Review

Novel Treatment Strategies for Cancer and Their Tumor-targeting Approaches Using Antibodies Against Tumor-associated Antigens

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Abstract. *Novel treatment strategies for cancer that are based on a more detailed understanding over the tumor biology are based on the latest new technology and are expected to improve the current treatment outcome for patients with cancer. However, many of these strategies still have one common and critical problem, being their limited specificity for tumor cells. In this context, antibodies against tumor-associated antigens (TAAs) are used in several ways to increase the tumor specificity of these novel strategies. Firstly, photodynamic or sonodynamic therapy using anti-TAA antibodies conjugated with new sensitizers offers additional therapeutic approaches. Secondly, re-targeting of T-cell immunotherapy using an anti-TAA antibody fusion protein was shown to be useful for the success of cancer immunotherapy, because the down-regulation of HLA class I molecules in tumor tissues constitutes a major tumor escape mechanism associated with tumor-specific cellular immunity. Thirdly, in oncolytic virotherapy, targeting viral vectors carrying cytolytic activity against tumor tissues by modifying the tropisms with anti-TAA antibodies is also very promising from a practical point of view.*

The three major types of cancer treatment, i.e. surgery, chemotherapy and radiotherapy, are powerful, but are associated with risks of injury or toxicity to normal tissues. Therefore, many other treatment methods that can kill tumor cells without these side-effects are being tried in research and clinical studies. Among them, photodynamic therapy (PDT), sonodynamic therapy (SDT), T-cell immunotherapy and oncolytic virotherapy are expected to improve current treatment outcomes due to their tumor cell killing mechanisms, which are different from those of the three major methods (1-5). However, these relatively new strategies are still associated with one common and critical problem: their limited specificity for targeting tumor cells (3). In this context, antibodies against tumor-associated antigens (TAAs) are being used in several ways to increase the tumor specificity of these new strategies (6).

Many TAAs have been identified, and monoclonal antibodies (MAbs) targeting these TAAs have been widely assessed for cancer therapy. Several specific immunotherapies using anti-TAA antibodies themselves seem to be very promising, because the actual expression of antibody-recognized TAAs on target tumor cells is easily confirmed by immunostaining or flow cytometry with the corresponding antibodies. A clinical benefit is more likely to occur if the TAAs are expressed only on cancer cells and not on vital tissues. The major antibody-recognized TAAs used or tried as the targets of cancer therapy include CD19 for B-cell leukemias and lymphomas (7), CD20 for B-cell non-Hodgkin's lymphoma (8), CD30 for Hodgkin's lymphoma and systemic anaplastic large-cell lymphoma cells (9), CD33 for acute myeloid leukemia (AML) (10), CD52 for chronic lymphocytic leukemia (11), human epidermal growth factor receptor-2 (HER2) for breast cancer (12), epidermal growth factor receptor (EGFR) for colorectal or lung cancer (13), vascular endothelial growth factor (VEGF) for colorectal cancer (14), carcinoembryonic antigen (CEA) for various types of cancer, including gastrointestinal (15, 16), and epithelial cell adhesion molecule (EpCAM) for colorectal cancer (17). The objective of this short review is to provide a brief overview of the possible applications of antibodies to TAAs in novel treatment strategies for cancer (Table I).

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Targeting of Photodynamic or Sonodynamic Therapy

MAbs that are not capable of directly eliciting antitumor effects can still be effective against tumors as selective carriers for delivering cytotoxic agents, such as toxins (18), chemotherapeutics (9, 19), radioisotopes (20), photosensitizers (PSs) (21) or sonosensitizers (SSs) (22). PDT and SDT are relatively new treatment modalities for various diseases, including cancer. These techniques involve the topical or systemic administration of a PS or an SS, followed by selective irradiation of the target lesion with a specific wavelength of non-ionizing light or ultrasound, which triggers oxidative photodamage or sonodamage and subsequent death of the targeted cells, respectively. Due to this two-step therapeutic process, PDT and SDT are relatively safe and non-invasive treatment modalities. Nevertheless, classical non-targeted PSs or SSs lack sufficient tumor selectivity and are taken up in the neighboring normal tissues, resulting in undesirable adverse effects. To overcome this obstacle, several tumor-targeting approaches using MAbs to TAAs have been developed (Table I) (21).

