Immunotoxin Therapy for Lung Cancer

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Introduction

Lung cancer is the leading cause for cancer-related deaths in both genders throughout the world. In the United States alone, there were 224,390 estimated new lung cancer cases and 158,080 estimated deaths in 2016.[1] As conventional chemotherapy has reached a plateau of effectiveness in lung cancers and fails in those tumors whose growth and metabolism can hardly be distinguished from normal tissues, innovative therapeutic strategies have been explored. The use of novel agents, for example, immunotherapies, has started to show promising potential in the field.

Among immunotherapeutic agents, immunotoxins are a class of antibody-conjugated agents that have been developed for clinical application in many malignancies. Immunotoxins usually contain a bacterial or plant toxin payload to induce cell killing, and a targeting domain with an antibody or its fragment to achieve specific binding capacity. Immunotoxins exert the function of cell killing by inhibiting protein synthesis, which is toxic to both dividing and nondividing cells.[2] In this article, we summarize the properties of the commonly used immunotoxins and their application in recent preclinical and clinical studies in lung cancer.

Immunotoxin-based therapeutics

Immunotoxin refers to a toxin with the targeting part being either an intact monoclonal antibody (mAb) or its fragment. The main function of an immunotoxin is to target specific cell surface molecules using a cytotoxic agent, which can be internalized to induce cell death by protein synthesis inhibition. In general, the essential parts of an immunotoxin are the binding unit (mAb or its fragment) and the cytotoxic unit (engineered toxin), which can be recombinantly linked together. Since immunotoxins function by directly killing the cells instead of inhibiting receptor-mediated signaling pathways, there can be less chance for tumor cells to upregulate rescue mutations or alternative signaling pathways to resist the immunotoxin therapy.[3]

Plant toxins can be obtained from nature in the form of holotoxins and hemitoxins. Holotoxins contain a binding domain and an enzymatic domain linked by a disulfide bond. These toxins include ricin, modeccin, mistletoe lectin, and abrin. Compared to holotoxins, hemitoxins contain an enzymatic domain without a binding domain, which includes pokeweed antiviral protein, gelonin, and saporin.[4] It has been proven that both holotoxins and hemitoxins are able to remove the base of A^{4324} in 28s rRNA so as to preclude the combination of elongation factor (EF)-1 and -2 with the 60s ribosomal subunit.[5,6]

Bacterial toxins are somewhat different. An important requirement of fusion toxins is that the catalytic domain has to be separated from the other parts intracellularly. The two commonly engineered bacterial toxins are *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT).[7] Both consist of three functional domains that can be produced as single polypeptide chains. The binding domain, Domain I, is located at the N-terminus, while domains II and III are located at the C-terminus. Domain II has translocation activity. Domain

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III catalyzes adenosine diphosphate-ribosylation and EF2 inactivation, to inhibit protein synthesis and ultimately causes cell death.[7,8] It is reported that up to 300 ribosomes can be irrecoverably inhibited in 35 minutes by a single toxin molecule, which is toxic enough to destroy a cancer cell.[9-12]

After binding to the specific receptor, internalization of PE occurs through clathrin-coated pits into the endocytic compartment, and then PE is proteolytically cleaved in between amino acid (AA) 270 and 280, with the reduction of the disulfide bond connecting residues 265 and 287, which results in a fragment of 37,000 (AA280-612) at the C-terminus. With the translocation domain, the fragment is then transported to endoplasmic reticulum (ER), from which the catalytic domain is released into cytosol to ribosylates EF-2, leading to its inactivation. DT's killing mechanism is similar but has fewer steps: from the endocytic compartment, DT directly goes into cytosol to function. DT has a different AA sequence with a different enzymatic domain at the N-terminus.[2,13]

Development of immunotoxins

In the early 1980s, as mAbs began showing promise in the field of cancer therapy, Blythman *et al*.[14] first reported a novel immunotoxin that could kill cancer cells. However, the first-generation immunotoxin chemically conjugated a whole toxin to mAbs, which failed to distinguish the target of cancer cells from normal cells due to multiple potential chemical conjugation sites and the existence of the cell-binding domain of a whole toxin, showing unfavorable results in animal models.[15] The second-generation immunotoxins removed the cell-binding domain from the toxin part, thus affecting a much smaller amount of normal cells in animal models.[7] Nevertheless, the products were expensive for manufacturing, and not efficient enough to penetrate large and heterogeneous tumors, although the potency was proven in this generation of immunotoxins.[2]

The third and latest generations of immunotoxins are designed to contain only the variable fragment (Fv) portion of a mAb for binding and the translocation and catalytic domains of toxins to kill tumor cells. The current production method for immunotoxins is to massively and cost-effectively use *Escherichia coli*. A disulfide bond or a peptide linker is engineered to link the heavy and light chain of the antibodies to form a single-chain variable fragment (scFv) or disulfide-stabilized scFv (scdsFv).[2,7] To date, more novel immunotoxins are increasingly developed with features such as stronger potency, higher affinity and specificity, and less immunogenicity.

