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T cell derived exosome for cancer immunotherapy

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Abstract:

To date, many approaches to cancer treatment have been developed, such as adoptive cellular immunotherapy and cancer vaccines. However, these methods often have certain limitations, such as low response rates and the risk of autoimmune attacks. Using exosomes for cancer intervention is currently a very promising therapeutic approach, especially for drug delivery or direct cancer cell targeting through exosomes. In this study, we focused on the extraction of exosomes derived from T cells to induce apoptosis in tumors, as well as conducting fundamental tests on their characteristics. We successfully extracted a certain number of exosomes from mouse T cell line CTLL-2 cells and examined their properties, including particle size, zeta potential, electron transmission microscopy, and western blot analysis. Finally, we confirmed the viability of the extracted exosomes in vitro.

Keywords: Exosome, T cells, Cancer immunotherapy, Drug delivery

Introduction

Cancer immunotherapy is advancing rapidly as a novel approach to treating tumours, in addition to more conventional methods like surgery, radiotherapy, and chemotherapy, offering promising prospects for clinical application[1]. When tumor cells infiltrate tissues, the immune system detects tumor-associated antigens (TAAs) present on the surface of the tumor cells, triggering specific humoral and cellular immune responses to eliminate the invading cells[2]. Simultaneously, the tumor cells can evade immune surveillance and suppress the body's immune response through various pathways. Immunotherapy for tumors involves external manipulating the immune system to reinitiate and sustain the 'tumor-immunity' process, enhance and revive the body's anti-tumor immune response, and bolster the detection and destruction capability of tumor cells. In comparison to conventional therapies like surgery, radiotherapy and chemotherapy, tumour immunotherapy boasts the benefit of being extremely precise and having minimal side effects[2]. Tumor-infiltrating lymphocytes (TILs)[3], chimeric antigen receptor T cells (CAR-T), and T-cell receptor-modified T cells (TCR-T) are among the most effective methods currently in clinical use. Passive tumor immunotherapy involves adoptive cellular immunotherapy techniques, where immune cells obtained from the patient's tumor are modified, stimulated, and expanded outside the body, then reintroduced into the patient to target the tumor. The most significant obstacles to the clinical application of adoptive cellular immunotherapy are

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its complex preparation process and high cost. However, glucocorticoids or Tocilizumab can be used to mitigate cytokine release syndrome and neurotoxicity, which are the primary side effects of these therapies. A variety of novel tumor immunotherapeutic approaches are currently being developed and tested, primarily focusing on the discovery of new immune checkpoints (including LAG3, TIM3, VISTA, and B7-H3, among others). To address the complexity of cancer progression in the medical field, CTLA4 and PD-1 inhibitors may be used in combination. Additionally, various types of tumor vaccines are under development. These vaccines utilize TAAs, peptides, or cell lysates to induce a tumor-specific immune response, thereby protecting the body from tumor invasion and aiding in both the prevention and treatment of cancer.