Tumor targeting of PDT with a PS-conjugated antibody. In PDT for cancer, MAbs to TAAs or their single-chain variable fragments (scFvs) conjugated with PSs are utilized to increase the tumor specificity, and this approach is called photoimmunotherapy (PIT) (23). Unlike conventional antibody-based immunotherapies, the MAbs used in PIT do not necessarily need to have an intrinsic effector function. However, different antibody formats significantly affect the pharmacokinetic profiles *in vivo*. The use of an intact MAb slows blood clearance, leading to an increased chance of the agent accumulating in vital organs; moreover, these agents have a poor ability to diffuse throughout the tumor mass due to their large molecular size. In contrast, the scFv format exhibits rapid blood clearance and easily diffuses within the tumor (24).

The proteins in the EGFR family are often overexpressed in many types of human tumors, and are known to be internalized by endocytosis, making them one of the molecular targets for intracellular drug delivery. Savellano *et al*. conjugated a clinically-approved benzoporphyrin derivative (verteporfin) to the MAb to EGFR, cetuximab, and showed that the conjugate effectively targeted and photodynamically killed EGFR-overexpressing A431 (epidermal carcinoma) and Ovcar5 (ovarian cancer) human cancer cells, whereas free verteporfin exhibited no specificity (25). One of the most striking studies on PIT against the EGFR was reported by Mitsunaga *et al*. (26). In their study, a near infrared phthalocyanine dye, IRDye700DX (IR700), was conjugated to an MAb to HER2 (trastuzumab) and MAb against EGFR (panitumumab). Target-specific photodynamic effects were observed in both *in vitro* and *in vivo* experiments. Importantly, unlike conventional PIT agents, which usually require translocation into the cytosol in order for them to exert their phototoxicity, the IRDye700DX–MAb conjugates were substantially effective when bound to the target cell membrane, with no need for internalization. These characteristics of IRDye700DX were further supported by a recent report of our group, in which the dye was conjugated to an MAb to human CEA (Figure 1) (27). Thus, IRDye700DX-based PIT seems to be promising and may be applicable for cancer treatment, especially for drug-resistant tumor cells (*e.g*. cancer stem cells) with a high drug efflux capacity.

Tumor targeting of SDT with a SS-conjugated antibody. In SDT for cancer, focused ultrasound can penetrate deeply into tissues and can be focused into a small region of a tumor to activate the cytotoxicity of the SSs (28). This is a unique advantage in the non-invasive treatment of non-superficial tumors compared to the laser light used for PDT. Given the similarity of the mechanisms of PDT and SDT, SDT may be exploited for the generation of effective therapeutic immunoconjugates, like PDT. In a previous study, we synthesized a conjugate between a novel mouse MAb against CEA with a new PS (ATX-70), which also has the properties of an SS (29). This conjugate, designated F39/ATX-70, retained immunoreactivity against CEA-expressing cells. The cytotoxicity of F39/ATX-70 against CEA-expressing human gastric carcinoma cells *in vitro* was found to be greater than that of ATX-70 when applied in combination with ultrasonic irradiation. When the *in vivo* anti-tumor effects in a mouse xenograft model were assessed, the intravenous administration of F39/ATX-70, followed by ultrasonic irradiation, produced a marked growth inhibition of the tumor in comparison to irradiation-alone or irradiation after the administration of ATX-70. These results suggest that the immunoconjugate between MAb to CEA and ATX-70 may have applications in SDT where destruction of the CEA-expressing tumor is required, and this approach might be called sonoimmunotherapy (SIT) (29). SIT using novel SSs (22, 28) may be a promising systemic treatment modality, not only for superficial cancers, but also for deep-seated tumors, which would make it more widely applicable than PIT.

