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Review Article

Bispecific antibodies as emerging pharmaceuticals for tumor immunotherapy

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Abstract

Bispecific antibodies consist of a targeting arm specific to the tumor antigen and a functional arm triggering an antitumor response through cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells, macrophages, etc. Bispecific antibodies prepared by conventional hybrid hybridoma or chemical conjugation methods have been tested in both the preclinical and clinical setting for the treatment of various cancers. The results from these studies revealed several major limitations of these molecules, such as difficulty in large-scale production of bispecific antibodies, immunogenicity due to murine components of the antibodies and severe side effects caused by the mass release of inflammatory cytokines. Rapid progress in the field of antibody engineering has made recombinant bispecific antibodies a paradigm for overcoming these obstacles. Some recombinant bispecific antibodies have already shown promising results and are expected to enter clinical trials over the next few years. This review focuses on the recent progress in the preclinical and clinical evaluation of recombinant bispecific antibodies.

Introduction

A bispecific antibody (bsMAb) is a unique type of antibody with two different binding specificities within a single molecule. The unique two-arm structure of bsMAbs allows scientists to place a therapeutic reagent on one arm while allowing the other to specifically target a disease site. The therapeutic reagents include radionuclides, drugs, toxins and enzymes. Another important application focuses on the use of bsMAbs to redirect cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells or macrophages to the tumor site and destroy the tumor cells.

In the past two decades, many bsMAbs prepared by traditional hybrid hybridoma or chemical conjugation methods have been tested in both the preclinical and clinical setting for the treatment of various hematological malignancies (*e.g.*, leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma) or solid tumors (*e.g.*, breast, ovarian, lung, renal and prostate cancer and melanoma). Unfortunately, progress has been very slow and major limitations include the difficulty in large-scale production of bsMAbs, lack of therapeutic efficacy, unsuitability for repeated administration due to immunogenicity and severe side effects caused by the mass release of inflammatory cytokines (1).

The ideal bsMAbs as potential pharmaceuticals for cancer immunotherapy should be humanized antibodies that can be produced at a reasonable cost, and their size should be small enough to access the tumor site easily and, at the same time, large enough to be retained at the tumor site to destroy tumor cells. The rapid and significant progress in the field of antibody engineering has made the recombinant bsMAbs (such as diabodies and bispecific single-chain Fv fragments) the paradigm for meeting these requirements. So far 18 monoclonal antibody-based products have been approved by the FDA for therapeutic applications, including 7 for oncology indications (Table I). This has generated enthusiasm for continuing the development of bsMAbs as potential pharmaceuticals

Table I: Monoclonal antibodies currently available for the treatment of cancer.

Antibody	Trade name(s)	Target	Indication
Rituximab	Rituxan, MabThera	CD20	Non-Hodgkin's lymphoma
Gemtuzumab ozogamicin	Mylotarg	CD33	Acute myeloid leukemia
Alemtuzumab	Campath, MabCampath	CD52	Chronic lymphocytic leukemia
Y-90 Ibritumomab tiuxetan	Zevalin	CD20	Non-Hodgkin's lymphoma
I-131 Tositumomab	Bexxar	CD20	Non-Hodgkin's lymphoma
Cetuximab	Erbitux	EGFR	Metastatic colorectal cancer
Bevacizumab	Avastin	VEGF	Metastatic colorectal cancer

for cancer patients. This review focuses on the recent progress in the preclinical and clinical evaluation of recombinant bsMAbs.