Exosomes are small lipid-filled vesicles ranging from 30 to 150 nanometers in size that are released by cells through exocytosis and can be found in various bodily fluids, including plasma, urine, serum, saliva, and cerebrospinal fluid. Exosomes are composed of a lipid bilayer and transport various transmembrane proteins, receptors, adhesion molecules, lipid raft-associated proteins, and immunomodulatory molecules. As key messengers in intercellular communication, exosomes deliver bioactive molecules to recipient cells, thereby participating in a wide range of pathophysiological processes, including tumors, cardiovascular diseases, and neurodegenerative diseases. A series of studies has reported that tumor-derived exosomes play an important role in tumor progression, immunomodulation, and metastasis. Exosomes can be used as potential biomarkers for the diagnosis and prognosis of various diseases, including neurodegenerative disorders, traumatic brain injury, stroke, and cancer, with potential therapeutic implications. Moreover, increasing evidence suggests that exosomes have the potential to be utilized in the early detection of cancer, predicting its progression, and aiding in determining the most suitable treatment. Exosomes are believed to play a role in the progression of various diseases, including immune and inflammatory diseases, cardiovascular diseases, neurological disorders, and tumors. The delivery of proteins, metabolites, and nucleic acids to recipient cells can effectively alter their biological responses, potentially influencing the development or suppression of disease through exosome-mediated mechanisms. As a therapeutic tool, exosomes avoid the safety issues associated with cell therapy, such as uncontrolled cell division, tumorigenicity, and vascular embolism. Exosomes are endogenous particles with a small size, offering advantages such as target tissue specificity, achieved through surface proteins, stability in body circulation, and good biocompatibility. Exosomes, loaded with various drugs through experimental drug-loading techniques, can be transported to target tissues through natural tropism, such as affinity for source cells and homing effects. The loading of additional drugs into exosomes, or engineering modifications, can enhance their selectivity for specific tissues and receptor cells, alter their in vivo distribution, and achieve more efficient drug-targeted delivery, thus improving therapeutic efficacy. Exosomes derived from osteoarthritic chondrocytes can stimulate inflammatory vesicle activation via miR-449a and increase mature IL-1β production in macrophages, potentially exacerbating synovitis and accelerating the progression of osteoarthritis[4].

CD8+ T cells play an essential role in protecting against infectious agents by killing infected cells following recognition of microbial peptides presented by MHC class I molecules on the surface of target cells. Major efforts are underway to harness tumor-specific CD8+ T cells to treat cancer cells[5]. After target cell recognition, cytotoxic granules are released into the immunological synapse formed between killer cells and their targets. The granules secreted by cytotoxic T lymphocytes (CTLs) contain perforins (which form pores in the target cell membrane), granulysin, and granzymes. The perforin pores allow granzymes to access the cytosol of target cells, where they activate cell death pathways. Exosomes derived from CD8+ T cells also contain granzymes and perforin. These granzymes are transferred either through exocytosis at the immunological synapse or via endocytosis by the target cells[6]. Typically, during immune synapse formation with target cells, exosomes may be released from CTLs to assist in killing target cells, although the precise mechanisms are still being studied[7].

Exosomes from CD8+ T cells not only modulate CD8+ CTL responses mediated by antigen-specific DCs by affecting the function of target cells through endocytosis by APCs and B cells, but they also inhibit antitumor immunity in an antigen-dependent manner. Activated CD8+ T cells can secrete exosomes that inhibit fibroblastic stroma-mediated tumor cell invasion and metastasis by inducing the apoptotic depletion of mesenchymal tumor stromal cells. However, it has been argued that exosomes from exhausted CD8+ T cells might still participate in the inhibition of tumor growth, invasion, and metastasis. Functional analysis has indicated that differently expressed lncRNAs from a variety of exosomal sources actively regulate CD8+ T cell responses by altering biosynthetic processes, gene expression, and metabolism[8].

The goal of this study is to isolate and characterize exosomes and microvesicles derived from PD-1-expressing T cells (PD-1-CTLL-2 cells) and evaluate their interaction with tumor cells. The focus is on the isolation, purification, and size characterization of PD-1-exosomes (PD-

1-Exo) and PD-1-microvesicles (PD-1-MV) using techniques such as dynamic light scattering or nanoparticle tracking analysis, followed by their functional evaluation in binding and interacting with tumor cells. It is expected that the PD-1-exosomes and microvesicles will effectively bind to tumor cells, potentially mediated by the interaction between PD-1 on vesicles and ligands on tumor cells. This will be demonstrated through analysis of their size, zeta potential, and the expression of key exosome markers like CD63, TSG101, and others. This study contributes to the understanding of T cell-derived exosomes in the context of cancer immunotherapy and provides a foundation for future exploration of their potential use in targeted therapeutic applications.