Retargeting of T-cell Immunotherapy

Cellular immunity, in which cytotoxic T-lymphocytes (CTLs) are the main effector cells, plays an important role in the antitumor defense mechanism. T-cell immunotherapy is based on the assumption that TAA peptides are correctly presented by HLA class I molecules on target tumor cells. However, human tumor cells are well-known to frequently

Table I. *Novel treatment strategies for cancer and their tumor targeting approaches with anti-TAA antibodies.*

A. Photodynamic or sonodynamic therapy
1. Cell-killing mechanism
Tumorcidal effect of photo- or sonosensitizer: reactive oxygen toxicity, *etc*.
2. Targeting approaches
i) Photosensitizer-conjugated antibody
ii) Sonosensitizer-conjugated antibody
B. T-cell immunotherapy
1. Cell-killing mechanism
T-cell-mediated cytotoxicity: perforin and granzyme pathway, etc.
2. Targeting approaches
i) Bispecific antibody
ii) Antibody-cytokine fusion protein
iii) Antibody-TCR chimeric antigen receptor
C. Oncolytic virotherapy
1. Cell-killing mechanism
Cell lysis activity of virus: disruption of cellular membranes, *etc*.
2. Targeting approaches
i) Antibody-directed vaccinia virus vector
ii) Antibody-directed herpes simplex virus vector

lose HLA class I molecules. This altered HLA class I expression constitutes a major tumor escape mechanism for tumors facing specific CTL-mediated responses. Therefore, endowing CTLs with the antigen-binding specificity of antibody to TAAs is promising for re-targeting the activities of these effector cells to tumor cells in an HLA-independent manner. The following strategies are currently being tried as fusion therapy using an antibody to TAA and cellular immunity (Table I) (30).

Retargeting of CTLs with a bi-specific antibody. The bi-specific antibody technology allows the generation of a single antibody directed against both a TAA and a given surface marker on effector cells, such as CD3 on T-cells (31). In a previous study, Reusch *et al*. generated a b-specific antibody consisting of anti-CD3 and anti-EGFR (cetuximab, Erbitux ™) (13). The bi-specific antibody was able to redirect T-cell activity to human EGFR-positive cancer cells *in vitro* and in an animal model. Recently, Aigner *et al*. produced an anti-CD3×anti-CD33 bi-specific antibody, designated AMG330, and showed that human T-cells armed with AMG330 were cytotoxic against AML cells in *ex vivo* experiments, as well as in a mouse xenograft model (32). More recently, Laszlo *et al*. also proved that the cytotoxicity of AMG330 against AML cells is proportional to the level of CD33 expression, but is not affected by adenosine triphosphate-binding cassette transporter activity (33). Taken together, these bi-specific antibodies may serve as a potentially useful immunotherapeutic reagents for human TAA-expressing cancers.

Retargeting of CTLs with an antibody–cytokine fusion protein. Treatment with cytokines holds great potential for cancer therapy, but is generally restricted by systemic toxicity. Therefore, the fusion of MAbs to TAAs and cytokines is an efficient technique to target cytokines to tumor cells, and hence focuses the killing activity of CTLs on the target cells *via* cytokine receptors (34). In order to generate antibody fusion proteins, cytokines such as interleukin-2 (IL2), interleukin-12 and interleukin-15 were generally fused to the C-terminus of IgG3 (heavy chain) or to the C-terminus of a diabody or scFv targeting TAAs, such as CD20, HER2, CEA or EpCAM, etc. (35, 36). In a previous study, we also genetically fused human IL2 to an antibody to CEA scFv (37). The resulting fusion protein effectively targeted IL2 to the surface of CEA-expressing tumor cells, and consequently introduced specific cytotoxicity of these human CTLs to the tumor cells. Recently, Ding *et al*. generated an anti-HER2–IL2 fusion protein, and covalently attached it to the non-toxic polymalic acid backbone to target HER2-expressing tumors and ensure the delivery of IL2 to the tumor microenvironment (38). Antisense oligonucleotides were also conjugated to the nanodrug to inhibit the expression of vascular tumor protein laminin-411 in order to block tumor angiogenesis. The nanobioconjugate exhibited marked antitumor activity manifested by significantly longer animal survival and a significantly increased anti-HER2 immune response in immunocompetent mice bearing D2F2/E2 murine mammary tumors that expressed human HER2. In addition, several antibody–cytokine fusion protein candidates are being evaluated in clinical trials (35). Therefore, antibody–cytokine fusion proteins may be promising immunological agents that will be especially useful for combinatorial cancer therapies.

