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ORIGINAL ARTICLE

Exosomes Derived from Irradiated Esophageal Carcinoma-Infiltrating T Cells Promote Metastasis by Inducing the Epithelial–Mesenchymal Transition in Esophageal Cancer Cells

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Abstract Exosomes are nanovesicles derived from tumor and normal cells that are detectable in human biological fluids, such as plasma, and cell culture supernatants. The function of exosome secretion from "normal" cells is unclear. Although numerous studies have investigated exosomes derived from hematopoietic cells, little is known regarding exosomes fromT cells, even though these cells play significant roles in innate and acquired immunity. A CCK-8 assay was used to examine the ability of exosomes to inhibit TE13 cell proliferation. In vitro invasion and wound healing assays were conducted to explore the effects of exosomes on TE13 cell migration and invasion. A Western blottinganalys is was performed to investigate the effects of exosomes on the expression of the EMT-related moleculesβ-catenin, NF-κB and snail. This study aimed to investigate the effects of exosomes from irradiated T cells on the human esophageal squamous cell carcinoma (ESCC) cell line TE13 and revealed that exosomes inhibit the proliferation but promote the metastasis of TE13 cells in a dose-and time-dependent manner. Furthermore, exosomes significantly increased the expression of β-catenin, NF-κB and snail in TE13 cells. The results of this study suggest an important role for T cell-derived exosomes in the progression of esophageal carcinoma: T cell-derived exosomes promote esophageal cancer metastasis, likely by promoting the EMT through the upregulation of β-catenin and the NF-κB/snail pathway. Moreover, this study supports the use of exosomes as a nearly perfect example of biomimetic nanovesicles that could be utilized in future therapeutic strategies against various diseases, including cancer.

Keywords T cells · Radiotherapy · Exosome · Esophageal carcinoma · Epithelial–mesenchymal transition · Metastasis

Introduction

Esophageal cancer (EC) is the sixth most common cause of cancer-related deaths world wide, and squamous cell carcinoma of the esophagus (ESCC) is the main pathological type of EC [1]. Although radiotherapy plays a significant role in the local control of EC, the survival rate of esophageal cancer patients remains less than 20% because radiotherapy may not hinder the metastasis of the primary tumor and tumor metastasis leads to death [2]. Recent studies have indicated that the epithelial–mesenchymal transition (EMT) plays a critical role in the progression of cancer radioresistance. The EMT is the process through which epithelial cells change from an epithelial cobblestone phenotype to an elongated fibroblast phenotype. At present, the EMT is known to be involved in the movement of tumor cells to distant areas and the development of treatment resistance [3, 4].

Over the last century, the notion that the immune system can control cancer has been the topic of much debate. In the early 1900s, Paul Ehrlich was perhaps the first to suggest that cancer would be rampant in longevous organisms without protection from the immune system [5]. Nevertheless, the immune system can also accelerate cancer progression by provoking chronic inflammation and by promoting factors that

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drive tumor growth, angiogenesis, and survival [6]. Accumulating evidence suggests that radiation therapy can trigger animmune response [7]. A series of reactions occur after radiotherapy, beginning with tumor cell damage via multiple mechanisms and leading to positive feedback from the immune system to the cancer. It is also known that ionizing radiation (IR) leads to an increase in tumor-infiltrating lymphocytes [8, 9]. Therefore, we hypothesize that one factor that enables the immune system to fight cancer is the secretion of exosomes from tumor-infiltrating lymphocytes. Exosomes are small membrane vesicles (50–100 nm in diameter) of endocytic origin that are formed through the fusion of the plasma membrane with multivesicular endosomes (MVEs) followed by exocytosis. Many types of cells, including dendritic cells (DCs), T cells, macrophages, B cells, mastocytes, reticulocytes and tumor cells, can inductively or constitutively secrete exosomes [10].

