이학석사 학위논문

표적 약물 전달을 위한 췌장 성상세포 유래 엑소좀의 표면 단백질 공학

Surface engineering of pancreatic stellate cell derived exosome for targeted drug delivery

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문보현의 이학석사학위 논문을 인준함

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ABSTRACT

Exosomes are small vesicles that carry biomolecules, such as proteins, microRNAs, and metabolites, and have great potential as therapeutic delivery vehicles. Chemotherapeutic agents used in cancer therapy can cause severe side effects, making the need for improved selectivity and stability of these drugs imperative. Exosomes provide an attractive platform for loading different types of therapeutics, and their surface can be modified by specific surface proteins that increase their local concentration at target cells or disease sites, leading to improved therapeutic outcomes with reduced toxicity.

Keywords: Exosome, Pancreatic cancer, Surfacesome, Drug delivery, Cancer therapy

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy that is frequently diagnosed at an advanced stage, and treatment options include surgery, chemotherapy, and radiation therapy or a combination of these modalities. Chemotherapy is widely used in the treatment of PDAC, but despite recent developments in chemotherapy, the overall survival of patients has not improved for decades. One of the main reasons for the limited success of pancreatic cancer therapy is drug resistance, and the nonselective nature of chemotherapeutic agents, which can cause severe undesired side effects leading to high mortality rates. [1], [2] PDAC is characterized by dense desmoplastic stroma and extensive fibrosis, which act as a barrier to drug delivery, making it difficult to penetrate the cancerous tissue. Pancreatic stellate cells (PSCs) are myofibroblast-like cells that become activated during inflammation and fibrosis, leading to increased production of collagen, movement, attachment, and proliferation. [3, 4] Several studies have shown that PSCs produce extracellular matrix components, such as desmoplastic stroma and highly interact with pancreatic cancer cells, making them an attractive target for therapeutic intervention. [5, 6] Recently, potential use of exosomes as a novel drug delivery vehicle for the for the treatment of pancreatic cancer by targeting PSCs or pancreatic cancer cells have been actively conducted. In this review, we will focus on the engineering of exosomes for targeted drug delivery and explore the candidates for exosome surface modification to enhance the delivery of drugs specifically to PSCs and pancreatic cancer cells.

GENERAL BACKGROUND

1. Exosomes and their role in intercellular communication

Exosomes are small 30–100 nm vesicles that are secreted by various cell types, and they play a crucial role in several processes, including cell–cell communication, immunomodulation, antigen presentation, tumor growth suppression, endothelial cell migration, and inflammation.[7, 8] The primary function of exosomes is intercellular communication by transferring lipids, RNA, and cytosolic proteins.[9] The small size of exosomes allows them to passively target and selectively extravasate into tumor or inflamed tissues owing to the enhanced permeability and retention effect.[10] Also, exosomes have certain advantages over synthetic nanoparticles as drug delivery vehicles due to their biogenic nature, lack of immune stimulant activity, and excellent physicochemical stability, making them well-tolerated in the body. As natural nanovesicles, exosomes have remarkable potential as a delivery vehicle due to low immunogenicity and their ability to enter tissues. [11]

2. Artificial exosomes as drug delivery vehicle

Exosomes have emerged as promising drug delivery systems with potential applications in various pathologies, including cancer. However, the production of exosomes derived from cells is associated with limitations such as low yield, high cost, and labor-intensive methods. Additionally, the lack of standardized processes further hinders their widespread use. To address these drawbacks, artificial exosomes have been developed as novel theragnostic biomaterials, offering a solution to the challenges associated with natural exosomes.

These artificial exosomes hold promise for future clinical applications. Various techniques have been developed to facilitate the isolation of exosomes from biological sources. The gold standard method is ultracentrifugation, which separates exosomes from

other extracellular components based on their sedimentation coefficients. However, density gradient ultracentrifugation, while providing the purest exosome samples, is time-consuming due to the need for equilibrium of solutions. In contrast, ultrafiltration is a membrane-based technique that separates exosomes from larger macromolecules using pores equivalent to the exosome size. This method significantly reduces processing time and does not require specialized equipment, making it an attractive alternative to ultracentrifugation. Another approach is immunological separation, which captures exosomes based on the antigen-antibody reaction. Antibody-coated plates, chromatography matrices, or beads are used to selectively capture exosomes, offering high purity and shorter processing times. However, this method can be expensive due to the need for special reagents and cell-free samples, limiting its applicability for large-scale samples. Researchers often combine these methods or employ additional techniques to optimize exosome isolation based on factors such as purity requirements, time constraints, available resources, and the scale of the experiment.

