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Review

Re-targeting of Cytotoxic T Lymphocytes and/or Natural Killer Cells to CEA-expressing Tumor Cells with Anti-CEA Antibody Activity

MASAHIDE KUROKI, KEN HACHIMINE, JIAN HUANG, HIROTOMO SHIBAGUCHI, TETSUSHI KINUGASA, SHIN-ICHI MAEKAWA and MOTOMU KUROKI

Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka, Japan

Abstract. *Cellular immunity, in which cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are main effector cells, plays an important role in the antitumor defense mechanism. T cell immunotherapy is based on the assumption that tumor antigen (TA) peptides are correctly presented by HLA class I molecules on target tumor cells, while NK cell immunotherapy is based on the hypothesis that cell surface TAs or ligands for NK receptors are widely expressed in tumor cells. However, human tumor cells are well known to often lose HLA class I molecules, and target cell ligands for NK receptors are not always expressed in human tumor cells. This altered HLA class I expression and non-ubiquitous distribution of NK receptor ligands constitute the major tumor escape mechanism facing tumor-specific CTL and/or NK cell-mediated responses. These facts also indicate that it is not easy to eliminate the target tumors only by activating tumor-specific CTLs or NK cells. On the other hand, although the protective role of humoral immunity in cancer seems not to be imperative, it is easily confirmed by immunostaining whether or not antibody-recognized TAs such as carcinoembryonic antigen (CEA) exist on the cell surface of target tumor cells. Therefore, endowing CTLs or NK cells with antigen-binding specificity of anti-TA antibody is promising for re-targeting the activities of these effector cells to tumor cells in an HLA-independent manner. This mini-review provides a brief overview of the following four technologies for re-targeting CTLs or NK cells to CEA-expressing tumor cells with anti-CEA antibody activity: i) bispecific antibody technology, ii) antibody-cytokine fusion protein technology, iii) chimeric immune receptor technology, and iv) antibody-HLA/peptide complex technology.*

Correspondence to: Prof. Masahide Kuroki, MD, Ph.D., Department of Biochemistry, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan. Tel: +81-92-801-1011 Ext. 3240, Fax: +81-92-801-3600, e-mail: kurokima@fukuoka-u.ac.jp

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Tumor antigens (TAs) are often used as targets for therapeutic approaches against cancer (1, 2). In this context, TAs can be categorized into three types; (a) TA peptides that are presented by HLA class I molecules on the tumor cell surface and are capable of evoking cellular immunity, especially cytotoxic T lymphocytes (CTLs), by being presented on HLA class II molecules of antigen-presenting cells such as macrophages and dendritic cells; (b) target molecules or ligands recognized by natural killer (NK) cell receptors; and (c) TAs that are directly expressed on the tumor cell surface and are identified by autoantibodies or heteroantibodies, *i.e.*, by humoral immunity. The major antibody-recognized TAs are carcinoembryonic antigen (CEA) for various cancers including gastrointestinal cancer (3), carbohydrate antigen 19-9 (CA 19-9) for digestive cancer (4), carbohydrate antigen 125 (CA 125) for ovarian cancer (5), prostate-specific antigen (PSA) for prostate cancer (6), squamous cell carcinoma antigen (SCC) for uterine cervical cancer (7), 17-1A for colorectal cancer (8), mucin-1 (MUC-1) for breast cancer (9), human epidermal growth factor receptor 2 (HER2) for breast cancer (10), CD20 or CD22 for B-cell non-Hodgkin's lymphoma (11) and CD52 (gp20) for chronic lymphocytic leukemia (12), *etc*.

T cell immunotherapy is based on the assumption that the TA peptides, recognizable by T cell receptors (TCRs), are correctly presented by HLA class I molecules on target tumor cells (2), while NK cell immunotherapy is based on the hypothesis that cell surface TAs for NK receptors are widely expressed in tumor cells (13). However, HLA class I

molecules are frequently down-regulated or lost during the development of malignancies (14, 15), and the ubiquitous expression of NK receptor ligands on tumor cells has not been identified (13). This altered HLA class I expression and the non-ubiquitous distribution of the target molecules for the NK receptor constitute the major tumor escape mechanism facing tumor-specific CTL- and/or NK cell-mediated responses. These facts imply that it is not easy to eliminate the target tumors only by activating tumor-specific CTLs or NK cells in patients with cancer vaccine treatments (16) or by adoptively transferring *ex vivo* activated CTLs or NK cells into patients (2). In contrast, the expression of antibody-recognized TAs such as CEA on the cell surface of target tumor cells is easily confirmed by immunostaining and/or flow cytometry using the corresponding antibodies (17), and current data suggest that specific immunotherapies using antibodies to TAs are promising (2). In this context, re-targeting of CTLs or NK cells with the antigen binding specificity of the anti-TA antibody shows potential for re-targeting the activities of these modified effector cells to TA-expressing tumor cells in an HLA-independent manner (18, 19). This review summarizes four novel strategies for re-targeting CTLs or NK cells with anti-CEA antibody activity.