Exosomes play a significant role in intercellular communication [11], which occurs via cell-cell synapses, direct cell-cell contacts and receptor-mediated events. Exosome-mediated intercellular communication represents a new mechanism of communication between cells. Exosomes can shuttle proteins, mRNAs and microRNAs between cells [12]. The biological functions of DC-derived exosomes (DCex) on tumor cells have been widely studied [13–15]. Stephan Munich reported that DCex not only express co-stimulatory molecules and functional transmembrane major histocompatibility complexes (MHCs) that enable them to indirectly stimulate an adaptive T cell response [16] but also express functional transmembrane TNF superfamily ligands (TNFSFLs) that enable them to directly mediate innate immune functions, such as NK cell activation, and tumor cell apoptosis [17]. However, the effect of immune cells on the invasion and migration of tumor cells has not been reported. Therefore, in this article, we discuss whether exosomes from tumor-infiltrating T cells in esophageal carcinoma promote the migration and invasion of esophageal cancer cells in vitro. Cells from the human ESCC cell line TE13 and immortalized T lymphocytes (Jurkat cells) were cultured.

Materials and Methods

Cell Culture and Reagents

The Shanghai Institute of Cell Biology provided the human ESCC cell line TE13, and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Carlsbad, CA,USA) supplemented with 10% fetal bovine serum (HyClone,GE Healthcare, Little Chalfont, UK) and 1% penicillin/streptomycin (Invitrogen, Life Technologies). Immortalized T lymphocytes (Jurkat cells, KG053) were kindly provided by KeyGen Biotech and were cultured in RPMI 1640 medium (Gibco, Barcelona, Spain) supplemented with 10% fetal calf serum using standard cell culture procedures. The cells were cultured in an incubator at 37 °C in an atmosphere containing 5% CO_2.

Cell Viability Assay

Cell proliferation was assessed using a cell counting kit-8 (CCK-8) assay. We seeded TE13 cells onto 96-wellplates at a density of 5×10^3 cells/well for 24 h and then incubated the cells with different concentrations of exosomes derived from T cells exposed to different doses of radiation (0,2,4,6,and 8Gy). After 24 h of incubation, we used a CCK-8 cell proliferation and cytotoxicity assay kit (Obio Technology, Shanghai, China) to analyze the cells and measured the absorbance at a wavelength of 490 nm.

Preparation of Exosomes.

Exosomes were isolated by ultracentrifugation. Tcells were cultured in RPMI 1640 medium without serum for 24 h after radiation. The cells and cellular debris were removed by centrifuging the media at 300 g for 5 min and at 2000 g for 20 min at 4 °C. Filters (0.22 μm, American Millipore Corporation) were then used to remove any remaining impurities before pelleting at 120,000 g and 4 °C for 2 h. The supernatant was then discarded, and the exosomes were resuspended in PBS and stored at −80 °C.

Nanoparticle Tracking Analysis

We identified the exosomes through nanoparticle tracking analysis (NTA). The exosomes were diluted in 5 ml of physiological saline and injected into a Nanosight NTA NS300 instrument (MalvernInstruments Ltd.) using asterile injection syringe. We then collected dynamic images and analyzed the concentration and distribution of the exosomes.

Migration Assay

Esophageal cancer cells were seeded in six-well plates and grown to80% confluence. We then treated each well with a different concentration of exosomes generated with different doses of radiation. We used a 200-μl pipette tip to draw a straight line in the middle of the cell monolayer. The cell migration distance after 0 and 24 h was observed and measured using an optical microscope.

Western Blot Analysis

The total protein from tumor cells was extracted using SDS Lysis Buffer (KeyGen, Nanjing, China). Equal amounts of protein from each lysate were separated by SDS-PAGE (10%acrylamide) and transferred to PVDF membranes (Millipore). The membranes were blocked and then probed

overnight with primary antibodies against NF-κB, β-cadherin, snail and β-actin, which was used as a loading control.