Retargeting of CTLs with a chimeric antigen receptor. The chimeric antigen receptor (CAR) technology also has the potential to re-target CTLs to tumor cells (39). Recombinant CARs encompass antibodies to TAAs that are genetically- fused to the signaling domains of either T-cell receptor (TCR). After transfection, CTLs expressing anti-TAA scFv/TCR-ζ(CD3ζ) receptors recapitulate the cytopathic effects mediated by the TCR, and allow the targeting of tumor cells in an HLA-independent manner (40). However, the length, flexibility and origin of the hinge domain is an important variable in the design of CARs, because T-cells require both primary and co-stimulatory signals for optimal activation, and because many tumors do not express critical co-stimulatory ligands (41). Thus, the 'generations' of CARs typically refer to the intracellular signaling domains. First-generation CARs include only CD3ζ as an intracellular signaling domain (39, 40), whereas second-generation CARs include a single co-stimulatory domain derived from either CD28 (Figure 2) or 4-1BB (41, 42), and third-generation CARs include two co-stimulatory domains, such as CD28, 4-1BB and other co-stimulatory molecules (43).

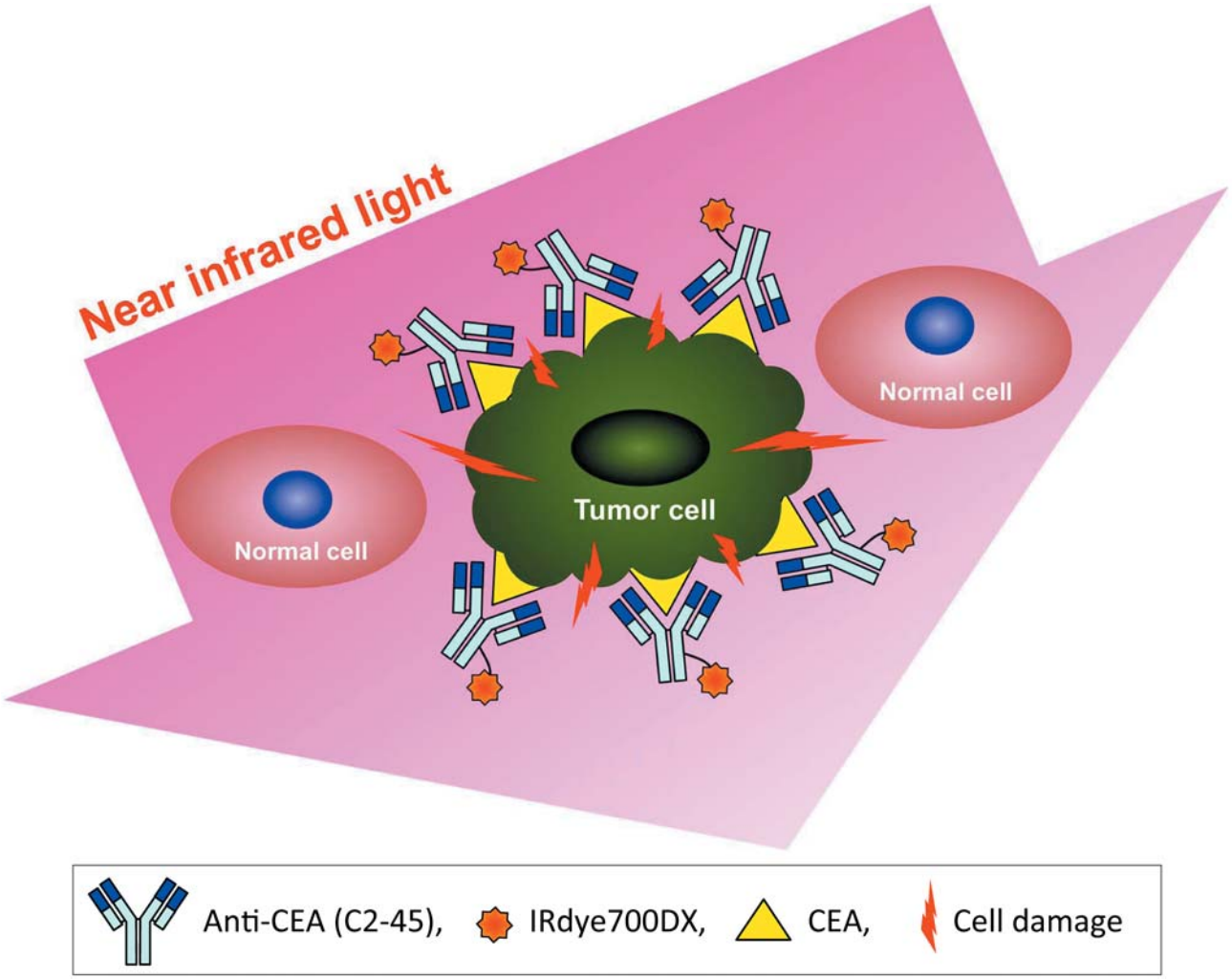

Figure 1. *Tumor-targeting of near infrared phototherapy with an antibody to CEA conjugated with a photosensitizer, IRDye700DX.*