Effector Cells

Lymphokine-activated effector cells. When peripheral blood mononuclear cells or tumor-infiltrating lymphocytes are cultured *in vitro* with interleukin-2 (IL-2), they become highly cytotoxic to a wide variety of tumor cells, many of which are resistant to freshly isolated NK cells. Such promiscuous killer cells are called lymphokine-activated killer (LAK) cells and consist mainly of NK (NK-LAK) cells and/or T (T-LAK) cells (20). To avoid histoincompatibility problems, the optimal source of LAK cells is the patient's autologous lymphocytes. Initial clinical experiments, in which these LAK cells were reinfused, have shown some good results, especially when IL-2 was given at the same time (21). However, well-controlled trials have produced less encouraging results and this non-specific therapy, involving high doses of IL-2, also has significant toxicity (22). The reason for this may be that neither NK-LAK nor T-LAK cells can efficiently reach their target cells (23), because target molecules for NK receptors are not well expressed in human tumor cells (13) and because T-LAK cells consist of a collection of T cells with different antigen specificities or different TCRs (23).

Genetically-engineered effector cells. Indirect cancer gene therapy involves the insertion of a gene that modifies or stimulates immunocytes to be more effective against tumor cells (17). Recent knowledge that T cell-recognized peptides are presented by HLA molecules and that the induction of immune responses is dependent on co-stimuli has led to the development of more rational strategies (23). Therefore, the genes used for this indirect gene therapy are cytokine genes, TA genes, costimulatory molecule genes, or HLA class I genes, which are often inserted into tumor cells as well as immunocytes (17). However, the tumor accumulation property of CTLs activated by the gene transfer is also unclear, similar to the case of LAK cells (see above).

Anti-CEA Antibodies

CEA. CEA, the most widely-used human TA, is a highly glycosylated, 180 kDa protein. It is overexpressed in about 95% of gastrointestinal and pancreatic cancers, as well as in most small cell and non-small cell lung carcinomas. It is also expressed in breast carcinoma and squamous cell carcinoma of the head and neck (24). Although the sensitivity for detecting early cancer by CEA is low, measurement of the CEA level in blood is considered to be a useful laboratory aid for the diagnosis and management of patients with various cancers, particularly in monitoring the response to cancer therapy, such as surgery, chemotherapy and radiotherapy (2). Immunohistochemical staining of CEA, using surgical or biopsy specimens, is also helpful as a diagnostic aid in patients with various cancers. However, it has been found that CEA is also expressed on mucosal epithelial cells of various normal tissues such as gastrointestinal and respiratory tracts (25, 26). In normal tissues, however, CEA is localized on the luminal surface of the single layer of columnar epithelial cells lining the upper parts of the crypts (27), so that normal CEA is not directly in contact with the blood flow or tissue fluid (28, 29). On the other hand, in tumor tissues that no longer conform to the single-layer organization by invading through the basement membrane in multicellular arrays, CEA is usually localized at all sides of the cell and directly faces blood flow or tissue fluid (28, 29). Hence, tumor CEA can still be a useful target molecule for immunodiagnosis and/or immunotherapy using an anti-CEA antibody (29).

Selection of anti-CEA antibodies. In recent years, instead of polyclonal antisera, monoclonal antibodies (mAbs) against the respective TAs are used as probes in the immunodiagnosis and/or immunotherapy of cancer (30). However, when CEA is used as a target, the presence of the CEA gene family proteins is a major problem, because they give rise to cross-reactivity of mAbs with normal tissues (31). These antigens were recently renamed the CEA-related cellular adhesion molecules (CEACAMs) (32). Among them, CEACAM1, CEACAM6 and CEACAM8 are a great impediment since they are often present in various types of normal tissues (28, 32, 33). It is, thus, indispensable to select anti-CEA mAbs specific for CEA by testing the

cross-reactivities with these CEACAMs, and several novel CEA-specific mAbs have been reported (34, 35).