3. Loading methods for drug into exosomes

There are two main types of methods for encapsulating cargo into exosomes: cell-based loading methods and non-cell-based loading methods. In the cell-based loading approach, the cargo is typically introduced into the donor cells first. Once packaged into extracellular vesicles, the cargo can be secreted and collected along with the EVs for therapeutic purposes. On the other hand, the non-cell-based loading approach involves direct loading of drugs into isolated EVs using techniques such as electroporation, sonication, incubation, and/or transfection.

Previous findings suggest that sonication is effective for macrophage-derived exosomes, while electroporation appears to be more suitable for primary dendritic cell (DC)-derived exosomes, based on measured efficiency. We plan to use incubation method for drug loading into exosomes.

4. Potential use of PSC derived exosomes in pancreatic cancer

PSCs play a critical role in the development and progression of pancreatic cancer. They secrete extracellular matrix components and growth factors that promote tumor growth, invasion, and metastasis. PSCs are also involved in the development of drug resistance in pancreatic cancer cells. Therefore, targeted delivery of anticancer drugs specifically to PSCs using PSC-derived exosomes holds great promise for the treatment of pancreatic cancer. In recent years, there has been growing interest in developing exosome-based therapies for pancreatic cancer by exploiting the unique properties of exosomes. PSC-derived exosomes have been shown to effectively target PSCs and deliver therapeutic agents, such as siRNAs and chemotherapeutic drugs. PSC-derived exosomes also have the potential to overcome the limitations of conventional drug delivery methods, such as poor pharmacokinetics and off-target effects.

5. Surface engineering of exosomes

In order to enhance the specificity of exosomes as targeted drug delivery vehicles, surface engineering techniques can be employed. One approach involves genetic engineering, whereby a guiding protein is fused with a membrane protein of the exosome to create an engineered exosome bearing a targeting protein. This can be achieved by transfecting donor cells with plasmids encoding the fusion proteins.[12]

LAMP-2B, a lysosome-associated membrane protein, is a commonly used surface protein for targeting. LAMP-2B is predominantly localized to lysosomes and endosomes, but also circulates to the cell surface. By fusing LAMP-2B to the membrane protein of exosomes, the resulting engineered exosome can specifically target cells that express a receptor for LAMP-2B. Chemical engineering approaches can also be used to modify exosome surfaces through conjugation reactions or by directly fusing other membrane structures with the exosome's lipid bilayer.[13] For instance, exosomes with the viral fusogen vascular stomatitis virus (VSV)-G protein can be fused directly to target cell membrane proteins, offering a new tool for membrane protein therapy.[14] Techniques such

as electrostatic interactions, incubation, and bioconjugation can also be used to facilitate spontaneous membrane protein fusion. These surface engineering strategies hold promise for the development of novel and effective targeted drug delivery systems using exosomes.

Table 1. Application of surface modification of exosomes for targeted drug delivery

Cancer	Therapeutic agent	Target ligand	Target cells	Method	Function	Reference
Lung cancer	SOX2 siRNA	Tlyp-1	Non-small cell lung cancer, A549 stem cells	Genetically engineered	Targeted drug delivery	[15]
Breast cancer	Doxorubicin	iRGD peptide	Breast cancer cell	Genetically engineered	Targeted drug delivery	[16]
Breast cancer	Paclitaxel	AS1411	Breast cancer cell(MDA-MA-231)	Chemically modified	Targeted drug delivery	[17]
Leukemia	Doxorubicin	Sgc8 aptamer	Leukemia cells	Chemically modified	Targeted drug delivery	[18]

MATERIALS AND METHODS

1. Cell cultures

Human primary PSCs was cultured in RPMI culture medium containing 5% fetal bovine serum, 1% penicillin/streptomycin, 4ug/mL transferrin, 4ug/mL hydrocortisone, 20ng/mL EGF. Cell lines were maintained in DMEM culture medium containing 10% fetal bovine serum, , 100U/ml penicillin and 100mg/ml streptomycin.

