exosomes was confirmed using the dot-blot analysis (Figure 2b). The results showed that the EL4 cells without the need for stimulation produced exosomes that carried the TRAIL protein. As a negative control, we analyzed the expression of *TRAIL* of 4T1cells

EL4-exosomes induced apoptosis and necrosis in 4T1 cells

To evaluate whether tumor-derived exosomes could induce apoptosis, the exosomes were harvested from the cultured EL4 cells, and their effect on 4T1 cells was evaluated. Following the treatment by TRAIL-containing exosomes, annexin /PI and AO/EB staining were used to determine the apoptosis rates in 4T1 cells. In a pilot study, several

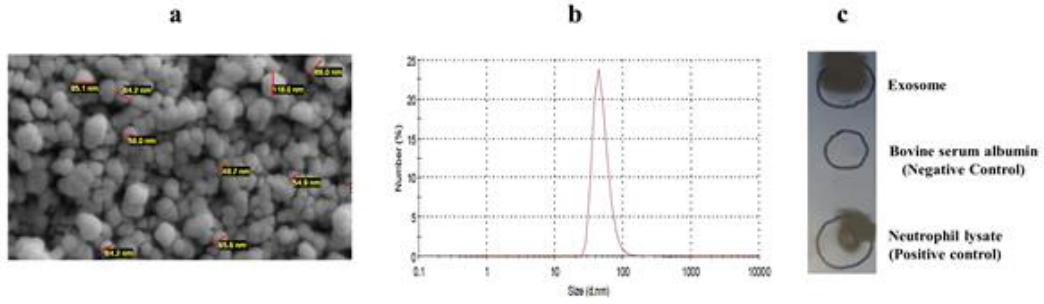

Fig. 1. The characteristic of isolated exosomes. (a) The shape and size of isolated exosomes were evaluated by scanning electron microscopy. The results demonstrated that most of the exosomes had a spherical shape with a size of less than 100 nm; **(b)** The histogram shows the distribution of the size of exosome which has been determined by using DLS; **(c)** Dot-blot analysis was used to demonstrate the presence of CD63 protein in the separated exosomes. Three biological and two technical replicates were assessed.

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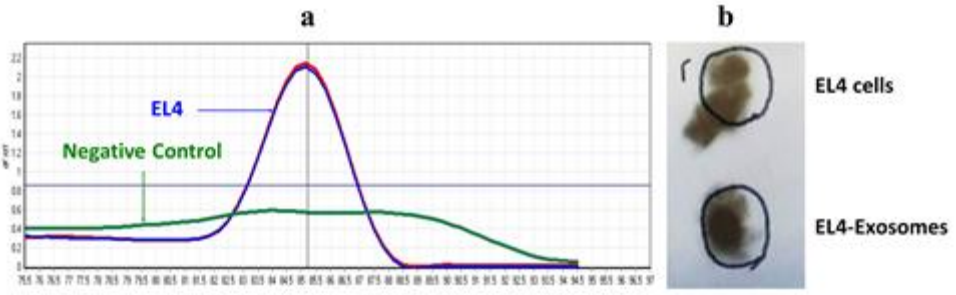

Fig. 2. Expression of TRAIL by EL4 cells and EL4-exosome. (a) The expression of TRAIL-mRNA in EL4 cells was evaluated using qRT-PCR. Melt curve analysis showed a peak at 85.5 °C, which was according to the expected melting point of the PCR product of TRAIL. The 4T1 cells were used as negative control. (b) Dot-blot analysis was performed in order to assay the TRAIL expression in EL4 cells and also in EL4-exosomes. The experiments were performed in duplicate.