Antibody engineering. There are three major limitations to antibody therapy (23). First, antibody penetration into large tumor masses is often poor. This might be overcome by smaller molecules that retain specific antigen binding, such as Fab' fragment or genetically-engineered single-chain variable fragmented (scFv) antibodies (36). Second, mouse mAbs are immunogenic in humans and may, therefore, be attacked by the host immune system. Moreover, even genetically-engineered chimeric or humanized antibodies may induce an immune response to their remaining antigenicity. In this context, it is very interesting to note that it has become easy to generate human mAbs to any human TAs using the KM mouse™ and the usual hybridoma technique (37). The KM mouse™ has been produced by the combination of mouse immunoglobulin knockout and human immunoglobulin transgenic technologies (37). Recently, we generated 46 fully human mAbs to CEA using the KM mouse™ (38). Among them, 22 clones reacted with CEA, but not with other CEACAMs. Considering their lack of immunogenicity to humans, these CEA-specific human mAbs may be useful for immunotherapeutic approaches. Finally, antibodies are bound by other cells including any normal cells expressing the target antigen, and non-specifically by cells bearing Fc receptors. Thus, either the chemical modification or genetic engineering of the antibody molecules may be needed to overcome these difficulties.

Strategies for Re-targeting CTLs or NK Cells with Anti-CEA Activity

Bispecific antibody technology. Bispecific antibody technology allows the generation of a single antibody directed against both a TA and a given surface marker on effector cells, such as CD3 on T cells or CD16 on NK cells (39). In theory, these antibodies should help to localize the LAK cells on the tumor *via* their anti-TA activity. In a previous study, we generated a mouse/human-chimeric bispecific antibody, designated CBA-CEACD3, with dual specificities for CEA and CD3 by chemical cross-linking of a chimeric antibody specific for CEA to another chimeric antibody against CD3 (40). Flow cytometric analysis showed that CBA-CEACD3 could bind specifically to cells expressing CEA and to normal human T cells bearing CD3, respectively. Furthermore, a cell to cell adhesion analysis demonstrated that CBA-CEACD3 could bind CEA-producing cells to CD3-expressing cells, suggesting that both arms of CBA-CEACD3 are simultaneously working and can re-target T cells to the tumor. Furthermore, this antibody was shown to effectively mediate CEA-expressing tumor cell killing by freshly isolated T cells. Baba *et al*. also developed a bispecific antibody, OH1, which simultaneously recognizes CD3 on T cells and CEA on tumor cells (41). T-LAK cells from patients pretreated with OH1 showed significantly increased cytotoxicity against CEA-expressing tumor cells compared with non-treated T-LAK cells. Taken together, these bispecific antibodies may serve as a potentially useful immunotherapeutic reagent for human CEA-producing cancers. One problem, however, is that it is difficult to produce and purify these bispecific antibodies in clinically appreciable amounts (42). In this context, it is of interest that several studies have recently reported on genetically-engineered bispecific diabodies (the smallest functional bispecific antibodies) with anti-CEA activity as well as anti-CD3 activity (Figure 1) (43-45). One of these bispecific diabodies inhibited the growth of established human colon carcinoma xenografts in combination with a fusion protein of anti-CEA diabody and B7.1 (45).

Antibody-cytokine fusion protein technology. The fusion of anti-TA mAbs and cytokines is an efficient technique to target cytokines to tumor cells and, hence, focuses the killing activity of LAK cells *via* cytokine receptors to the target cells (46). In a previous study, Xu *et al*. generated an anti-CEA scFv-IL-2 fusion protein for selective tumor targeting of cytokines (47). The variable domains of a high affinity anti-CEA antibody, T84.66, were used to form an scFv joined to the crystallizable fragment, Fc (scFvFc). The fusion protein, designated scFvFc.IL-2, consisted of mouse IL-2 fused to the C-terminal end of the scFvFc. Biodistribution and tumor-targeting studies were carried out in immunocompetent CEA-transgenic mice bearing CEA-positive murine tumors as well as the antigen-negative parental tumor. The scFvFc.IL-2 fusion protein had tumor localization properties similar to those of the intact mAb. The growth of CEA-expressing syngeneic tumor cells in the CEA-transgenic mouse model was inhibited after treatment with scFvFc.IL-2. We have also genetically fused human IL-2 to the F11-39 scFv antibody to CEA (Figure 2) (48). The resulting fusion protein, designated F39scFv/IL-2, effectively targeted IL-2 onto the surface of CEA-expressing tumor cells and consequently introduced a specific cytotoxicity of human NK-LAK cells to the tumor cells. On the other hand, the bacterial superantigen staphylococcal enterotoxin A (SEA) is an extremely potent activator of T lymphocytes when presented on HLA class II molecules. To develop a tumor-specific superantigen for cancer therapy, several recombinant fusion proteins of SEA and an anti-TA scFv antibody have been reported (49, 50). The resulting fusion protein introduced a specific cytotoxicity of T-LAK cells to TA-expressing tumor cells, and consequently suppressed the tumor growth in a SCID mouse xenograft model (50). Anti-CEA scFv might also be applicable for this kind of fusion protein. These studies indicate that anti-CEA antibody-