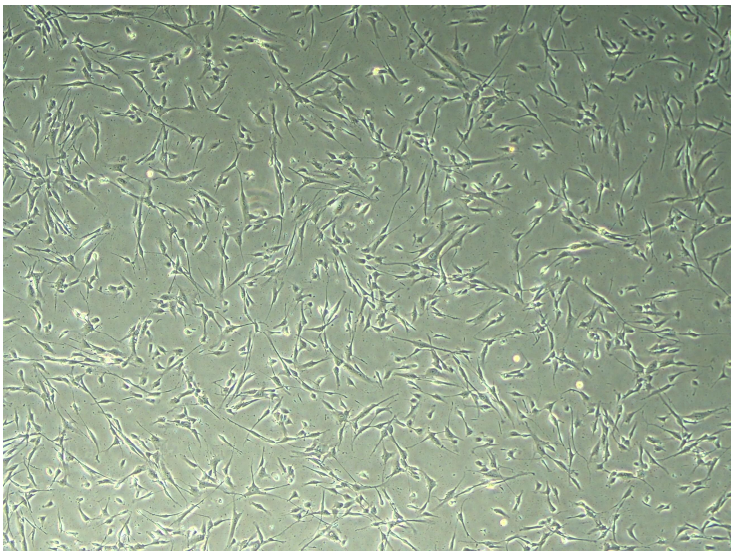

Figure 1. The culture of pancreatic stellate cells.

2. Isolation of artificial exosomes and drug loading into exosomes

We used ultrafiltration method to isolate exosomes from PSCs. The cell suspension was sequentially extruded three times through 5 μm, 1 μm, and 0.4 μm polycarbonate membrane filters using a mini-extruder. We plan to use incubation method for drug loading into exosomes.

3. Identification of surface protein for exosome engineering

In order to enhance the targeting efficiency of exosomes towards pancreatic cancer cells, we aim to identify potential candidate proteins that are highly expressed on the

surfaces of both pancreatic cancer cells and pancreatic stellate cells. These proteins will serve as promising targets for exosome surface engineering. Our objective is to thoroughly investigate and explore these candidate proteins in order to gain a better understanding of their roles and potential implications in improving exosome targeting towards pancreatic cancer cells.

RESULTS

1. Surface proteome in pancreatic stellate cells

1) Cadherin-11 (Cad-11)

Cadherin 11 is a cell adhesion molecule that is primarily expressed in mesenchymal cells such as pancreatic stellate cells (PSCs). [19], [20] It has been shown to play a crucial role in promoting tumor progression and invasion in various types of cancer, including pancreatic cancer. [21] The overexpression of Cadherin 11 has been linked to the activation of several signaling pathways involved in cancer cell growth and survival, including the Akt and ERK pathways. Recent studies have suggested that Cadherin 11 can be utilized as a potential target for cancer therapy. Micalizzi at.el showed the use of Cadherin 11-targeting antibodies or small molecules that can selectively bind to and inhibit the function of Cadherin 11.[12] By engineering the surface of exosomes with Cadherin 11-specific peptides or antibodies, it is possible to enhance the delivery of drugs specifically to PSCs and pancreatic cancer cells.

2) Toll-like receptors (TLR)

TLRs are proteins that recognize foreign pathogen-associated molecular patterns (PAMPs) and activate innate immunity. TLRs are expressed in various immune cells, including macrophages, dendritic cells, and B cells, and even in nonimmune cells such as fibroblasts and epithelial cells.[22] In the case of pancreatic stellate cells (PSCs), they express TLR2, 3, 4, and 5, as well as the associated molecules CD14 and MD2. PSCs express a variety of TLRs and respond to TLR ligands, leading to the activation of signaling pathways and proinflammatory responses.[23]

TLR activation in PSCs is believed to contribute to the development and progression of pancreatic cancer by promoting inflammation and fibrosis in the tumor microenvironment.

Therefore, TLRs on the surface of PSCs may be a potential target for the development of novel cancer therapies.