Transwell Invasion Assay

Invasion assays were performed using transwell chambers (Corning, MA, USA) with polyethylene terephthalate filters (8-μm pore size), which were covered with 2 mg/ml of reconstituted basement membrane (Matrigel,Corning, MA, USA). We added 500 μl of medium supplemented with 20% fetal bovine serum (FBS) to the bottom chamber as the chemotactic factor and then seeded 1×10^5 tumor cells in 500 μl of DMEM (without FBS) into the topchamber. Subsequently, different concentrations of exosomes generated with different doses of radiation (0,2,4,6,and 8Gy) were added to the top chambers, and the cells were cultured for 24 h. The invasive cells were fixed with cold 4% paraformaldehyde, stained with 0.1% crystal violet for 15 min, and then washed three timeswith PBS. The fixed cells in three random fields were photographed under a light microscope (×200).

Data Analysis

We calculated the means ± standard deviations (SDs) from triplicate assays and determined the differences between the treatment groups by ANOVA. The statistical analyses were performed using Prism 5.0 software (GraphPad, La Jolla, CA, USA) and STATA 11.0 software (StataCorp, College Station,TX, USA). $P < 0.05$ was considered statistically significant.

Results

Irradiated T Cell-Derived Exosomes Affect the Growth of TE13 Cells

Before discussing the biological function of exosomes, we first wanted to determine whether radiation can stimulate T cells to release more exosomes. Previous studies have demonstrated that radiation can stimulate glioma cells, prostate cancer cells and lung cancer cells to release exosomes [18, 19]. The NTA results showed that exosome secretion was increased by radiation and was positively correlated with the radiation dose. Moreover, radiation did not change the size or diameter of the exosomes (Fig. 1a), confirming the results obtained in previous studies. Based on the finding that T cells secreted different concentrations of exosomes in response to different doses of radiation, a CCK-8 assay was used to explore whether exosomes directly affect TE13 cells. The CCK-8 assay revealed that higher concentrations of exosomes were associated with greater inhibitory effects on the growth of TE13 cells (Fig. 1b).