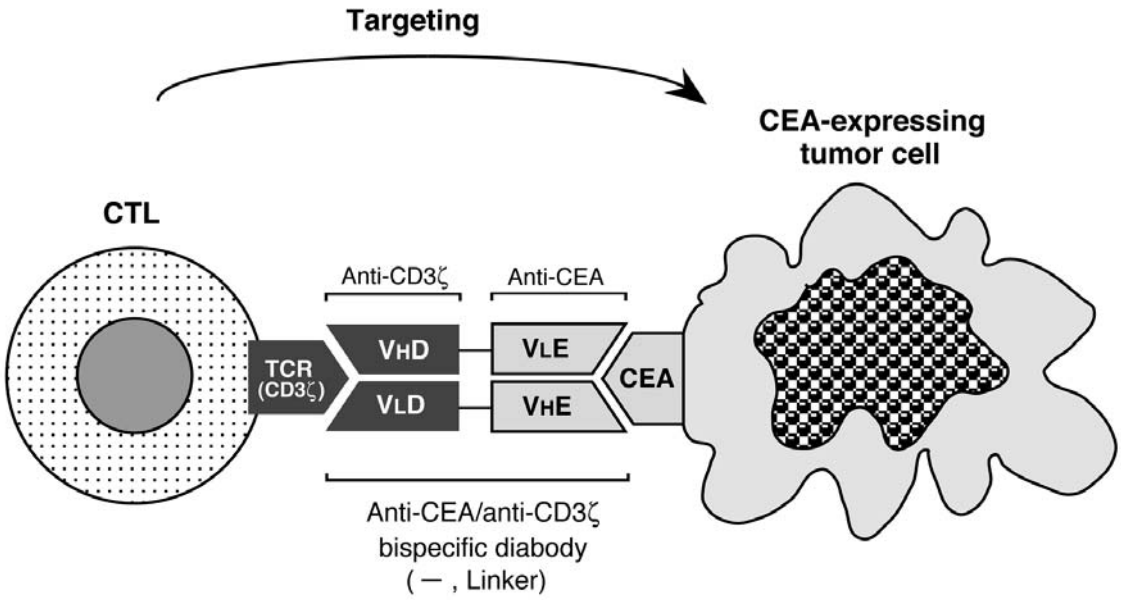

Figure 1. *Anti-CEA and anti-CD3ζ bispecific diabody. Diabody comprises a heavy-chain variable (V_H) domain connected to a light-chain variable domain (V_L) on the same polypeptide chain connected by a peptide linker that is too short to allow pairing between the two domains on the same chain. This forces paring with the complementary domains of another chain and promotes the assembly of a dimeric molecule with two functional antigen-binding sites. To construct bispecific diabody, the V-domains of anti-CEA (V_LE and V_HE) antibody and the V-domains (V_HD and V_LD) of anti-CD3ζ antibody are fused to create the two chains V_HD-V_LE, V_HE-V_LD. Each chain is inactive in binding to antigen, but recreates the functional antigen-binding sites of anti-CEA and anti-CD3ζ antibodies on pairing with the other chain. Diabody is usually produced in bacteria (E. coli) or yeast (Pichia pastoris) in functional form.*

directed cytokine targeting may also offer an effective treatment for CEA-expressing carcinomas.