Improving potency, affinity, and specificity

There are several main methods to improve the potency of immunotoxins, which include changing or mutating the toxin structure, assembling different fragments of antibodies, and changing the conjugation between the two parts.[8] Point mutation techniques are used in remodeling original toxins.[16] The binding domain of the toxin is removed or mutated to be non-effective, which results in much smaller constructs, such as PE38 (AA253-364 and AA381-613) and DT_{388} or DAB_{389} (the first 388 AA).[17-20] When the construct PE38 translocates into cytosol, it transforms to components including AA280-364 and AA381-613 with only one cysteine residue at position 287. Moreover, with the modification of the antibody from full size to scFv, the tumor penetration is further improved, leading to increased access of the tumor mass.[21] Nevertheless, a reduced binding stability is found in this altered form, which leads to the application of an intrachain disulfide bond connecting only the two Fvs of immunoglobulin (heavy-chain variable domain and light-chain variable domain), to maintain stability and affinity.[3] In addition, PE obtained increased potency with the carboxyl terminus sequence REDLK replaced with KDEL. The KDEL residue improves the cytotoxicity of PE by increasing binding to a sorting receptor that retrogradely transports the toxin from the trans-Golgi apparatus to the ER.[3]

Decreasing immunotoxin immunogenicity

One of the biggest challenges for immunotoxin therapy is its potential immunogenicity, which can originate from either antibody part or toxin part.[16] To avoid the generation of host anti-murine antibodies against the antibody part of an immunotoxin, this part can be further humanized or replaced by a fully human counterpart.[22] As for the more immunogenic toxin domain, it can be reengineered by combining mutations that decrease lymphocyte epitopes to significantly minimize the immunogenicity induced by a foreign toxin protein.[23] Immunotoxins with mutations at both B- and T-cell epitopes can theoretically eliminate the issue of immunogenicity. Mazor *et al*.[23] engineered the mesothelin-targeting immunotoxin, LMB-T14, for patients with lung cancer by removing both B- and T-cell epitopes to achieve reduced immunogenicity while maintaining cytotoxicity.

In addition, studies using immunosuppressant regimens along with the immunotoxins also showed some benefit.[3,24,25] Other regimens, including a lymphocyte-depleting regimen, which consists of pentostatin and cyclophosphamide, were also found promising in delaying the stimulation of neutralizing anti-immunotoxin antibodies, thus allowing repetitive immunotoxin treatments for patients with solid tumors.[25] Pentostatin and cyclophosphamide selectively suppress the effect of T- and B-cells while largely sparing myeloid cells.[26]

Reducing adverse effects

Immunotoxin-induced toxicity is either targeted or nonspecific. Vascular leak syndrome (VLS) is a typical nonspecific toxicity, which is caused by endothelial cell damage from a high concentration of immunotoxins. In this case, capillaries are injured with fluid leakage. Fluid retains in tissues and causes edema in tissues, and serum albumin level falls. VLS can usually be managed by adequate hydration.[16] It is reported that high-dose ricin-based immunotoxins induce severe vascular collapse.[2] Bacterial toxins may be better in terms of VLS, given the fact that

compared to ricin toxin A chain (RTA) that can directly binds to endothelial cells, a ligand is required for modified PE to connect with the endothelium. In an animal model, a mutation on RTA can decrease the occurrence of VLS.[27]

Another inducement of toxicity is the unpredicted target effect due to same-target antigens also expressed on normal tissues. It has been reported that if organs with crucial functions, including the liver, neurons, and kidneys, express the same antigens targeted by immunotoxins, they will undergo immunotoxin-induced injury.[28-30] Thus, the selected target antigen should be highly specified to avoid targeting normal cells.[2]

Application in Lung Cancer Treatment

Although there are accumulating data of immunotoxins targeting hematologic tumors, solid tumors (e.g., lung cancer) are much more difficult to treat. The tumor cells are highly condensed with tighter junctions in between cells. Furthermore, some researchers suggest that patients with these cancers are less immunosuppressed, less likely to become immunosuppressed with systemic treatment, and more likely to derive neutralizing antibodies to immunotoxins.[27]