Immunotherapy

Cytotoxic T- lymphocytes as effector cells

bsMAbs are able to target and activate the cellular immune system to destroy tumor cells. A number of different effector cells have been used in this application, including CTLs, NK cells and macrophages. CTLs were initially believed to be the most suitable candidate for retargeting cytotoxicity, since they participate in the recognition and subsequent killing of tumor cells, virus-infected cells and allogeneic targets. The antitumor effect of CTLs reacting against tumor antigens is well established in experimentally induced tumors. In humans, CTLs play a protective role against virus-associated neoplasms (such as Epstein-Barr virus-induced Burkitt's lymphoma and human papillomavirus-induced tumors) and they have been detected in the blood and tumor infiltrates of cancer patients. CTLs kill target cells by bypassing upstream signaling events and directly inducing the effector phase of apoptosis. Upon recognition of foreign antigens, CTLs secrete perforin, a transmembrane pore-forming molecule, which allows entry of the CTL granule serine protease known as granzyme B. Granzyme B has the ability to cleave proteins at aspartate residues and is able to activate a variety of cellular caspases (2). CTLs also express Fas ligand (FasL) on their surface and initiate apoptosis of target cells by binding to Fas receptors.

The primary cytotoxic trigger molecule on CTL is the TcR/CD3 complex, which is antigen-specific and major histocompatibility complex (MHC)-restricted. However, bsMAbs can react with the TcR/CD3 complex and initiate retargeted cytotoxicity bypassing MHC restriction. Unfortunately, the targeting of TcR can produce disparate outcomes depending on the subset and differentiation state of T-cells being stimulated. The naïve CTLs ($CD8^+$) cannot lyse target cells since they require preactivation by cytokines such as IL-2 in order to become cytotoxic. Based on experience from clinical trials with the first generation of anti-TcR/CD3 bsMAbs, these antibodies can only produce antitumor responses when given locally; they fail to present therapeutic efficacy when administered intravenously and frequently induce severe toxicity due to the mass release of inflammatory cytokines. In addition to many potential factors, careful selection of the tumor target could be critical in this approach.

A broad range of tumor targets have been applied in bsMAb-based immunotherapy, including carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), HER-2/*neu*, Mov-18, CD19, CD20, CD52, etc. One of the relatively new targets is epithelial cell adhesion molecule (EpCAM; also known as GA733-2, KSA, 17-1A antigen), a human cell-surface glycoprotein expressed on some normal and most neoplastic epithelial cells, including ovarian, breast, lung, prostate and colorectal carcinoma cells. It is now widely recognized as playing an important role in tumor biology (3). Initial studies of monoclonal antibodies directed against EpCAM demonstrated the presence of anti-idiotype networks involving both B- and T-cells, antibody-dependent cell cytotoxicity and complement-mediated cell death as mechanisms of tumor growth inhibition. Recently, a novel receptor for EpCAM has been described that is a member of the inhibitory group of immunoglobulin-like receptors and is present on lymphocytes, monocytes, dendritic cells and NK cells. Neoplastic cells that interact with this receptor may enact immunological escape, conferring a selective advantage for their growth and spread. A bsMAb with affinity for both EpCAM and TcR/CD3 may block the binding of EpCAM to its inhibitory receptor and thereby enhance T-cell-mediated cytotoxicity.

Wimberger *et al.* have developed such a bispecific single-chain Fv (bscFv) binding to both EpCAM and TcR/CD3, which was tested using primary ovarian cancer tissue (4). A total of 17 of 21 (81%) patient samples showed concentration-dependent tumor cell elimination by this bscFv. Specific tumor cell lysis was seen at low bscFv concentrations, at low effector/target ratios and in the absence of T-cell co-stimulation. Recently, another research group also developed a similar molecule which can mediate the specific lysis of human colon and ovarian cancer cell lines. The bscFv was able to induce a co-stimulation-independent polyclonal activation of $CD4^+$ and $CD8^+$ T-cells and secretion of interferon gamma, tumor necrosis factor-α (TNF-α) and IL-2, IL-4 and IL-10. $CD8^+$ T-cells were the major player in redirecting tumor cell lysis by the bscFv. With a delay, $CD4^+$ cells could also contribute, presumably as a consequence of marked upregulation of granzyme B expression. In studies with

NOD/SCID mice, this bscFv was shown to be highly effective in inhibiting colon and ovarian cancer growth (5).