Chimeric immune receptor technology. The chimeric immune receptor (CIR) technology also has the potential to re-target CTLs or NK cells to tumor cells (51, 52). Recombinant CIRs encompass anti-TA antibodies genetically-fused to the signaling domains of either TCR or Fc receptor (FcR) (53). After transfection, CTLs or NK cells expressing anti-TA scFv/TCR-ζ (CD3ζ) or anti-TA scFv/FcRγ receptors recapitulate the cytopathic effects mediated by TCR or FcR and allow the targeting to tumor cells in an HLA-independent manner (19, 53). Furthermore, given that T cells require both primary and costimulatory signals for optimal activation and that many tumors do not express critical costimulatory ligands, modified CIRs have been engineered to co-deliver CD28 costimulation (54, 55). Thanks to this CIR technology, large numbers of CTLs or NK cells with redirected anti-CEA specificity have thus been generated (18, 52, 54-57). We have recently constructed a CIR gene that encoded the F11-39 scFv antibody to CEA, the human CD8α hinge region, the CD28 transmembrane and cytoplasmic domains, and the human CD3ζ-chain (58) (Figure 3). The resulting CIR gene, F39scFv/CIR-2, was transfected into human T cells. When incubated with CEA-expressing tumor cells, the transfected T cells formed rosette-like aggregates around the tumor cells, thus indicating the cell-specific re-targeting of T cells. Haynes *et al*. have compared the antitumor potency of primary T lymphocytes expressing CEA-reactive CIRs that incorporate either TCR-ζ(CD3ζ) or CD28/CD3ζ signaling (54). Although both receptor-transduced T cell effector populations demonstrated cytolysis of CEA-expressing tumors *in vitro*, T cells expressing the scFv/CD28/CD3ζ chimera had a far greater capacity to control the growth of CEA-expressing xenogeneic or syngeneic colon carcinomas *in vivo*. Overall, this study illustrated the ability of a chimeric scFv receptor capable of harnessing the signaling machinery of both TCR-ζ and CD28 to augment T cell immunity against tumors that have lost expression of both MHC/peptide and costimulatory ligands *in vivo*. Recently, Sheen *et al*. have transduced T lymphocytes isolated preoperatively with recombinant retroviruses encoding a CIR consisting of an anti-CEA scFv and CD3ζ (59). T cells expressing the CIR were specifically activated by coculture with a disaggregated CEA-positive tumor, whereas control non-CEA-targeted T-cell populations failed to be activated. These studies emphasize the potential of this CIR strategy as a new therapy for CEA-expressing primary tumors and their metastases.

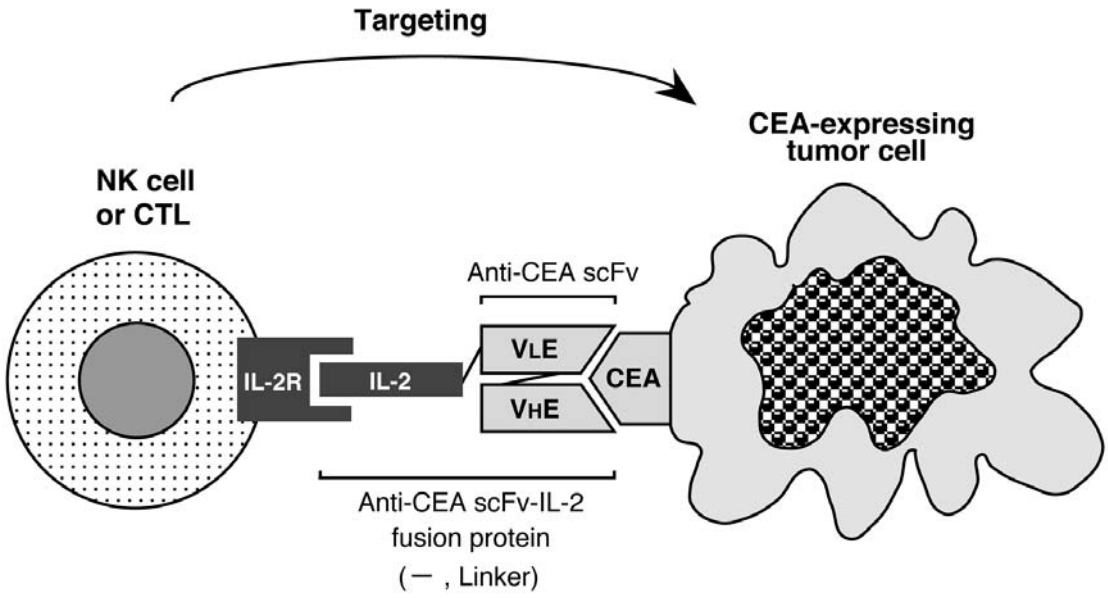

Figure 2. *Anti-CEA scFv-IL-2 fusion protein. Antibody-cytokine fusion protein is also produced in bacteria or yeast in functional form. IL-2R, IL-2 receptor. For V_LE and V_HE, see Figure 1 legend.*