MATERIALS AND METHODS

Materials: PD-1-CTLL-2 cells, Exosome-free FBS, Phosphate Buffered Saline (PBS), RIPA buffer (Beyotime), Alexa Fluor 647 NHS Ester, Dimethyl sulfoxide (DMSO), Confocal microscope (Zeiss), B16F10 cells, Dynamic light scattering (DLS) instrument, Zetasizer, Copper grids (carbon and formvar coated), Poly-l-lysine, Uranyl acetate solution (1%), Primary anti-PD-1 antibody, Secondary antibody linked to 5-nm gold particles, BCA Protein Assay Kit (Sigma-Aldrich), SDS-PAGE gel, Polyvinylidene fluoride (PVDF) membranes, Methanol, PBS-T solution, Horseradish peroxidase-linked secondary antibodies, Chemiluminescence substrate (Thermo Fisher Scientific), Tanon chemiluminescence/fluorescence image analysis system

Isolation and extraction PD-1-MV and PD-1-Exo from PD-1-CTLL-2 cells

As shown in Fig. 1, the isolation protocol was based on well-established methods. PD-1-MV and PD-1-Exo were isolated and purified from the cell culture medium. Exosome-free FBS was prepared by centrifugation at 100,000g overnight to deplete serum-derived EVs. The cells were first cultured in standard media as previously described. When they had reached 60-70% confluence, they were centrifuged and washed with PBS at 300g, then transferred into exosome-free medium and cultured for 72 hours. Subsequently, the cell culture medium was collected and centrifuged at 800g for 10 minutes and 2000g for 30 minutes to remove the debris. Next, the supernatant from the previous centrifugation was further centrifuged at 10,000g for 30 minutes to harvest the microvesicles. Finally, the supernatant was centrifuged again at 100,000g for 70 minutes to isolate exosomes by pelleting them. All centrifugation steps were carried out at 4°C. Microvesicles and exosomes were washed and resuspended in PBS, then stored at -80°C for further analysis[9].

Characteristics of T cell exosomes, and killing tumor cells in vitro

Furthermore, microvesicles and exosomes were stored at -80°C for subsequent analysis. Freshly prepared exosomes and microvesicles were examined for particle size using a dynamic light scattering instrument. C-MV and C-Exo were previously labeled with Alexa Fluor 647. Then, PD-1-Exo, PD-1-MV, C-Exo, and C-MV (100 μg/mL) were each incubated with B16F10 cells, which had been previously seeded in confocal wells, at 37°C for 3 hours. Afterwards, the cells were observed using a confocal microscope (Zeiss). Cells, MVs, and Exos were lysed using RIPA buffer (Beyotime) to prepare protein samples. The lysates were then boiled with loading buffer (Beyotime) and separated on a 15% or 10% SDS-PAGE gel under 60-90V. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with 5% milk at room temperature (RT) for 2 hours. After that, primary antibodies against PD-1, granzyme B, CD63, and TSG101 were applied to the membranes at 4°C overnight. Secondary antibodies were then applied to the membranes for 1 hour at RT. Finally, the results were detected using the Tanon chemiluminescence/fluorescence image analysis system with the enhanced chemiluminescence substrate.

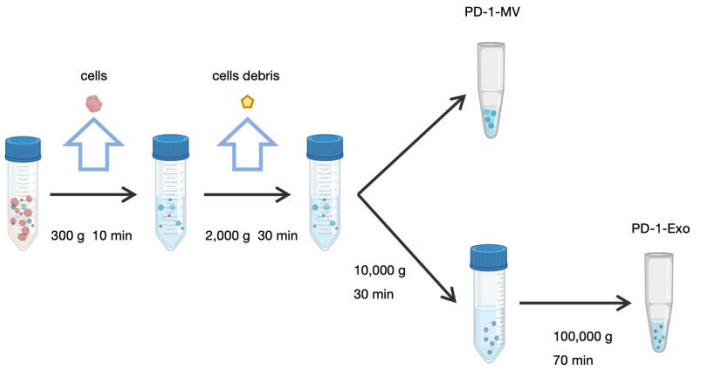

Fig.1. T cells exosome isolation and purifications

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Exosome Labeling with Alexa Fluor 647