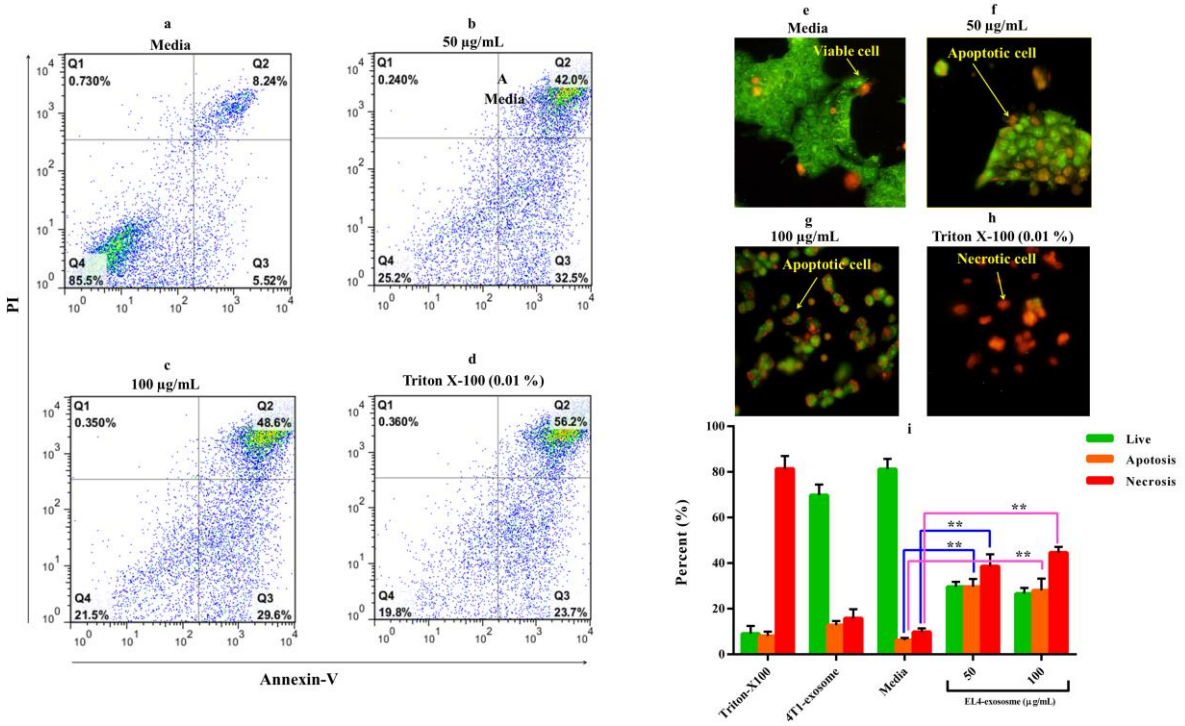

Fig. 3. Analysis of the apoptosis and necrosis rates in 4T1 cells treated with EL4-exosomes. Annexin /PI staining was used to evaluate cell death in 4T1 cells, which were cultured in the **(a)** absence of exosome; **(b)** in the presence of 50 µg/ml EL4-exosomes; and **(c)** in the presence of 100 µg/mL EL4-exosomes. Additionally, fluoromicrography (× 200) was performed after AO/EB staining in 4T1 cells, which were cultured in the presence of **(e)** media, **(f)** 50 µg/ml, and **(g)** 100 µg/mL EL4-exosome. **(d & h)** Triton X-100 treatment was used to induce apoptosis in EL4 cells; **(i)** in order to compare apoptosis rate in treated cells, the number of live, apoptotic and necrotic cells was determined in 100 cells. The experiment shown is representative of three different experiments. Kruskal-Wallis Test was performed to analyze the differences in the groups. **P ≤ 0.01. These assays were performed in triplicate.

concentrations of exosomes including 10, 20, 30, 50 and 100 µg/mL were examined. The best-obtained results were for concentrations of 50 and 100 µg/mL, which were considered for the subsequent steps of the study. As it is presented in Figure 3, TRAIL-containing exosomes at the concentrations of 50 and 100 µg/mL induced significant apoptosis and necrosis in 4T1 cancer cells, contrary to the control groups treated with PBS. The frequency of cells that were in early-apoptotic state or were necrotic, at the concentrations of 50 µg/mL EL4-exosomes was 32.80 ± 3.36% and 34.00 ± 3.49%, respectively, which was significantly higher than the control incubated with media (for apoptosis: 5.60 ± 1.07%, P = 0.009, and for necrosis: 11.10 ± 1.98%, P = 0.009; Figure 3a and b). In addition,

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EL4 exosomes at the concentration of 100 µg/ml caused a significant increase in apoptosis and necrosis rate in comparison with the control group (25.60 ± 4.98%, P = 0.009 and 48.60 ± 6.10%, P = 0.009, respectively; Figure 3c). However, the apoptosis or necrosis rate in 4T1 cells which were treated with 100 µg/mL EL4-exosomes was not significantly different in comparison with the cells treated with 50 µg/mL EL4-exosomes (P > 0.05).