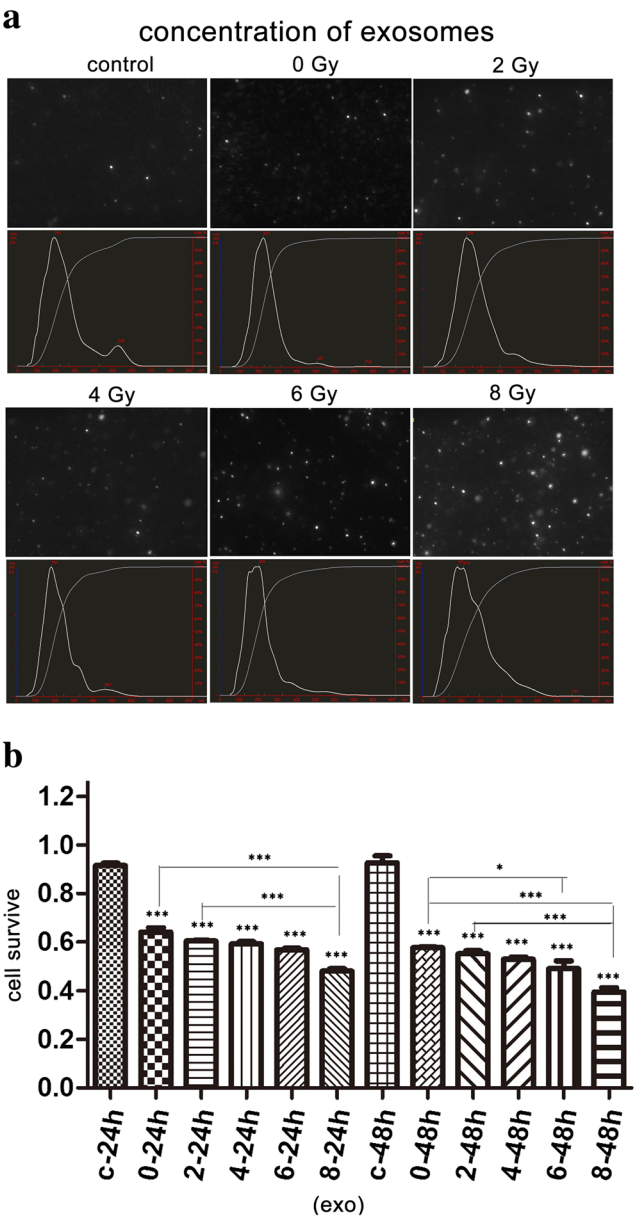


Fig. 1 Effects of exosomes on cell viability. **a** Extraction of exosomes released by T cells after exposure to different doses of radiation (0, 2, 4, 6, and 8Gy). The nanoparticle trace analysis indicated that increases in the radiation dose resulted in increases in the number of exosomes. The diameter of the particles was between 100 and 300 μm, further confirming their identity as exosomes. **b** When co-cultured,the exosomes inhibit the proliferation of TE13 cells. The effect of the exosome extracts increased with increasing doses of radiation. Each experiment was performed in triplicate. The results are shown as the means of three experiments, and the error bars represent the standard deviations (*$P < 0.05$)

Exosomes Promote the Migration and Invasion of Esophageal Carcinoma Cells, And this ability Is positively Correlated with the Radiation Dose

Transwell and wound healing migration assays were performed to further evaluate the effects of exosomes derived

from T cells exposed to different doses of radiation. The wound healing assays revealed that exosomes significantly promoted the migration of cancer cells (Fig. 2). Moreover, the transwell assays demonstrated that a higher radiation dose was associated with more invasive cells. The percentages are shown in Fig. 3. We found that exosomes secreted by cells exposed to 0-,2-,4-,6-,or 8-Gy irradiation significantly promoted the migration and invasion of TE13 cells.

Exosomes promote the EMT of TE13 Cells by upregulating the Expression of β-Catenin Andthe NF-κB/Snail pathway*in vitro*

Cells are potentially more highly invasive after the EMT [20]. To investigate whether exosomes promote the metastasis of esophageal cancer cells by inducing the EMT, we selected three representative molecules, namely snail, NF-κB and β-catenin, and assessed their expression levels in TE13 cells treated with exosomes. Snail is a transcription factor that plays key roles in the EMT. Similarly, NF-κB functions as an essential mediator of the EMT, and the NF-κB pathway regulates snail expression through transcriptional and post-translational mechanisms [21]. Moreover, a previous study showed that the translocation of β-catenin from the cytoplasm to the nucleus plays a crucial role in promoting the expression of EMT-related genes, particularly snail [22, 23].TE13 cells were treated with 0-, 2-, 4-,6-, and 8-Gy-radiated exosomes for 24 h and then subjected to western blotting. As shown in Fig. 4,exosomes upregulated the protein levels of snail, NF-κB and β-cadherin in a time-dependent manner.