In order to fully activate CTLs, additional co-stimulatory signals are often needed, including CD2, CD5, CD28, CD40, LFA-1, ICAM-1 and the presence of cytokines (*e.g.*, IL-2 or TNF-α). Interesting enough, targeting without cytokines may actually induce T-cell apoptosis, creating a major challenge for bsMAb therapy. TcR/CD3-specific bsMAbs will not only trigger $CD8^+$ T-cells, but also $CD4^+$ helper T-cells. Under such circumstances, co-administration of cytokines will influence the outcome of the TcR/CD3-targeting response. Inflammatory cytokines can induce naïve $CD4^+$ T-cells to differentiate into Th1 cells, whereas cytokines (*e.g.*, IL-4 and IL-10) will promote Th2 cell differentiation. It is still not known which type of response would be most beneficial to immunotherapy. Bearing this in mind, a recombinant interacting bscFv was produced recently by Willems *et al.*, which was capable of triggering both the T-cell receptor and the co-stimulatory molecule CD28. They introduced a peptide tag and its cognate single-chain variable fragment, respectively, into a pair of antitumor x CD3 and antitumor x CD28 bsMAbs. A 30-fold increase in tumor-dependent T-cell activation was observed as compared to a noninteracting bsMAb. The study indicates a large window of opportunity in which only tumor cell-dependent T-cell activation is induced and systemic tumor cell-independent T-cell activation is avoided. This will minimize potential severe side effects by allowing the use of lower concentrations of the activating bsMAb (6).

Noncytotoxic T-lymphocytes as effector cells

Since CTLs require complicated interactions to be fully activated, the use of CTLs as immune effector cells for bsMAb immunotherapy is more complex compared to NK cells and myeloid effector cells. NK cells are lymphocytes that are capable of destroying tumor cells without prior sensitization (7). In addition, many tumors downregulate the expression of class I MHC molecules as a way of evading immunity. NK cells are particularly effective against cells with reduced MHC expression. Myeloid cells are not generally considered effector cells since they do not specifically recognize tumor cells. However, in the presence of a bsMAb, myeloid cells were able to kill tumor cells. Furthermore, myeloid cells are known to be able to infiltrate tumors which are engineered to secrete cytokines (*e.g.*, IL-2, IL-4, IL-7, interferon gamma, TNF-α, granulocyte colony-stimulating factor [G-CSF]) and the induction of tumor immunity requires the communication between neutrophils and T-cells (8). NK cells, macrophages and neutrophils have gained much attention in bsMAb-based cancer immunotherapy because they can be readily activated and mobilized *in vivo*. Fcγ receptors (*e.g.*, CD64, CD16) are the most widely investigated trigger molecules on NK cells and myeloid cells. Compared to anti-TcR/CD3 bsMAbs, anti-Fcγ receptor bsMAbs have shown more positive responses with less toxicity.

Shahied *et al.* constructed a bispecific minibody (anti-HER2/*neu* x anti-CD16) in two distinct binding formats (9). The parent minibody was constructed such that the IgG_1 C_H3 constant domain serves as the oligomerization domain and is attached to an anti-CD16 and an anti-HER2/*neu* scFv via 19- and 29-amino-acid linkers, respectively. Cytotoxicity studies showed that the minibody can induce significant tumor cell lysis at low concentrations. A trimeric bispecific minibody that binds dimerically to HER2/*neu* and monomerically to CD16 induced equivalent cytotoxicity at even lower antibody concentrations than either the parent minibody or the corresponding single-chain dimer. Both minibody constructs were stable in mouse and human serum for up to 72 h at 37 °C. Bruenke *et al.* developed a bscFv (CD19 x CD16) for the treatment of leukemias and lymphomas (10). This bscFv was stable because the disulfide bonds bridging the respective variable light (V_L) and variable heavy (V_H) chains were introduced into both component scFvs. In cytotoxicity studies, the bscFv mediated specific lysis of both $CD19^+$ human lymphoma cells and tumor cells from patients with chronic lymphocytic leukemia (CLL) or acute lymphoblastic leukemia (ALL).