Thanks to this CAR technology, large numbers of CTLs with redirected anti-TAA specificity have thus been generated, and some of them, especially the first- and second-generation CARs, are currently being examined in clinical trials (44, 45). Overall, these studies have proven that CARs are capable of harnessing the signaling machinery of both TCR-ζ and co-stimulatory molecules to augment T-cell immunity against tumors that have lost the expression of both MHC/peptide and co-stimulatory ligands *in vivo* (46).

Targeting of Oncolytic Virotherapy

Oncolytic virotherapy is based on viruses whose replication is restricted to malignant cells. In general, this tumor selectivity can be achieved in one of the following two ways (47). The first way involves the use of viruses that normally do not cause disease in humans but can replicate in tumor tissues, where the interferon antiviral response is frequently non-functional because of the immunosuppressive environment. These are typically small viruses with fast replication cycles, such as reovirus, Newcastle disease virus or vesicular stomatitis virus. The second way is the development of oncolytic vectors that are based either on viruses that are used as vaccines against common disease-causing viruses, such as the vaccinia virus, or on viruses that themselves cause known disease in humans, such as adenovirus or herpes simplex virus (HSV). These tend to be larger viruses that are amenable to genetic engineering to produce or enhance their tumor selectivity. This increased selectivity is normally achieved through the deletion of viral virulence genes that are redundant for viral replication in tumor cells. As a result, tumor-selective replicating viruses seem to offer advantages