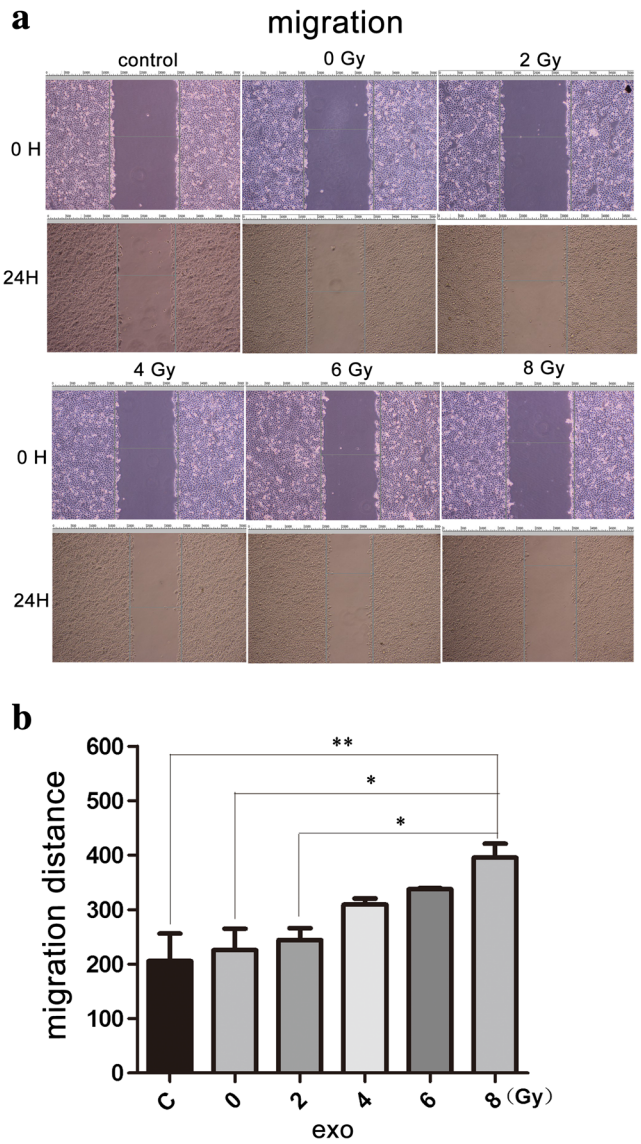
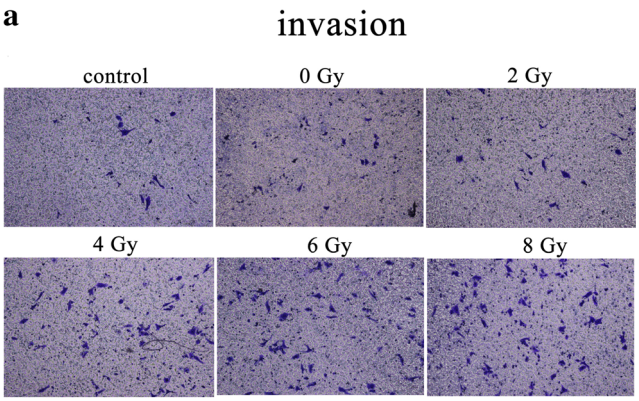


Fig. 2 Effects of exosomes on migration, as determined through a woundhealing migration assay. Exosomes extracted from T cells after radiation and co-cultured with TE13 cells promoted cellular migration in a manner that was directly associated with the radiation dose. A more significant effect was observed with increasing exosome concentrations. Each experiment was performed in triplicate. The results are shown as the means of three experiments, and the error bars represent the standard deviations (*$P < 0.05$)

Discussion

Accumulating evidence suggests that exosome transfer is an important mechanism of signal transduction between tumor cells. In particular, this mode of communication influences tumor biology through the following mechanism: exosomes can transfer biological factors that change the phenotypes of stromal or recipient malignant cells. Exosomes contain a variety of cell-specific proteins depending on the function of the putative target and their cellular origin. For example, DC-and B lymphocyte-derived exosomes carry MHC class-I and class-II proteins [24], and activated T cell-derived exosomes contain bioactive Fas ligand (FasL) that can induce apoptosis in T cells [25]. Additionally, some tumor exosomes carry metalloproteinases, adhesion molecules, and tissue-specific proteins related to metastasis and tumorigenesis [26–28]. Although studies on T cell exosomes are rare, we can learn more by studying exosomes from other APCs, such as DCs. It has been shown that DCex express co-stimulatory molecules and functional MHCs on their limiting membrane. The expression of these molecules enables DCex to efficiently present antigenic peptides to T cells in the background of MHC molecules and induce adaptive immune responses, similarly to their parental DCs [16, 29]. The immune mechanism appears to be mediated through host DCs that acquire functional MHC-peptide complexes from DCex [29]. Whether DCex can express other biologically active DC molecules or mediate immune functions directly has yet to be demonstrated. Stephan Munich first reported that DCex could activate NK cells and induce tumor cell apoptosis [17]. Considering the potential benefits of DCex as an immune therapy, DCex have been developed as cell-free cancer vaccines for clinical



