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Feasibility assessment of using CRISPR-Cas9 to improve the infiltration of CAR-T cells in solid tumors

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Abstract. Chimeric antigen receptor T-cell immunotherapy (CAR-T) has been developing for decades, CAR-T is playing an increasingly important role in tumor treatment. However, because fibroblasts (CAFs) in solid tumors secrete proteins and glycans to form ECM, CAR-T is faced with the challenge of improving tumor invasion. To this end, a new scheme was put forth to alter CAR T cells such that they release heparinase (HPSE) to break down heparan sulfate proteoglycan (HSPG), which is covered on the outermost layer of the cancerous cells by CAF and released. In order to solve the above problems, CRISPR-Cas9 was proposed to modify CAR T to enhance the secretion of HPSE. Although this method has the advantage of broad spectrum, it still has certain defects. The matrix characteristics of different solid tumors and the subpopulations and regulatory mechanisms of HPSE need to be further studied. The off-target effects of CRISPR-Cas9 gene editing technology and the high cost and time-consuming problems of flow cytometry also limit its application. It is anticipated to enhance cell infiltration in solid tumors, boost CAR-T therapy's effectiveness in treating solid tumors, and advance the field of CAR-T therapy.

Keywords: HPSE, CRISPR-Cas9, CAR-T cell tumor infiltration, solid tumor stroma.

1. Introduction

Zelig Eshhar introduced the idea of CAR in 1989, and Carl June was the first to advance CAR-T to human clinical trials in 2010, and successfully cured multiple leukemia (ALL) patients. Bill Ludwig, the first patient to receive CAR-T therapy, had no detectable signs of leukemia for up to 12 years after treatment [1]. Since the tumor matrix with HSPG as the main component is flooded around the solid tumor, the invasiveness of existing CAR-T tumors is reduced. The expression level of HPSE in human serum was detected by enzyme-linked immunosorbent assay (ELISA), and the results can indicate the degree of infiltration of CAR-T on solid tumor cancer cells. However, how to avoid or reduce the side effects caused by the high expression of HPSE, as well as the side effects of tumor cell re-growth or metastasis, has become the focus and difficulty of research. According to clinical data, CAR-T therapy for drug-resistant and recurrent blood system provides an alternative to chemotherapy for malignant tumor patients, the method of many patients to accept this kind of novel treatment and treatment effect is more outstanding. After decades of development, CAR-T cells have also undergone four generations of updates, and cutting-edge methods such as multi-target CAR-T cell culture, new targets, specific T

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cell subsets modified CAR-Ts, CAR-X cell therapy have been developed to improve the therapeutic effect.

Due to the physical barriers of solid tumors and the special tumor microenvironment (TME) of solid tumors, these factors make it difficult for CAR-T to infiltrate tumor tissues. It has been shown that enhancing tumor invasion requires the decomposition of HSPG as a major component of tumor matrix and other physical barriers. HPSE is a kind of enzyme involved in the key enzyme of ECM remodeling [2], HPSE decomposable HSPG, damage and change the ECM. Therefore, genetic engineering technology can be used to make CAR-T express and secrete HPSE, degrade ECM, and enhance the infiltration and anti-tumor effect of CAR-Ts in solid tumors [3]. Therefore, it is necessary to find a pathway to up-regulate the expression of HPSE, or to make CAR-T cells secrete HPSE through biological engineering technology to act on HSPG decomposition and increase the infiltration rate of CAR-Ts. The latest research by using ELISA to detect patients with lung cancer, according to the multiple ligands proteoglycan - 1 in serum (SDC), heparan sulfate (HS) and heparan enzyme expression level of components (HPA) system, to apply the results in [4] to diagnose lung cancer. According to the results of clinical experiments, the expression level of HPSE can be used as a basis for judging the infiltration effect of CAR-Ts. Previous studies have proved the feasibility of ELISA to detect the expression level of HPSE. The high expression of HSPE can make related cells secrete HPSE, thereby decomposing HSPG, increasing the infiltration rate of CAR-T by destroying the fibrous structure of solid tumors, and improving the efficacy of CAR-T in the treatment of solid tumors. In order to solve the challenges of CAR-T cell therapy for solid tumors, this paper provides a new solution. Based on the latest CRISPR-Cas9 technology, this scheme explored the HPSE gene sequence secreted by T cells, and used the self-repair mechanism of DNA to modify CAR-Ts to efficiently secrete HPSE and destroy ECM, so as to improve the tumor invasion effect of CAR-Ts. At the same time, based on the existing research, the feasibility of the scheme was evaluated to provide a new solution for the difficulties faced by CAR-T in the treatment of solid tumors.