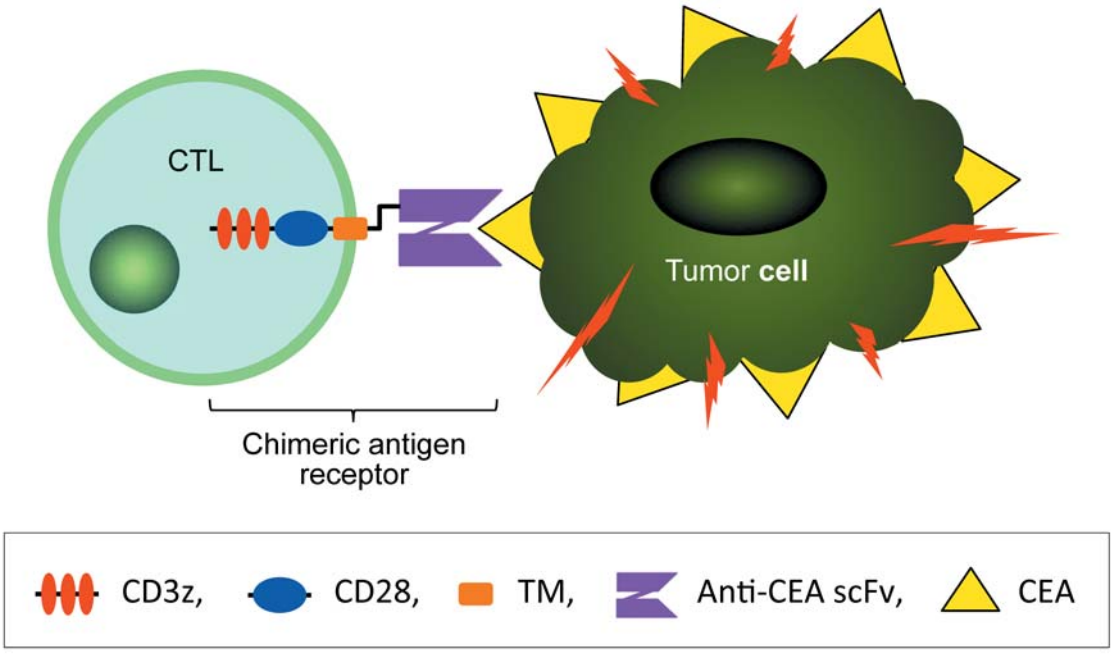

Figure 2. *Tumor-targeting of CTL with a chimeric antigen receptor consisting of an scFv antibody to CEA, a transmembrane domain (TM), CD28 and CD3ζ.*

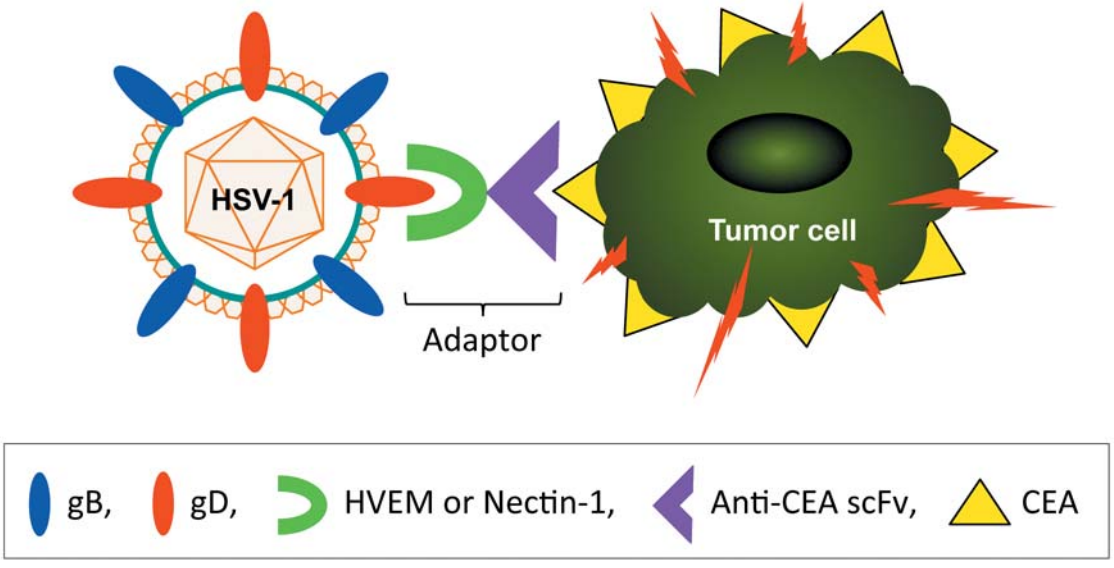

Figure 3. *Tumor-targeting of an oncolytic virus (HSV-1) with an adapter protein consisting of an scFv antibody to CEA and viral glycoprotein D (gD) protein. gB: Glycoprotein B; HVEM: herpes virus entry mediator.*

over conventional cancer therapy (48). However, the tumor-selective properties of these oncolytic viruses are relatively weak. Thus, to establish oncolytic virotherapy as a feasible treatment for cancer, more emphasis will be required on the development of optimal gene delivery systems with greater tumor tissue specificity. To improve the effectiveness of oncolytic virotherapy, several tumor-targeting approaches using MAbs to TAAs have been tried (Table I) (49, 50).