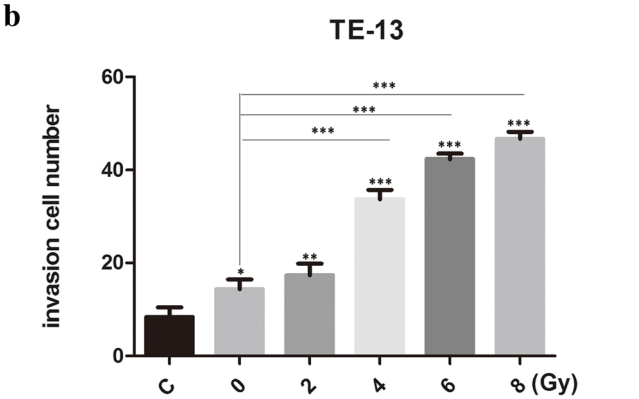


Fig. 3 Effects of exosomes on cell invasion. Transwell chambers were used for the invasion assay, and images were obtained at 200X magnification. TE13 cells were treated with exosomes extracted from T cells that received different doses of radiation (0, 2, 4, 6, and 8Gy). The exosomes extracted from T cells after radiotherapy with increasing radiation dose significantly increased the invasion of the treated cells. Each experiment was performed in triplicate. The results are shown as the means of three experiments, and the error bars represent the standard deviations (*$P < 0.05$)

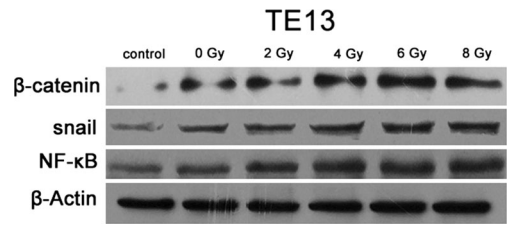


Fig. 4 Effects of exosomes on protein expression levels. A Western blot analysis showed that the exosomes extracted from T cells after radiation significantly increased the expression levels of β-catenin, NF-κB and snail in co-cultured cells. The effect was more significant with an increased radiation dose. Each experiment was performed in triplicate

use. Two phase I clinical trials [30, 31] using DCex have been completed in patients with advanced cancer, and these studies confirmed that the administration of DCex to patients is safe and highlighted the feasibility of massive DCex production. The early clinical trials of DCex as a cancer vaccine showed limited clinical efficacy in advanced cancer patients, although disease stability was observed in all of the studies. Additionally, the in vivo fate and trafficking of DCex in patients remain unknown [32].

Upon activation, peripheral blood T lymphocytes can differentiate into cytotoxic T lymphocytes (CTLs), which contain characteristic exosomes. Studies of the ultrastructure of human CTL-derived exosomes have indicated that cytotoxic exosomes are enveloped with a membrane and contain a dense core and many small internal vesicles. TCR/CD3 complex activation on CTLs triggers exosome redistribution and exocytosis to the target cell (TC) [33]. The actual mechanism through which CTLs damage TCs has been only partially elucidated. Some researchers have suggested that the contents of exosomes are transferred between TCs and CTLs [34, 35]. Perforin, proteoglycans, and granzymes are the primary components isolated from exosome fractions [33]. Under proper conditions, perforin can aggregate into supramolecular tubules, which insert into the TC plasma membrane to lead to TC death and tubular lesions [36]. Additionally, cytotoxic exosomes have been shown to contain specific serine esterases of unknown function. Purified intact exosomes also show potent lytic activity [35–39]. Our study showed that T cell-derived exosomes clearly inhibit the proliferation of TE13 cells (Fig. 1).

Over the past decade, the importance of the EMT in the progression of various cancers has been increasingly recognized [40]. The EMT occurs when epithelial cells transition to a mesenchymal phenotype during various pathological as well as physiological processes, such as fibrosis, wound healing, embryonic development, and tumor progression. The EMT promotes tumor metastasis and alters intercellular adhesion [41–43]. E-cadherin, claudin3, N-cadherin, and vimentin (EMT biomarkers) mediate cell adhesion. The downregulation of claudin3 and E-cadherin expression is necessary for the metastasis of human hepatocellular carcinoma (HCC) cells [44, 45]. Vimentin is an intermediate filament protein that forms the cytoskeleton along with actin filaments and microtubules [21]. The biological activities that initiate and advance the EMT include the activation of transcription factors, the expression of certain cell-surface proteins, the expression and recognition of cytoskeletal proteins, and the production of extracellular matrix-degrading proteases. The transcription factor snail has been shown to be a common downstream target of many signal transduction pathways that regulate the EMT. Snail induces the EMT by suppressing many epithelial markers, including claudins and E-cadherin (the most important protein component of tight junctions). E-cadherin is a molecular marker of the epithelial phenotype, and its expression decreases during the EMT in tumor progression, fibrosis, and embryonic development [23].NF-κB can bind to the human snail promoter and increase snail transcription. Our results revealed that NF-κB expression decreases following exosome treatment of TE13 cells, which is in accordance with the earlier finding that NF-κB is an upstream regulator of snail that indirectly mediates the EMT, and this finding suggests that NF-κB may play a key role in the ability of exosomes