2. Overview of CAR-T therapy

2.1. The construction and development of CAR-T

CAR is the core component of CAR-T. The external structure of CAR is a Single-Chain Fragment Variable (scFv), which is composed of variable heavy chain (VH) and variable light chain (VL). The antibody uses a synthetic peptide chain to link VH and VL to form a recombinant gene. In the actual treatment, the affinity of scFv plays a decisive role in the tumor killing ability of CAR-T. At present, CAR-T targeting CD19, CD38, HER2 and EGFR can solve the off-target problem in the treatment of solid tumors by adjusting the affinity of scFv under the premise of the killing ability of scFv [5]. The hinge region (also known as the spacer region) combines the antigen recognition structure outside the cell with the transmembrane domain. In order to overcome space steric hindrance and homologous antigen, hinge area needs to have a certain flexibility. At present, CAR-T with different hinge regions, such as CD8 hinge region and transmembrane region, have been designed to reduce the probability of cytokine storm [6]. The intracellular domain contains two parts, namely, the stimulus domain and the signal transduction domain. The role of the signal transduction domain is to provide a second activation signal to T cells, and the common domain includes CD28 and 4-1BB. CD28 and 4-1BB show complementary effects to achieve stronger and longer lasting activation. Currently, the signal transduction domain of a standard CAR molecule is considered to be the CD3ζ chain, and the CD3 dimer is part of a T-cell receptor (TCR) complex consisting of multiple polypeptide chains. Immune receptor tyrosine activation motif (ITAM) gets transported by the CD3 dimer, and the ITAM sequence on the CD3ζ chain is an essential signal module for T cell activation. When TCR binds to antigen, lymphocyte-specific protein tyrosine kinase (LCK) phosphorylates the ITAM sequence, which activates downstream signaling pathways and affects cell behaviors such as cell proliferation and cytokine release.

CAR-T therapy has been used in clinical practice and has been continuously developed and improved. So far, CAR-T has completed the fourth generation update. The first generation of CAR - T invented by

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Eshhar's team, the first generation of CAR by specific scFv fragments and CD3 zeta signal domain of two parts, and able to provide the first cell activation signals, which can realize activation of T cell toxicity reaction, achieve antitumor effect. The second and third generation of CAR-T in order to obtain more amplification and proliferation ability, on the basis of the previous added CD28 and CD137 or 4-1 bb stimulus signal domain. The next generation of CAR-T was mainly used for the treatment of solid tumors, called TRUCK-T. Compared with previous generations, TRUCK - T introduced IL - 12, IL - 23 proinflammatory factor, not only overcome the inhibition of TME, and TRUCK - T in the process of anti-tumor, release can make the phagocytes to the tumor site migration and accumulation of cytokines, which increase the anti-tumor breadth.

2.2. Challenges of CAR-T therapy in treating solid tumors

CAR-T treatment has shown promising results in clinical trials for b cell leukemia, lymphoma, and malignant tumor blood systems. However, there are challenges in treating solid tumors, such as identifying tumor-specific antigen targets, overcoming tumor antigen escape, improving CAR-T cell transport, infiltration, and proliferation at the tumor site, and ensuring their persistence and function in hostile TME [7]. Compared with hematological tumors, solid tumor antigens are mostly heterogeneous. Due to the genetic instability of tumors and the different expressed antigens during the development and metastasis of primary and metastatic tumors, the lack of ideal antigens directly limits the therapeutic effect of CAR-T.

Solid tumors differ from the fluid environment of hematologic tumors. Solid tumors lack blood vessels and TME, and these factors directly limit the tumor invasion effect of CAR-T. Even if CAR-Ts can reach the tumor, on the one hand, CAR-Ts need to cope with the inhibitory conditions such as acidic environment, hypoxia, and nutrient deprivation in the tumor. On the other hand, the efficacy of CAR-Ts is also inhibited by related cell-mediated immunosuppressive factors in TME, such as regulatory T cells (Tregs) and myeloid-derived cells (MDSCs) in TME. Potential impact on the patient's body in order to prevent the CAR - T, the side effects of major organ systems and even life safety can directly suppress the cells release cytokines such as TGF – beta [8]and IL - 10. MDSC, a typical immunosuppressive cell, not only accelerates the invasion of solid tumors by secreting proinflammatory factors, but also negatively regulates the anti-tumor activity of CAR-T [9,10]. All factors together inhibit the use of CAR-T the curative effect of treatment of solid tumors.