3) CD44

CD44 is a cell surface glycoprotein that plays a key role in cell adhesion and signaling processes. It is expressed in a wide range of cell types, including pancreatic stellate cells (PSCs). In the context of pancreatic cancer, CD44 has been implicated in promoting tumor growth, invasion, and metastasis. In particular, CD44 has been shown to interact with its ligand, hyaluronan, which is abundant in the tumor microenvironment of pancreatic cancer. This interaction promotes the activation of PSCs and the secretion of factors that support tumor growth and metastasis. By engineering exosomes to display CD44 on their surface, it may be possible to selectively target PSCs and deliver therapeutic agents directly to the tumor microenvironment. Thus, targeting CD44 on PSCs using exosome-based drug delivery systems holds promise as a potential therapeutic strategy for pancreatic cancer.

2. Surface proteome in pancreatic cancer cells

1) Transient receptor potential cation channel subfamily M member 7(TRPM7)

TRPM7) is a protein that is a member of the TRP superfamily of ion channels. TRPM7 is a unique ion channel because it has both ion channel and kinase domains within the same protein.[24, 25] TRPM7 is known to be involved in many cellular processes, including cell growth and survival, differentiation, and migration.[26] TRPM7 has been found to be overexpressed in pancreatic cancer cells, and it has been shown to play a role in promoting cancer cell growth and survival. In addition, inhibition of TRPM7 has been shown to decrease pancreatic cancer cell growth and promote cell death, making it a potential target for the development of novel pancreatic cancer therapies.[27] Several small molecule inhibitors of TRPM7 have been developed and tested in preclinical studies. These inhibitors have shown promising results in reducing pancreatic cancer cell growth and promoting cell death in vitro and in vivo. However, more research is needed to determine the safety and efficacy of TRPM7 inhibitors in humans, and to identify the optimal dosing and treatment regimens for these drugs.

2) CD90(Thy-1)

CD90, also known as Thy-1, is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that is expressed in a variety of cell types, including pancreatic cancer cells.[28] It has been found to be involved in several cellular processes such as cell migration, adhesion, and signaling.[29, 30] In pancreatic cancer, CD90 expression has been associated with cancer stem cells (CSCs) which are thought to play a role in tumor initiation, progression, and recurrence.[31, 32] Jianhui Zhu et al. revealed that the abundant CD90 expression was predominantly present in PDAC stroma, such as fibroblasts and vascular endothelial cells, which could serve as a promising marker to distinguish pancreatic adenocarcinoma compared to normal pancreas and non-malignant pancreatic diseases. The CD90+ stromal cells were clustered around malignant pancreatic ductal cells, indicating that CD90 may be involved in the tumor-stroma interaction and establish a favorable environment that promotes tumor progression.[33] Several studies have demonstrated that targeting CD90 can inhibit the growth and proliferation of pancreatic cancer cells in vitro and in vivo. For example, one study showed that targeting CD90 with a specific antibody reduced the viability and colony-forming ability of pancreatic cancer cells, and also inhibited tumor growth in a mouse xenograft model.[34]

3) Connective tissue growth factor (CTGF)

CTGF is a protein that plays a role in cell adhesion, proliferation, and differentiation. CTGF is secreted by pancreatic stellate cells and binds to various extracellular matrix proteins, including fibronectin and collagen. CTGF has been identified as a potential target for the development of novel pancreatic cancer therapies. In pancreatic cancer, CTGF expression is upregulated and promotes tumor growth and invasion. In preclinical studies, inhibition of CTGF expression or activity has been shown to inhibit pancreatic cancer cell growth and improve survival in mouse models. Several approaches have been developed to target CTGF in pancreatic cancer, including small molecule inhibitors, antibodies, and RNA interference. Some of these approaches have shown promising results in preclinical studies,

and some are currently being evaluated in clinical trials. Targeting CTGF may be a viable strategy for the development of novel pancreatic cancer therapies.