to inhibit tumor progression. β-Catenin participates in adherens junctions and increases cell adhesion by binding to the intracellular transmembrane domain [46]. In non-tumor cells and normal epithelial cells, β-catenin localizes to the cell membrane after the EMT. During the EMT, β-catenin mediates the interaction between the cytoskeleton and cadherins and forms the transcription factor coactivation complex (β-catenin/TCF/LEF complex) with T-cell factor (TCF/LEF), which directly regulates EMT-related genes [47, 48]. β-Catenin is also the crucial effector molecule in the Wnt/β-catenin signal transduction pathway. Our results showed that the snail levels in TE13 cells are decreased following exosome treatment. In summary, our findings suggest that T cell exosomes promote cancer metastasis by inducing the EMT through enhanced NF-κB-snail signaling. Our conclusions provide a new mechanistic basis for the therapeutic application of exosomes to esophageal cancer patients. Exosomes may be an effective alternative therapy for persistent carcinoma [21].

One of the limitations of this study is the use of the Jurkat tumor cell line instead of human T cell lines. Although Jurkat cells are characterizedas immortalized T lymphocytes, they secrete different exosomes. However, exosomes derived from tumors also possess immunosuppressive properties and promote metastasis, tumor growth and drug resistance. Therefore, attention should be devoted to distinguishing the exact composition of exosomes from Jurkat cells and T cells [32, 49, 50]. Proteomic analyses have been conducted on exosomes from DCs [51], B cells [52], intestinal epithelial cells [53], malignant pleural effusion [54], human urine [55] and biological fluids, as well as from exosomes many other cell types [56, 57]. Alberto Bosque performed a proteomic analysis of exosomes from human T cell blasts and compared them with those from the Jurkat tumor cell line, and the analysis revealed differential protein expression between the two types of exosomes. These researchers identified 359 and 418 proteins in exosomes from Jurkat cells and T cell blasts, respectively, but only 145 (approximately40%) of the proteins were shared. A further analysis demonstrated 30 additional proteins in exosomes from T cell blasts or Jurkat cells, revealing some specificities in their distribution. Therefore, 17 proteins were detected in exosomes derived from both cell types: annexin-I, annexin-VI, Na+/K+ ATPase, GAPDH, nucleolin, tropomyosin-3, three histones and eight ribosomal proteins. Three proteins were identified only in exosomes derived from Jurkat cells: CD3-ζ, Y-box binding protein-1 (YBX-1) and signal recognition protein (SRP). Ten proteins were only detected in exosomes derived from normal T cell blasts: CD3-d, β2 integrin, ADP-ribosylation factor6-interacting protein, HLA-I, β2-microglobulin, CD97, CD5, CD48, Arp2/3and interferon-induced transmembrane protein-1 (IFITM-1) [58]. Therefore, we do not know whether the experimental results are attributable to the use of exosomes from human leukemic cells.

Conclusions

In summary, this manuscript constitutes the first report describing that exosomes from irradiated T cells have the potential to promote TE13 cell metastasis in vitro, possibly by promoting the EMT through the upregulation of β-catenin and the NF-κB/snail pathway. These findings provide anovel perspective for the mechanisms underlying the pro-metastatic effect of exosomes and may be used as a theoretical basis for the therapeutic application of exosomes in anti-metastatic therapy for esophageal cancer [23].

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