First, prepare the Alexa Fluor 647 dye by dissolving it in anhydrous DMSO to create a stock solution, typically at 1 mg/mL. Then, add the dye solution to the exosome suspension in a ratio of 20 µg dye per 100 µg of exosomes. Gently mix the solution and incubate it for 1 hour at room temperature in the dark to prevent light exposure, allowing the dye to bind to the exosome membrane proteins. Once the incubation is complete, it is important to remove any unbound dye to ensure accurate labeling. The mixture is centrifuged at 100,000g for 70 minutes at 4°C to pellet the exosomes. After pelleting, resuspend the exosomes in PBS and store them at -80°C until further use.

Particle Size and Zeta Potential Measurement Procedure

First, the size of the exosome particles was measured using dynamic light scattering (DLS). This helps to determine the size distribution of the particles in the liquid. Next, the zeta potential was measured using a Zetasizer. Zeta potential is important because it helps to understand the stability of the exosomes in the solution. It shows how much the particles repel each other, which affects whether they will stay suspended or clump together.

TEM Procedure for Exosome Imaging

First, exosome samples were placed on copper grids that were coated with carbon and formvar. These grids were treated with poly-l-lysine for about 30 minutes. Then, the samples were stained with a 1% uranyl acetate solution for 1 minute. For gold labeling, the grids were placed in a solution with the primary anti-PD-1 antibody overnight at 4°C. After that, the grids were incubated with a secondary antibody linked to 5-nm gold particles for 2 hours at room temperature. After staining, the grids were gently dried with filter paper and allowed to air dry completely. Finally, the samples were examined using a TEM at 80 kV, and digital images were taken using the AMT Imaging System.

Western blot analysis

First, protein concentrations were measured using the BCA Protein Assay Kit from Sigma-Aldrich. Then, the proteins were separated by SDS-PAGE and transferred to PVDF membranes that had been activated with methanol. This process was done at 250 mA for 70 minutes. After that, the membranes were blocked with 8% fat-free milk in a PBS-T solution (which is a mix of phosphate-buffered saline and Tween 20) for one hour at room temperature. Next, the membranes were incubated with the primary antibodies for 16 hours at 4°C. The membranes were washed and then incubated with secondary antibodies that were linked to horseradish peroxidase for one hour at room temperature. Finally, the signals from the proteins were developed using a chemiluminescence detection method, and the images were captured using darkroom techniques.

RESULT

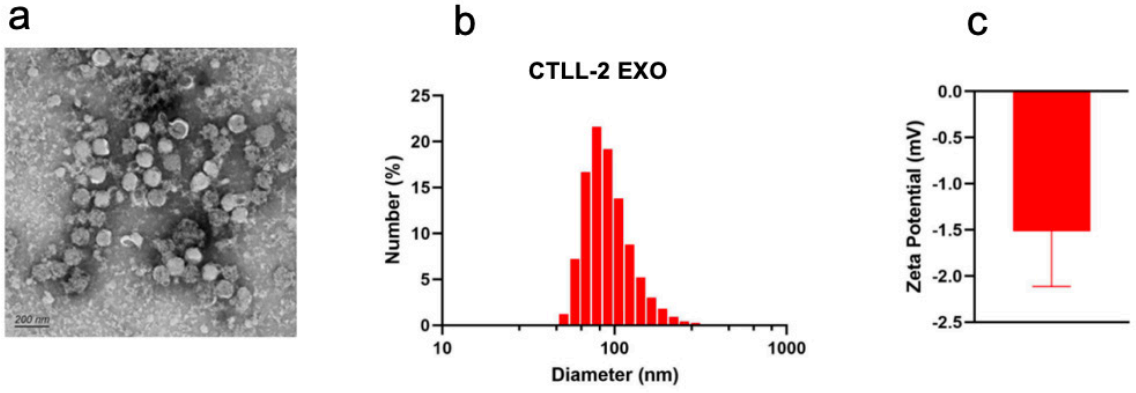

Fig.2. The process of isolation of exosomes and the measurement of their size and potentials. (a) Stable T cell lines expressing GFP-PD-1 and the production of PD-1-Exo and PD-1-MV.PD-1-Exo's TEM images(scale bar: 200 nm)are presented.(c) Size distribution of PD-1-Exo analyzed by dynamic light scattering (DLS) analysis.(d)Zeta potential of PD-1-Exo analyzed by Zeta potential Analyzer.