Furthermore, the rates of TRAIL-induced apoptosis and necrosis in 4T1 cells were studied by using AO/EB staining and fluorescence microscopy (Figure 3i). Generally, the apoptosis and necrosis rates which were recorded for the controls and treated cells were distinctly comparable in both methods (P > 0.05).

Discussion

In the current study, it was showed that EL4-derived exosomes carry functional TRAIL molecules, as an inducer of cell death for the sensitive cell line.

The expression of TRAIL on the surface of many cancer cells, such as EL4 was shown previously (11). Nevertheless, the number of studies showing that tumoral cells are capable to produce exosomes containing TRAIL protein is small. Cells that have been confirmed to be able to produce TRAIL-positive exosomes include human melanoma cell line (12), human colorectal cancer cells (13), and human Jurkat cells (14). In the current study, EL4 cell was chosen because of its ability to produce TRAIL protein, as well as, because of its resistance to TRAIL-mediated apoptosis. While other studies have used recombinant methods to produce TRAIL positive exosomes (6,7), we have shown that EL4 cells can release exosomes containing TRAIL without stimulation and continuously.

The production of TRAIL protein by tumor cells is one of the pivotal strategies for escaping from the immune response through inducing apoptosis in tumor-infiltrated lymphocytes (15). It seems that the delivery of TRAIL by exosomes enhances the cytotoxic potential of the TRAIL because it facilitates the multimerization of TRAIL that is a crucial step for apoptosis induction (12). In addition, exosomes are a natural delivery system that guarantees stable bio-distribution of their cargoes in the body (16). Furthermore, exosomes contain several types of proteins and genetic materials, which may act synergically with TRAIL to increase cell death in targets (17). In addition, TRAIL induces cell death in the endothelial cells in order to facilitate extravasation through the endothelium (18), thus TRAIL plays an important role in cancer metastasis (19,20). Similar to the soluble TRAIL, TRAIL-containing exosomes may employ another mechanism to suppress tumor-associated immune responses. It has been shown that TRAIL triggers cytokine secretion by TRAIL-resistant cancer cells. The secreted cytokines are capable of polarizing the monocyte to myeloid-derived suppressor cells and M2-like macrophages (1). In addition, tumor-derived exosomes as an independent entity are able to suppress many types of immune cells. A recent study showed that exosomes derived from tumor cells dampen the antigen-specific immune response, conversely augment the functions of regulatory T cells (8). Thus, TRAIL-armed exosomes could be considered as a potent weapon of tumor cells to kill the immune response.

Besides immune cells, tumor cells themselves may be targeted by TRAIL. Our results showed that EL4-exosomes also are able to induce apoptosis and necrosis in 4T1 cells. The necrosis induced by TRAIL was reported in different human cancer cell lines with distinct origins including leukemia, gallbladder adenocarcinoma, non-small cell lung carcinoma, and malignant melanoma (21). It should be pointed out that TRAIL, as well as FasL and TNF-α, could induce an alternative type of cell death called programmed necrosis or necroptosis

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(22–26). Besides the role of TRAIL-induced necrosis in eliminating cancerous cells, it has been elucidated that necrosis is an immunologic cell death which is able to initiate inflammation. Under this circumstance, macrophages uptake the necrotic cells by phagocytosis; afterward, stimulate T cells by presenting tumor-antigens (27,28). Thus, inducing necroptosis in cancerous cells can be quite beneficial in a therapeutic setting. In a recent study, the vaccination with necroptotic CT26 cells was introduced as an alternative approach in cancer therapy (28). Therefore, TRAIL-induced cell death is a complicated event that has both positive and negative effects on cancer development (20).

In conclusion, the results of the present study showed that EL4 cells release exosomes bearing the functional TRAIL, with no need for stimulation. The released exosomes were capable to induce apoptosis and necrosis in a TRAIL-sensitive cell line. Further studies are needed to elucidate the impact of TRAIL-containing exosomes on the immune suppression, as well as on the tumor metastasis process.

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Conflict of interest

The authors declare that there are no conflicts of interest related to this paper.

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