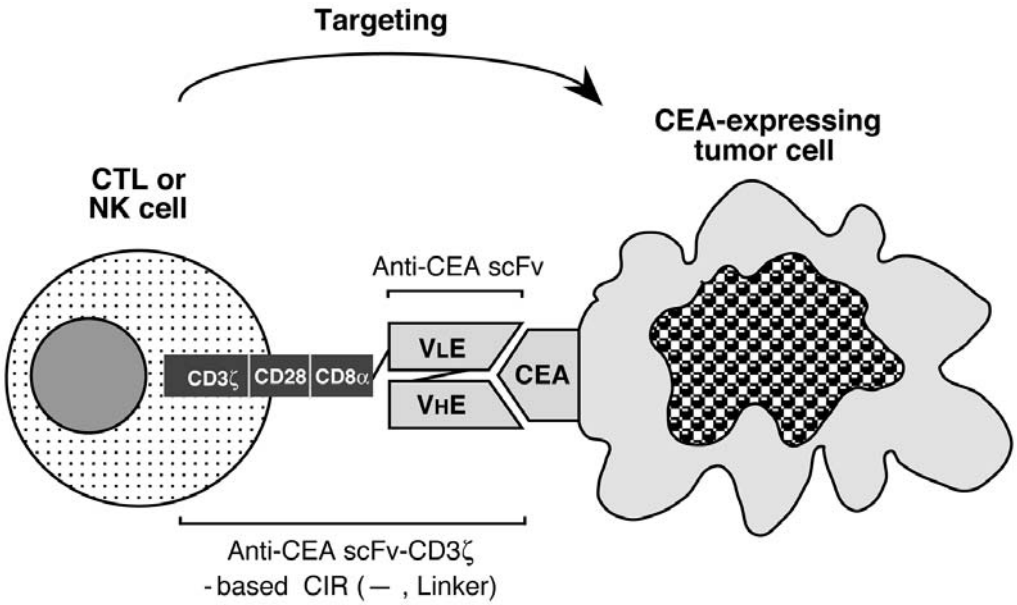

Figure 3. *Anti-CEA scFv-CD3ζ-based CIR. In this construct, the human CD8α hinge region gene, the CD28 transmembrane and cytoplasmic domain genes are inserted between anti-CEA scFv and CD3ζ genes. The final gene construct is usually transfected into human T or NK cells with viral vectors and expressed on the cell surface of these cells. For V_LE and V_HE, see Figure 1 legend.*

Antibody-HLA/peptide complex technology. Recently, several anti-TA antibody-HLA-restricted antigen peptide complexes have been constructed for antibody-guided re-targeting of relevant T-LAK cells towards tumors (60-62). First, Robert *et al*. have successfully targeted flu-sensitized T-LAK cells to tumor cells by pulsing with anti-TA Fab'-HLA/flu peptide complexes (60, 61). Single Fab' fragments from an anti-CEA mAb were coupled to either tetrameric or monomeric HLA-A2 complexes containing the immuno-dominant influenza-matrix peptide 58-66. When targeted to