Fig. 2 illustrates the process of isolating exosomes from CTLL-2 cells and analyzing their size and surface charge. In Figure 2(b), the TEM image shows the structure of exosomes. The exosomes appear to be mostly spherical and have a relatively uniform size, which fits the typical characteristics of exosomes. Figure 2(c) presents the size distribution of exosomes as measured by DLS. The majority of the exosomes are around 100 nm in size, which is consistent with the expected range for exosomes. The histogram shows that while there are some larger particles present, most of the exosomes fall within the expected size distribution. In Figure 2(d), the Zeta potential anal-

ysis indicates that the exosomes have a surface charge of around -1.5 mV. This negative charge is important because it affects the stability of the exosomes in solution. A higher negative charge usually helps to prevent the exosomes from clumping together, making them more stable in a biological environment.

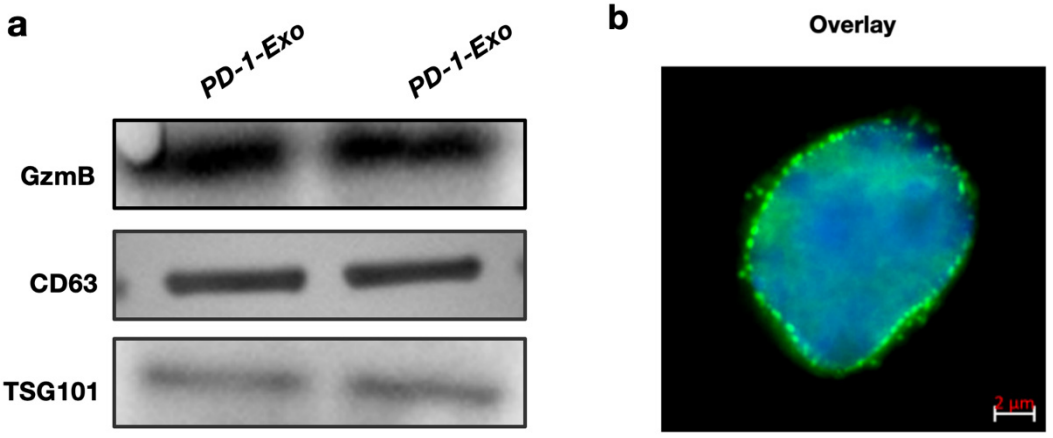

Fig.3. Exosome characterization and Tumor cells binding assays. (a)Western blot analysis of PD-1 and markers of EVs (CD63, TSG101 and GRANB) in PD-1 Exo. (b) PD-1-Exo binding on the surface of tumor cell. Scale bar: 2 μm

In Figure 3(a), Western blot analysis shows the expression of several key proteins in PD-1 exosomes, including GzmB, CD63, and TSG101, which are known exosome markers. The presence of Granzyme B, a protein that promotes cell apoptosis, suggests that PD-1 exosomes may have the potential to induce apoptosis in tumor cells. Both CD63 and TSG101 are common exosome markers. CD63 is often used as a marker of exosomal membranes, while TSG101 is involved in the exosome formation and secretion process. The detection of these proteins confirms the purity and characteristics of the exosomes.

In Figure 3(b), the binding of PD-1 exosomes to tumor cells is visualized. The green fluorescence represents exosomes labeled with Alexa Fluor 647, while the blue fluorescence represents the tumor cell nucleus stained with DAPI. The image shows the green signal surrounding the blue cell nucleus, indicating that PD-1 exosomes have successfully attached to the surface of the tumor cells. The images clearly demonstrate that PD-1 exosomes can bind to tumor cells, suggesting that these exosomes are capable of targeting tumor cells effectively.