3. Overview of altering solid tumor stroma to improve CAR-T cell infiltration

3.1. Solid tumor stroma

Both stromal and cancerous cells make up tumors. TME's key components include CAF, immune cells, inflammatory cells, adipocytes, glial cells, and some vascular cells. Activities related to metastasis, tumor growth, and interstitial tissue are closely related.

Collagen, proteoglycans (PGs) and glycosaminoglycans (GAGs), elastin and elastic fibers, laminin, fibronectin, etc. in the ECM are constantly synthesized and degraded [11], which puts the ECM in a complex dynamic balance. CAFs in the ECM can induce a profibrotic reaction. Massive fiber secretion leads to ECM precipitation, which hinders the migration of CAR-Ts to cancer cells and increases tumor drug resistance. On the contrary, the degradation of ECM will cause the fibrous structure to separate from the cancer cells and destroy the physical barrier formed by the proteins and glycans secreted by CAFs. Although the gap produced by the destruction provides a metastatic channel for cancer cells, it also removes the barrier for CAR-Ts and promotes the infiltration of CAR-Ts.

3.2. Solid tumor-associated fibroblasts

In ECM and relatively rich species and content of CAF in primary and metastatic tumors, which are involved in the formation of tumor matrix microstructure, not only provide physical support for tumor, but also have close relationship to development and metastasis of tumor.α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP), two markers expressed by CAF in cancer therapy, have been

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demonstrated to bind CD3 to T cells (called mesoFAP CAR-TEAM cells), target CAFs through FAP, and improve CAFs' capacity to respond to tumor therapy. It may successfully remove pancreatic ductal adenocarcinoma (PDAC) [12], reorganize the tumor cell matrix, and destroy and modify the PDAC stroma. Based on this study, this article focuses on HSPG produced by CAFs, and by HPSE to degrade heparan sulfate proteoglycans, thereby improving the feasibility of CAR-T cell tumor infiltration.

3.3. HSPG

HSPG, which can be produced by CAF and is involved in maintaining the structural integrity of the ECM, consists of a core protein and multiple heparan sulfate chains. CAFM synthesises core proteins and adds HS chains to them to form HSPG, which at the same time can bind cytokines, chemokines, growth factors and morphogens to protect them from hydrolysis. These growth factors signal and affect cell proliferation, differentiation, and tissue growth. HPSE part can be decomposed HSPG carbohydrates, realizes the remodeling of the ECM.

According to recent studies, this section found that solid tumors are composed of stromal cells and cancer cells, and their TME has multiple cell components, such as CAFs, immune cells, inflammatory cells, etc., which are closely related to tumor growth and metastasis. Collagen and other components in ECM are in dynamic balance. CAFs cause ECM precipitation by secreting a large number of fibers, which hinders the infiltration of CAR-Ts into cancer cells. On the contrary, the degradation of ECM can promote the infiltration of CAR-T cells. CAFs are abundant in ECM and tumors, and signature proteins such as α-SMA and FAP can enhance the therapeutic effect of tumors. In particular, HSPG produced by CAFs can play an important role in maintaining the structure of ECM. HPSE can achieve ECM remodeling by decomposing HSPG, thereby improving the infiltration ability of CAR-T cells in tumors. Therefore, using HPSE to degrade HSPG to transform ECM, thereby improving the infiltration of CAR-T cells, can provide some new ideas for CAR-T therapy to treat solid tumors.

4. Modifying CAR-T to express HPSE to improve the efficacy of solid tumors

4.1. Overview of HPSE functionality

HPSE is an endogenous β-glucuronidase in the human body. HPSE can perform the degradation of HSPG to achieve the destruction and reorganization of the ECM. Destruction of HPSE be HSPG fiber structure of ECM, prompted the possibility of metastasizing, therefore clinical study, will be the expression of HPSE level as a basis for the evaluation of cancer development stage one of [5].

As an enzyme, HPSE can reflect the degree of inflammatory response. Several pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, have been shown to up-regulate the expression and activity of HPSE. Carcinogenic signaling pathways, such as the Ras/MAPK pathway and the PI3K/Akt pathway, can upregulate the expression of heparinase [7]. Existing findings have shown that when HPSE degrades HSPG, it will release chemokines in advance to guide T cells to migrate to the ECM. These mechanisms provide ideas for modifying CAR-Ts through bioengineering methods to express HPSE.