4) MUC1

MUC1 is a transmembrane glycoprotein that is overexpressed on the surface of pancreatic cancer cells. It plays a critical role in the progression and development of pancreatic cancer by promoting tumor growth, metastasis, and invasion. The aberrant expression of MUC1 is associated with poor prognosis and is considered a potential target for pancreatic cancer therapy. The extracellular domain of MUC1 is heavily glycosylated, which creates a steric hindrance that prevents antibodies and other therapeutic agents from effectively binding to the protein. Therefore, strategies to target MUC1 in pancreatic cancer are focused on identifying novel approaches to overcome this glycosylation barrier. Recent studies have shown that MUC1-targeted therapy using monoclonal antibodies, peptides, and small molecules has shown promising results in preclinical studies and is currently being evaluated in clinical trials for the treatment of pancreatic cancer. Wu et al. shows that MUC1-specific monoclonal antibody-drug conjugates effectively bind to MUC1 on the cell surface of pancreatic cancer cells. And it significantly reduced the growth of pancreatic xenograft tumors by inhibiting cell proliferation and enhancing cell death.[35]

DISCUSSION

Exosomes have advantages with low immunogenicity, safety, and lack of cytotoxicity.[36]. Exosomes derived from pancreatic stellate cells offer a promising targeted drug delivery system for selective PDAC treatment. However, there are still challenges that need to be addressed, such as low delivery efficiency, drug resistance, and limited clinical-grade production.[37] To overcome these challenges, we propose the use of surface engineering with specific proteins to increase delivery efficiency and reduce drug resistance. By identifying surface proteins highly expressed on PSCs or closely interacting with pancreatic cancer cells, we can modify exosomes to better target the tumor microenvironment. Although promising, further in vivo studies are needed to validate the efficiency of surface-modified exosomes in clinical applications. This review provides insight into the emerging therapeutic applications of exosome labelling via surface modification and highlights the potential of novel artificial exosomes as an effective drug carrier for improved healthcare. Future research should focus on developing novel strategies for exosome-mediated therapies, particularly for cancer. Another potential avenue of research is the optimization of exosome production and purification methods for clinical-grade production. This could involve the development of scalable production methods and the implementation of quality control measures to ensure consistent and safe drug delivery. Additionally, more comprehensive preclinical and clinical studies will be required to evaluate the safety and efficacy of exosome-based therapies in human patients. This could involve the testing of different exosome types, doses, and delivery routes, as well as the identification of potential side effects and toxicity concerns. Overall, the potential of exosome-mediated therapies for cancer is promising, and continued research and development in this field could lead to significant improvements in cancer treatment and patient outcomes.

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국문요약

췌장암은 침습성이 강하고 병이 많이 진행이 되기까지 증상이 없어 진행된 병기로 진단이 되는 경우가 높아 완치율이 낮고 사망률이 높습니다. 다양한 항암제의 개발에도 수십년간 전체 생존율이 개선되지 않았습니다. 췌장암의 치료제 개발의 낮은 성공 원인 중 하나는 약물의 내성과 세포독성 항암제의 부작용입니다. 체내의 특수부위에만 약물을 도달시키는 방법을 통해 약물의 부작용을 줄이고 효능 및 효과를 극대화 시켜 필요한 양의 약물을 효율적으로 전달할 수 있도록 약물 전달시스템에 대한 연구가 진행 중이고 최근 엑소좀이 각광을 받고 있습니다. 엑소좀은 단백질, 마이크로 RNA, 대사물질과 같은 생체분자를 운반하는 작은 소포로 높은 생체적합성 및 표적 지향적 전달이 가능하기 때문에 치료용 전달체로서 막대한 잠재력을 갖고 있습니다. 자연 엑소좀은 세포에서 자연적으로 분비되는 것으로 그 생성량이 적고 분리 및 정제의 어려움이 있어 추후 약물 전달 시스템으로 사용하기 위해서는 대량 생산의 문제점이 존재합니다. 본 연구에서는 압출법이라는 방법을 통해 인공 엑소좀을 대량으로 생산할 수 있는 방법을 제시하였습니다. 엑소좀을 이용한 약물 전달 시스템은 순환 반감기가 짧고, 표적화 및 효율성이 낮아 응용이 제한되는 여러가지 도전 과제가 있습니다. 본 연구에서는 표적 세포나 질병 부위에서의 약물 농도를 증가시킴으로써 치료 효과를 향상시키고 독성을 감소시키기 위한 엑소좀 표면 단백질 공학 후보 물질들을 발굴하였습니다. 향후 화학적 공학 기법 또는 유전적 공학 기법을 통해 엑소좀 표면에 후보 물질들을 부착하여 췌장암 세포에 효과적으로 약물을 전달을 하는지 및 충분한 치료효과가 있는지에 대한 추가적 연구가 진행이 될 예정입니다.