DISCUSSION

As important mediators of intercellular communication, exosomes have attracted much attention in the field of tumor research in recent years. Their unique structural and functional properties make them significant targets for tumor disease research. In this paper, we will discuss both the extraction methods of exosomes and the detection of their functional properties, with a particular focus on their role in tumor biology. Exosomes, as crucial carriers of intercellular communication, show significant research advantages and challenges in tumor studies. Regarding research advantages, exosomes have become a hotspot in tumor research due to their unique structure and function. Firstly, exosomes are rich in a variety of biologically active molecules, such as proteins, RNA, and DNA, which reflect the physiological state and gene expression of tumor cells, providing potential biomarkers for the early diagnosis of tumors. Secondly, exosomes have ideal biocompatibility and potential as drug carriers, which can be exploited for targeted drug delivery, offering new therapeutic strategies for tumor treatment. In addition, exosomes are involved in intercellular communication and immune regulation. In-depth studies of their functional properties can help to reveal the mechanisms of tumorigenesis and development, providing new targets for tumor control.

However, there are certain challenges in the extraction of exosomes and the detection of their functional properties. Firstly, the extraction and purification of exosomes is complex and labor-intensive, requiring various technical methods such as ultracentrifugation and size exclusion chromatography, which are time-consuming and may affect the purity and biological activity of the exosomes. Secondly, the size and density of exosomes are similar to other extracellular vesicles, making the separation process challenging and prone to contamination and batch-to-batch variation. Furthermore, the detection of the

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functional properties of exosomes requires high-precision techniques such as mass spectrometry and sequencing, which are costly and technically demanding, limiting their widespread use in clinical and scientific research.

In examining exosomes, TEM is the gold standard for identifying the morphology, size, and internal structure of exosomes, but it is complicated to perform and does not allow for quantitative analysis. In contrast, protein immunoblotting (Western Blot) and high-throughput sequencing technologies can reveal the protein composition and genetic information of exosomes, providing important insights into their function and origin. In tumor research, exosomes are not only involved in the immune escape and invasive metastasis of tumor cells but also serve as potential biomarkers for the diagnosis and prognosis of tumors. For instance, changes in the expression of specific miRNAs, such as miR-150 and miR-21 in colorectal and lung cancers, offer new avenues for disease diagnosis.

Conclusion

The extraction and detection of T cell-derived exosomes are of great importance in the field of tumor research. These exosomes are vesicles secreted by T cells that play a crucial role in intercellular communication and can carry a variety of biomolecules, including proteins, lipids, and RNA. As we delve deeper into their function, we are beginning to recognize their potential to influence tumor behavior and the immune response to cancer. As technology continues to advance, particularly in nanotechnology and molecular biology, we are gaining a deeper understanding of the mechanisms by which these exosomes operate. This understanding may lead to the identification of specific biomarkers that could aid in the early diagnosis of tumors and ultimately improve patient therapies[10]. In addition, T cell-derived exosomes may offer new avenues for tumor treatment, providing a way to deliver targeted therapies directly to the tumor site, thereby improving the efficacy of existing therapies while minimizing the side effects of drugs on the human body. We hope that this type of drug delivery system could eventually replace traditional chemotherapy, which is often very damaging to the body. T cell-derived exosome-based therapies are more targeted and could also significantly improve patient survival rates. As researchers utilize advanced technologies such as next-generation sequencing and high-resolution imaging, we expect to discover new ways in which exosomes regulate tumor immunity and progression. The ultimate goal is to translate these discoveries into innovative strategies for diagnosis and treatment, opening new avenues for more personalized cancer therapy. In summary, the study of T cell-derived exosomes represents a promising frontier in oncology research with the potential to revolutionize cancer diagnosis and treatment in the near future.

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