4.2. Design of gene editing technology to modify CAR-T to promote HPSE secretion

The CRISPR is considered to be a groundbreaking gene editing technology. Le Cong et al. have realized the editing of multiple sites in the mammalian genome using CRISPR/Cas9. Existing studies have compared freshly isolated T cells, T cells that have undergone transient activation and T cells that have been cultured in vitro for a long time (LTE-T). The ability of freshly isolated T cells to perform ECM decomposition is significantly stronger than the latter two. The expression of HPSE is lost in T cells that have been cultured in vitro for a long time. The reason for the loss is believed to be the accumulation of the HPSE gene promoter and the p53 protein. It has been proven that when CAR-Ts are cultured in vitro and injected into the human body, they actually lack the expression of HPSE.

At present, through the successful design the expression retroviral gene transfection HPSE LTE-T cells, T cells attack ability is also improved. The results showed that after more than 10 days of starvation, the expression of HPSE in LTE-T was still stable, and the ability of LTE-T to degrade ECM was still

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better than that of the control (LTE-T without transduction). In addition, co-expressing HPSE and anti-tumor LTE-T showed more enhanced anti-tumor activity in the presence of ECM and was able to lyse the human neuroblastoma cell line GD2.In mice implanted with NB tumor cells and injected with a co-expression of anti-tumor specific CAR and retroviral gene transfer HPSE (CAR I HPSE LTE-T), survival was significantly improved in the experimental group compared with the control group (CAR LTE-T). Observation showed that the infiltration rate of T cells in the tumor tissue of the experimental group was higher, and the tumor mass was reduced. In conclusion, it is feasible to engineer the gene sequence of CAR-Ts to express HPSE and enhance tumor invasion.

First, it is necessary to design a guide RNA (gRNA) that can target and bind to the gene that secretes HPSE in CAR-T to guide Cas9. After the CRISPR/Cas9 component is inserted into the CAR-Ts, Cas9 binds to the HPSE secretory gene under the guidance of gRNA and generates a double-strand break (DSB) at the target site. After using the cell's DNA repair mechanisms, secretion of HPSE gene sequences in the repair process integrated into the genome of the CAR-Ts. This process is called homologous recombination (HDR). In addition, the sequence can be inserted or deleted at the target site through non-homologous end joining, that is, the original sequence is destroyed to make CAR-T secrete HPSE.

After gene editing is completed, the technique of flow cytometry can be used to detect cells that express the HPSE gene, screen the modified CAR-T and conduct quantitative analysis. The screened CAR-Ts are cultured, and the level of heparinase in the cell culture supernatant is measured by ELISA to evaluate the activity of the modified CAR-Ts and the expression level in the human body.

Existing research shows that through gene editing technology, such as CRISPR/Cas9, CAR-T can be modified to express HPSE and enhance its ability to infiltrate tumors. Studies have shown that HPSE-expressing CAR-T cells have significant effects in degrading ECM, enhancing anti-tumor activity and improving survival rate. Specific operations include designing gRNA to target the HPSE gene and integrating the HPSE gene into the CAR-T genome through NHEJ. Gene-edited CAR-T cells showed higher HPSE expression levels and anti-tumor activity in in vitro and in vivo experiments, proving that this modification method has application prospects.

5. Conclusion

CAR-T should be designed and improved for different tumors, and their excellent effect has been seen in hematological tumors. After a series of updates and upgrades, CAR-T has begun to develop in the direction of solid tumor treatment. The efficacy of CAR-T is limited due to the extracellular matrix surrounding solid tumors. Through the analysis of ECM components, it is found that HPSE makes one of the important components of the matrix, and can improve the tumor killing effect of CAR-T by degrading HSPG. Existing studies have shown that T cells that have been cultured in vitro for a long time lose HPSE expression. Cells expressing HPSE were designed by retroviral gene transfection. It has been confirmed that HPSE expression is stable and can achieve the function of decomposing ECM, which not only improves T cell infiltration, but also eliminates tumor cells. Although this scheme has the advantage of broad spectrum, it still has defects. First, due to the characteristics of different solid tumor matrices, the subpopulations and regulatory mechanisms of HPSE need to be explored in detail. Secondly, due to the limitations of CRIPR-Cas9 gene editing technology, Cas9 enzyme may perform editing outside the target site, resulting in off-target and other mutations. On the other hand, flow cytometry is relatively more expensive and sorting takes a long time. ELISA may be interfered when detecting microbial serum samples, which may affect the accuracy of detection. The use of CRISPR/Cas9 technology to remodify CAR-Ts to express HPSE has not made substantial contribution, and the tumor killing ability and actual effect of modified cells by this technology remain to be explored. Expected future more used for solid tumor therapy CAR - T drugs can approve, provide more treatment options for patients.

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