Immunotoxin therapy for nonsmall cell lung cancer

MAb L6 is an immunotoxin that targets antigens expressed on human lung, breast, colon, and ovarian cancers. The antibody is chemically conjugated to the whole structure of ricin. In mice studies, mAb L6 showed cell-killing effects in xenograft human lung adenocarcinoma.[31]

Mesothelin has a strong expression in many solid tumors, including lung adenocarcinoma and mesothelioma, but has low expression in mesothelium.[32-41] SS1P is an immunotoxin combining the SS1 anti-mesothelin antibody and PE38. It is currently combined with pentostatin and cyclophosphamide in a phase II study for immune depletion to reduce its immunogenicity in patients with lung adenocarcinoma and other mesothelin-positive cancers (NCT01362790).[42] The pilot study of SS1P showed that 3 of 10 treatment-refractory mesothelioma patients had major responses persisting more than 18 months.[25] The immunotoxin RG7787, combined with the humanized Fv fragment of SS1 and a modified PE fragment, has been reported to decrease tumor size in a xenograft mesothelin-expressing lung model.[16,43]

High expression of the Lewis Y antigen (Le^y) is found in many epithelial tumors. For instance, the Le^y antigen was found to be expressed in 80% of lung adenocarcinomas and 42% of squamous cell lung carcinomas in an immunohistochemistry analysis.[44] LMB-1 is an immunotoxin of mAb B3 (which reacts with the Le^y antigen) and PE38.[45] It is effective in colon cancer and breast cancer patients, with toxicity due to limited specificity involving endothelial cells.[46] Based on LMB-1, its derivative, LMB-9, yet was developed by combining scdsFv of mAb B3 and PE38, which was utilized to treat several different types of advanced solid tumors including recurrent nonsmall cell lung cancer (NSCLC) expressing the Le^y antigen (https://clinicaltrials.gov/ct2/show/NCT00019435). However, results from a phase I study did not show significant effectiveness.[2,47]

Naptumomab estafenatox, also known as ABR-217620, is an immunotoxin consisting of the fragment of the antigen-binding part of a mAb targeting 5T4 and the superantigen *Staphylococcal* enterotoxin A. Over 95% of tumors from patients with NSCLC, renal cancer, and pancreatic cancer have the expression of the 5T4 antigen. Thirty-one patients, including 19 NSCLC patients, were enrolled in a phase I study and had moderate and tolerable side effects. Most patients in this study achieved stable disease.[48] Results from the updated MONO study as well as another phase I study combining docetaxel (COMBO study) revealed that 36% and 38% of patients in the MONO study (with 51% of those being NSCLC patients) and the COMBO study (with 100% of those being NSCLC patients), respectively, reached stable disease (15% had a partial response in COMBO) at a 2-month follow-up with a maximum-tolerated dose of 26 μg/kg and 22 μg/kg, respectively.[49]

Immunotoxin therapy for small cell lung cancer

Similarly, immunotoxins based on mAbs SWA11 and SWA20, which target human small cell lung cancer (SCLC) antigen clusters w4 and 5A, respectively, conjugated with RTA, has shown promising activity against SCLC in mice.[50-53] The mouse mAb BrE-3 targeting the polypeptide core of antigen MUC1[54] is combined with RTA to form another immunotoxin, which has been reported to be effective in SCLC.[55]

CD56, an antigen of the neural cell adhesion molecule family, is the SCLC cluster 1 antigen. The immunotoxin N901-bR, fused by the anti-CD56 antibody N901 and modified ricin, was reported to be potent against SCLC expressing CD56.[56] In a phase I trial, N901-bR was administered in a group of 21 relapsed or refractory SCLC patients. One partial response was reported.[57-59] However, the future use of this drug is limited by the results of a phase II study using the same regimen, in which one fatal progressive VLS was found and all patients developed anti-immunotoxin antibodies, despite one stable disease and one complete remission for 3–4 months.[60]

HuD is a neuronal RNA-binding protein detected in all SCLC cells. Ehrlich *et al*.[61] assembled a type of immunotoxin (BW-2) containing the mouse anti-human-HuD mAb and streptavidin/saporin complexes. It was reported that the intratumoral injection of immunotoxins decreased the local tumor progression in six xenograft mouse models of human SCLC without toxicity.[61,62]