Others

Both CTLs and non-CTLs (NK cells or macrophages) have shown effective killing of tumor cells in bsMAb-based approaches. Can bsMAbs activate both CTLs and NK cells or macrophages simultaneously? This concept was explored by Heiss *et al.*, who designed a new class of bsMAbs with the ability to activate not only T-cells but also Fcγ receptor type I/III^+ cells (macrophages, NK cells and dendritic cells) (11). This trifunctional antibody (trAb), which bound either the EpCAM or HER2/*neu* antigen, was capable of killing tumor cells without any pre- or co-stimulation. The effect of this trAb was further investigated in 8 patients with malignant ascites due to peritoneal carcinomatosis. Treatment consisted of 4-6 applications within 9-23 days with a total amount of 145-940 μg. Seven of 8 patients required no further paracentesis during follow-up or until death, with a mean paracentesis-free interval of 38 weeks (median = 21.5 weeks; range = 4-136 weeks). Tumor cell monitoring showed a complete elimination of tumor cells in ascites already at total doses as low as 40-140 μg. All patients had disappearance of ascites, which was correlated with elimination of tumor cells (p = 0.0014). Severe adverse events were not observed. This treatment may offer a new therapeutic option for patients with peritoneal carcinomatosis.

Trifunctional antibodies can also be designed to bind two different tumor targets and one effector trigger molecule. Investigators at the University of Munich performed a pilot study that demonstrated the feasibility of combining high-dose chemotherapy (HDCT) with such a trAb (anti-EpCAM x anti-CD3 and anti-HER2/*neu* x anti-CD3) in the treatment of metastatic breast cancer (12). After leukapheresis and cryopreservation of T-cells, patients received 2 cycles of induction chemotherapy (ET; epiru-

bicin/paclitaxel) and 1 cycle of epirubicin/ifosfamide (EI), followed by G-CSF and stem cell harvest. After a final cycle of ET, responders underwent HDCT (thiotepa 600 mg/m^2 + melphalan 140-180 mg/m^2) and stem cell transplant. Once reconstitution was achieved, T-cells were re-infused, followed by administration of trAbs. Thirty-three patients were recruited into the study and 19 who had responded to initial chemotherapy underwent HDCT and stem cell transplant (4 complete responses, 15 partial responses). Two early deaths were observed (1 due to toxicity and 1 due to early progression). T-cell re-infusion and trAbs were given to 17 patients. Side effects were mild with trAb treatment. Patients who received 3 trAb doses showed improved overall survival (47.2 months *vs.* 22.4 months).

BsMAbs can also simultaneously block two signaling pathways with one molecule. Both the EGFR and the insulin-like growth factor receptor (IGFR) have been implicated in the tumorigenesis of a variety of cancers. The EGFR-targeting monoclonal antibody Erbitux™ (cetuximab) has been approved by FDA for the treatment of colon cancer. Lu *et al.* proposed that simultaneous targeting of both EGFR and IGFR with a bsMab would lead to enhanced antitumor activity. They produced a recombinant human IgG-like bispecific antibody, a di-diabody, using the variable regions from two antagonistic antibodies: IMC-11F8 to EGFR and IMC-A12 to IGFR. The di-diabody binds to both EGFR and IGFR and effectively blocks both EGF- and IGF-stimulated receptor activation and tumor cell proliferation. The di-diabody also inherited the biological properties of both of its parent antibodies; it triggered rapid and significant IGFR internalization and degradation and mediated effective antibody-dependent cellular cytotoxicity in a variety of tumor cells. Finally, the di-diabody strongly inhibited the growth of two different human tumor xenografts *in vivo* (13).

Conclusions

With their unique two-arm structure, bsMAbs are able to activate the immune defense system by artificially combining humoral and cellular components to retarget them to tumor cells. The unique biological properties of bsMAbs spur their continued development. Although tumor immunotherapy using recombinant bsMAbs is still in an early stage, promising results have already been obtained. A major advantage of using recombinant bsMAbs is to overcome the problem of large-scale production of bsMAbs. In addition, using humanized recombinant bsMAbs will not evoke an antibody response, allowing multiple-dose administration. However, questions regarding the therapeutic efficacy and immunogenicity of these new recombinant constructs remain to be answered with large-scale clinical trials.

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