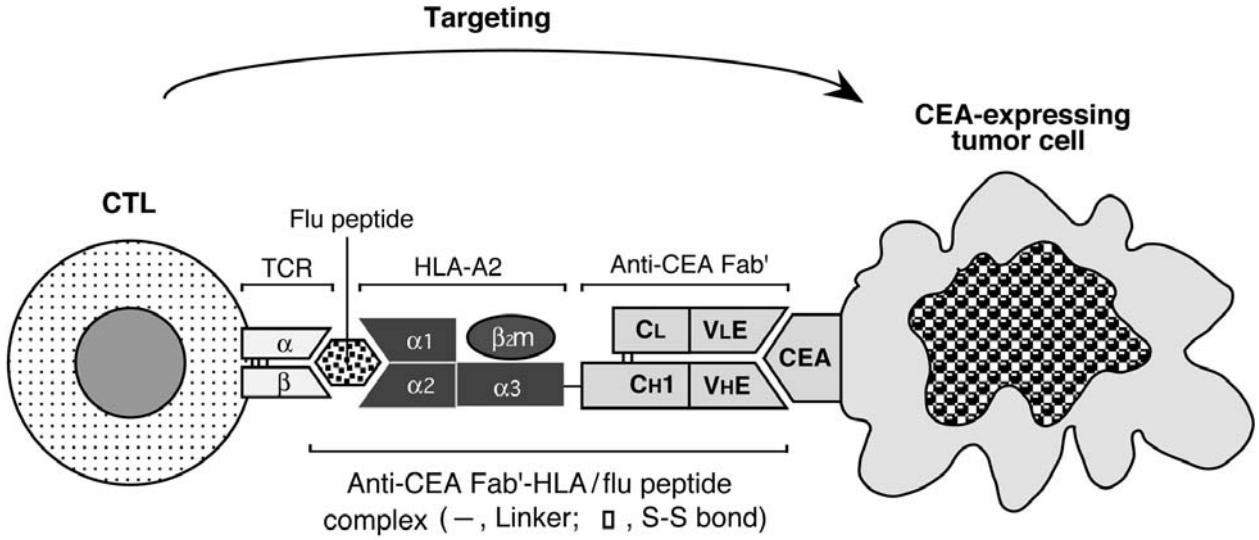

Figure 4. *Anti-CEA Fab'-HLA-A2/flu peptide complex. The free complexes should not bind to TCR due to the low affinity of the monomeric HLA/peptide complex-TCR interaction. The advantage of the complexes is that their antibody site can associate with highly expressed, non-HLA restricted CEA, while their HLA/peptide site, when coated at high density on the target cell surface, can induce the binding and effector function of specific CTLs. The antibody-HLA conjugates were originally generated by chemical linkage, but have also been produced by genetic engineering. β_2m, β_2-microglobulin; C_L, light-chain constant domain; and C_H1, heavy-chain constant domain-1. For V_LE and V_HE, see Figure 1 legend.*

CEA-expressing tumor cells, the conjugates induced CTL activation and efficient tumor cell lysis *in vitro*, as a result of HLA/flu peptide surface oligomerization, independent of the HLA class I molecule expression on target tumor cells (Figure 4). Furthermore, *in vivo* targeting of the anti-CEA Fab'-HLA/flu peptide complexes induced specific growth inhibition and regression of established syngeneic tumor grafts (63). More recently, Lev *et al*. have demonstrated that targeting anti-TA scFv-HLA/peptide complexes to tumor cells can function *in vitro* and, most significantly, *in vivo* in a human solid xenograft tumor model (64). Anti-CEA scFv might also be applicable for this kind of antibody-HLA/peptide complex. These results suggest that injection of Fab'-HLA/flu peptide conjugates could represent a new form of immunotherapy, bridging antibody and flu-sensitized CTL attack on cancer cells.

Conclusion

The development of T or NK cell populations with anti-TA antibody activity may be important for the success of cancer immunotherapy because the down-regulated HLA class I molecules and the non-ubiquitous expression of NK receptor ligands in tumor tissues constitute the major tumor escape mechanism facing tumor-specific CTL- and/or NK cell-mediated responses. Here, four novel strategies to endow T or NK cells with anti-CEA antibody activity were briefly reviewed. However, a common problem for these strategies is the existence of soluble, circulating CEA in patients with CEA-expressing tumors. In this context, the polyvalent reaction of cellular CEA with anti-CEA designer CTLs would be resistant to competition by the necessarily monovalent reaction with soluble CEA, in accordance with the well-known affinity enhancement available to multivalent cell-cell interactions (65). Under the multivalent binding interactions between modified T cells and targets, the inhibitory capabilities of soluble CEA are dulled since the presence of even 10,000 ng/ml of soluble CEA does not block designer T cells for purposes of binding, activation of IL-2 secretion or cytotoxicity against tumor targets (57). Soluble CEA in patient sera only infrequently exceeds 1,000 ng/ml and, thus, would be unlikely to be a factor in inhibiting anti-CEA designer T cells in *in vivo* therapies. Therefore, endowing CTLs or NK cells with the antigen-binding specificity of anti-CEA antibody is promising for re-targeting the activities of these effector cells to CEA-expressing tumor cells in an HLA-independent manner.

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