Future Directions

Improvements in therapeutic techniques that focus on the specific targeting potency and adverse effects of immunotoxins will be useful for lung cancer treatment. First, it is critical to identify new specific tumor antigens

on lung cancer cells that can become potential targets. Fortunately, previous studies have revealed many tumor antigens expressed on lung cancer cells that can be potential targets,[63,64] including glycoproteins such as epithelial cell adhesion molecules, carcinoembryonic antigen, mucins, podoplanin (PDPN), and tumor-associated glycoprotein 72; growth and differentiation signaling receptors such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER) 2, HER3, hepatocyte growth factor receptor, insulin-like growth factor 1 receptor, ephrin receptor A3, and tumor necrosis factor-related apoptosis-inducing ligand receptor 1; and stromal and extracellular matrix antigens such as fibroblast activation protein. Antibodies have been developed in previous studies for these potential targets and can be utilized to synthesize targeting immunotoxins to treat those cancer cells expressing specific antigens. For example, NZ-1 and D2C7 immunotoxins have been developed to specifically target PDPN and EGFR overexpressed on the tumor cell surface, respectively, both of which show a robust antitumor efficacy in the preclinical studies.[65,66]

Currently, most literature about immunotoxins focuses on hematological malignancies and tumors restricted to a certain area (such as malignant brain tumors) due to adverse effects of systemic administration of immunotoxins (e.g., the immunogenicity of the immunotoxin, off-target toxicity, and VLS).[16] Thus, it is important to optimize the method for safer and more efficient delivery of immunotoxins in lung cancer treatment. As an initial step, orthotopic murine lung cancer models have been established using either human xenograft lung cancer cells or Lewis lung carcinoma cells, building a platform to investigate novel immunotoxins in orthotopic animal models.[67,68] Due to the rapid clearance of immunotoxins and potential immunogenicity, immunotoxins are usually administered by locoregional delivery into the tumor site instead of via systemic delivery.[16,69] Thanks to the development of therapeutic and imaging techniques, Niu *et al*.[70] successfully injected antitumor agents percutaneously into the lung tumor site using a fine needle under the guidance of computed tomography without any serious adverse event in patients. With the application of locoregional administration, immunotoxin therapy through intratumoral delivery has already been used to successfully treat patients with glioblastoma to increase the local drug concentration and minimize the systemic toxicity and immunogenicity.[71,72] Locoregional administration of immunotoxin therapy for the treatment of lung cancer can now move forward based on modern technique improvements.

Although immunotoxin monotherapy has been proven to be effective for the treatment of many malignant tumors, its antitumor efficacy can further be enhanced by the appropriate combination strategies with other agents. Studies have shown that a type of newly developed immunotoxin may have better potency by exerting its cytotoxic moiety effects based on human-derived endogenous proteins, such as pro-apoptotic proteins or RNase.[73] Sensitivity of cancer cells to apoptosis will largely affect the cytotoxicity of these sorts of immunotoxins, and inactivation of p53 and upregulations of apoptosis inhibitors (e.g., B-cell lymphoma [Bcl]-2 and Bcl-xL) will lead to drug resistance. Thus, these immunotoxins may be more effective if combined with small molecule inhibitors of anti-apoptotic proteins to sensitize cancer cell apoptosis.[73] Besides, Leshem *et al*.[74] reported that the combination therapy of RG7787 immunotoxin with anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibody (an immune checkpoint inhibitor) led to a high rate of complete remissions in their breast cancer model, indicating that the combinatorial therapy of immunotoxin and immune checkpoint inhibitors may promote the activation of the antitumor immunity to achieve a long-term tumor elimination. Currently, two anti-programmed cell death protein 1 (PD-1) immune checkpoint inhibitors, nivolumab and pembrolizumab, were approved by the US Food and Drug Administration (FDA) to treat NSCLC, which can be potential candidates to be combined with immunotoxins to treat advanced NSCLC in the future.

In conclusion, the latest immunotoxins have emerged from many studies involving engineered immunotoxins that bind to tumor-surface epitopes with reduced *in vivo* toxicity and immunogenicity. In many preclinical and clinical studies, immunotoxins have displayed a different mechanism of tumor cell killing than traditional chemotherapy or radiation therapy. Further progress and improved clinical response of immunotoxin therapy against lung cancer depends on the identification of new tumor targets and optimized administration methods to promote its specificity and potency while minimize the adverse effect. Furthermore, immunotoxins may synergistically work with other therapeutics to enhance the antitumor efficacy as a combinatorial therapy.

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Conflicts of interest

Darell D Bigner owns stock in Istari Oncology and is a consultant to Genetron Health.

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