Tumor targeting of HSV vectors with an antibody against TAA. The safety and efficacy of oncolytic HSV type-1 (HSV-1) for solid tumors can be enhanced by redirecting the virus infection to tumor-specific cell-surface markers. Successful retargeting of HSV-1 has been achieved using vectors that carry a modified envelope glycoprotein D (gD) engineered to interact directly with novel receptors (49). In addition, soluble bridging adapters have been used to link gD indirectly to cell-specific receptors (51). In a recent study, we developed an adapter connecting gD to CEA (Figure 3) (52). The adapter consisted of an antibody to CEA scFv fused to the gD-binding region of the gD receptor, a herpes virus entry mediator (HVEM). This adapter was used in combination with a vector that was detargeted for recognition of the widely expressed gD receptor nectin-1, but retained an intact binding region for the less common HVEM. The adapter enabled infection of HSV-resistant Chinese hamster ovary cells expressing ectopic CEA and nectin-1/CEA-bearing human gastric carcinoma cells that were resistant to the vector alone. In addition, cell-to-cell spread following adapter-mediated infection *in vitro* and reduced tumor growth *in vivo* were observed.

More recently, Uchida *et al*. established an HSV retargeting system that relies on the combination of two engineered viral glycoproteins, gD and glycoprotein B (gB), to mediate highly efficient HSV infection exclusively through recognition of the abundantly expressed EGFR on glioblastoma cells (53). They demonstrated its efficacy *in vitro* and in a heterotopic tumor model in mice. Treatment of orthotopic primary human glioblastoma xenografts demonstrated prolonged survival of animals showing a complete response, confirmed by magnetic resonance imaging. Thus, these methods involving vector retargeting may provide a novel strategy for tumor-specific delivery of tumoricidal HSV and may be applicable to the treatment of a broad range of tumor types.

Tumor targeting of vaccinia virus vectors with an antibody to TAA. In a previous study, Yu *et al*. reported that the replication-competent vaccinia virus (VACV), GLV-1h68, showed remarkable oncolytic activity and efficacy in different animal models (54). They also reported the construction of three VACV strains encoding an antibody to VEGF scFv (55). The replication efficiency of all the svFv-expressing VACV strains in cell culture was similar to that of the parental GLV-1h68 virus. Successful tumor-specific delivery and continued production of functional scFv derived from individual VACV strains were obtained in tumor xenografts following a single intravenous injection of the virus. The VACV strains expressing the scFv exhibited significantly enhanced therapeutic efficacy in comparison to treatment of human tumor xenografts with the parental virus GLV-1h68 (55).

Recently, Buckel *et al*. tested one of the VACV strains expressing the scFv, designated GLV-1h164, in combination with fractionated irradiation in a subcutaneous model of U-87 glioma, and showed that it enhanced tumor growth inhibition compared to the parental virus and irradiation (56). Irradiation of tumor xenografts resulted in an increase in VACV replication of both GLV-1h68 and GLV-1h164. However, the treatment with GLV-1h164 in combination with irradiation resulted in a dramatic decrease in the intra-tumoral VEGF levels and tumor vessel numbers in comparison to GLV-1h68 and irradiation. These findings demonstrated the successful incorporation of GLV-1h164 into a fractionated radiation scheme to target tumor cells by enhanced VACV replication in irradiated tumors, as well as to radiosensitize the tumor endothelium, which resulted in enhanced efficacy of the combination therapy for human glioma xenografts. Taken together, these findings indicated that the VACV-mediated delivery and production of scFv antibodies to VEGF may provide a unique therapy concept: tumor-specific, locally amplified drug therapy in humans, and the added element of the antibody to TAA may increase the effectiveness of the combination viral and radiation therapy (56).

Conclusion

Several novel treatment strategies for cancer, including PDT, SDT, T-cell immunotherapy and oncolytic virotherapy are being examined in research and clinical studies. However, these strategies have been limited by their specificity for targeting tumor cells. Antibodies against TAAs are used in several ways to increase the tumor specificity of these strategies. These approaches seem to be very promising, because the recent studies using antibodies to TAA as tumor-targeting tools showed that they were effective, and because the actual expression of the TAAs on patient tumor cells can be easily confirmed by immunostaining or flow cytometry with the corresponding antibodies.

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