

CAR Mechanics: Driving T Cells into the MUC of Cancer

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Abstract

Several monoclonal antibodies bind in a highly selective manner to tumor-associated glycoforms of MUC1. We set out to exploit this by developing a MUC1-specific chimeric antigen receptor. Difficulties were encountered in this endeavor, owing to MUC1-imposed steric hindrance and antigenic heterogeneity. These issues were addressed by the iterative engineering of all components of the fusion receptor. Our experience underlines the need for careful individual optimization of immunotherapeutic reagents as dictated by the molecular vagaries of the target under study. [Cancer Res 2009;69(11):4559–62]

Introduction: Cancer, as Common as MUC

The MUC1 glycoprotein has attracted considerable interest as a target for tumor immunotherapy. Aberrant expression of MUC1 has been reported in most solid tumors and many hematologic malignancies. However, little about the structure, function, or immunology of this molecule has proven to be straightforward. To reflect this, the authors have identified 18 synonyms for this prototypic mucin!

Several MUC1 variants have been described in cancer. The best studied is a heterodimer in which a large extracellular mucin (MUC1-N) is noncovalently linked to a membrane-spanning signaling polypeptide (MUC1-C) (ref. 1). MUC1-N protrudes well beyond the glycocalyx of the cell, owing to a variable number tandem repeat (VNTR) domain that is copied up to 125 times. Each VNTR can carry five *O*-linked glycans, causing MUC1-N to assume a filamentous tertiary structure, which extends up to 0.5 μ m and is heavily coated in sugar. Importantly, in healthy cells, these glycans are large, branched structures that effectively shield the underlying peptide backbone.

In cancer, three key changes occur to MUC1 that together render it a potential "holy grail" among immunotherapeutic targets. First, *MUC1* undergoes transcriptional upregulation (2). Second, polarity of expression is lost, making native MUC1 more accessible to the immune system (3). However, the most critical change results from dysregulated *O*-linked glycosylation. Owing to altered glycosyltransferase expression, tumor-associated MUC1 carries a preponderance of shorter glycans, including the classical tumor antigens T (Thomsen-Friedenreich), Tn, sialyl-T (ST), and sialyl-Tn (4). Because these truncated glycans expose and alter the conformation of the VNTR, several monoclonal antibodies bind

preferentially to tumor-associated MUC1 (5–7). Foremost among these is SM3, which reacts with over 90% of breast and ovarian carcinomas, but does not bind appreciably to healthy breast tissue (5).

Immunotherapeutic Targeting: Where There's MUC, There's Brass?

Monoclonal antibody therapy would appear to be the most logical strategy by which to target aberrantly glycosylated MUC1. However, therapeutic trials have not yet achieved meaningful results for patients. In part, this may reflect the difficulty in harnessing antibody effector function against this unwieldy and cumbersome target (8). An alternative way forward involves the convergent application of antibody and cellular immunotherapy, by using chimeric antigen receptor (CAR) or "T-body" technology. In this approach, T cells are engineered to express modular fusion receptors that comprise a targeting moiety (commonly an antibody-derived single-chain fragment [scFv]), hinge, spacer, membrane-spanning element, and signaling endodomain. With efficient gene transfer and optimized expansion protocols, large numbers of CAR-grafted T cells can be rapidly generated. A key advantage is that CARs are targeted against native rather than processed antigen; consequently, function is not hampered by the frequent occurrence of HLA downregulation in human cancer.

Steric Hindrance: Struggling to Become Stuck in the MUC

Against this background, we set out to engineer a CAR with specificity for tumor-associated MUC1. An SM3 scFv was substituted for that within the highly active P28z CAR, directed against prostate-specific membrane antigen (9). However, the resultant S28z proved extremely poor in retargeting human T cells against a variety of MUC1-expressing tumor cells (7). Two types of steric inhibition were implicated in these disappointing results, imposed by the large size of immobilized MUC1 and its heterogeneous glycosylation. The precise binding properties of SM3 for tumor-associated MUC1 were next analyzed and found to be suboptimal. In particular, SM3 did not bind to MUC1-ST, which is widely expressed by many tumor cells (10). Owing to this combination of factors, we reasoned that S28z struggled to bind bivalently, a prerequisite to achieve CAR cross-linking and T-cell activation. To rectify this, two independent approaches were pursued.

First, we reasoned that greater flexibility and reach were required within S28z, to provide improved access to epitope and thereby overcome steric inhibition. Nature provides an attractive solution to this problem in the form of the IgD hinge. Immunologists have long wondered why naïve B cells coexpress IgD and IgM antibody of identical specificities on the cell surface. Løset and colleagues provided evidence that the elongated and

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monomeric hinge that is unique to IgD favors bivalent binding when epitope is present at low density (11). Insertion of the IgD hinge into S28z enabled T cells to proliferate and produce interferon- γ upon engagement with MUC1-expressing tumor cells (7).

Antigenic Heterogeneity: Not All MUC Is the Same

In light of the inability of SM3 (and S28z) to bind MUC1-ST, we next reevaluated our choice of targeting moiety. Binding of SM3 and other MUC1 antibodies was compared by using a panel of recombinant MUC1-IgG fusion proteins that carry individual tumor-associated glycans (ref. 7 and data not shown). This analysis revealed that HMFG2 was clearly superior in achieving the strongest and broadest binding capacity to diverse MUC1 glycoforms, including the challenging MUC1-ST. We cloned the HMFG2 scFv and observed that it differs in only 5 amino acids from SM3. In keeping with this striking similarity, both antibodies achieve a high degree of tumor selectivity and bind to overlapping epitopes within the MUC1 VNTR. Importantly, however, we observed that HMFG2-based CARs were less susceptible to steric hindrance and consequently exhibited improved antitumor function.

An Optimized CAR: The Model T Body

Next, additional CARs were engineered, which demonstrated that incorporation of the IgD hinge and HMFG2 scFv both contribute independently to improved CAR-based targeting of MUC1. Clinical immunologists have long been familiar with the poor stability of IgD paraproteins in patients with myeloma. In keeping with this, we found that IgD hinge-containing CAR expressed poorly at the cell surface, a deficiency that was corrected by using IgG1 Fc as a stabilizing factor.

Finally, the endodomain was modified by insertion of OX40 sequences between CD28 and CD3 ζ . In agreement with others (12), this led to enhanced activation of CAR⁺ T cells upon engagement by tumor cells that express intermediate levels of MUC1. The resultant CAR (named HOX; Fig. 1) enabled human T cells to achieve delayed tumor growth in mice bearing an established MUC1⁺ xenograft (7). Importantly, HOX⁺ T cells spared an immortalized (nontransformed) human mammary epithelial cell line, which expresses low levels of HMFG2 immunoreactivity. We also found that soluble tumor-derived MUC1 could engage with HOX-expressing T cells, to stimulate their proliferation *in vitro*. In principle, engagement of a soluble target could reduce the efficacy of CAR-based immunotherapy, as a result of impaired binding of

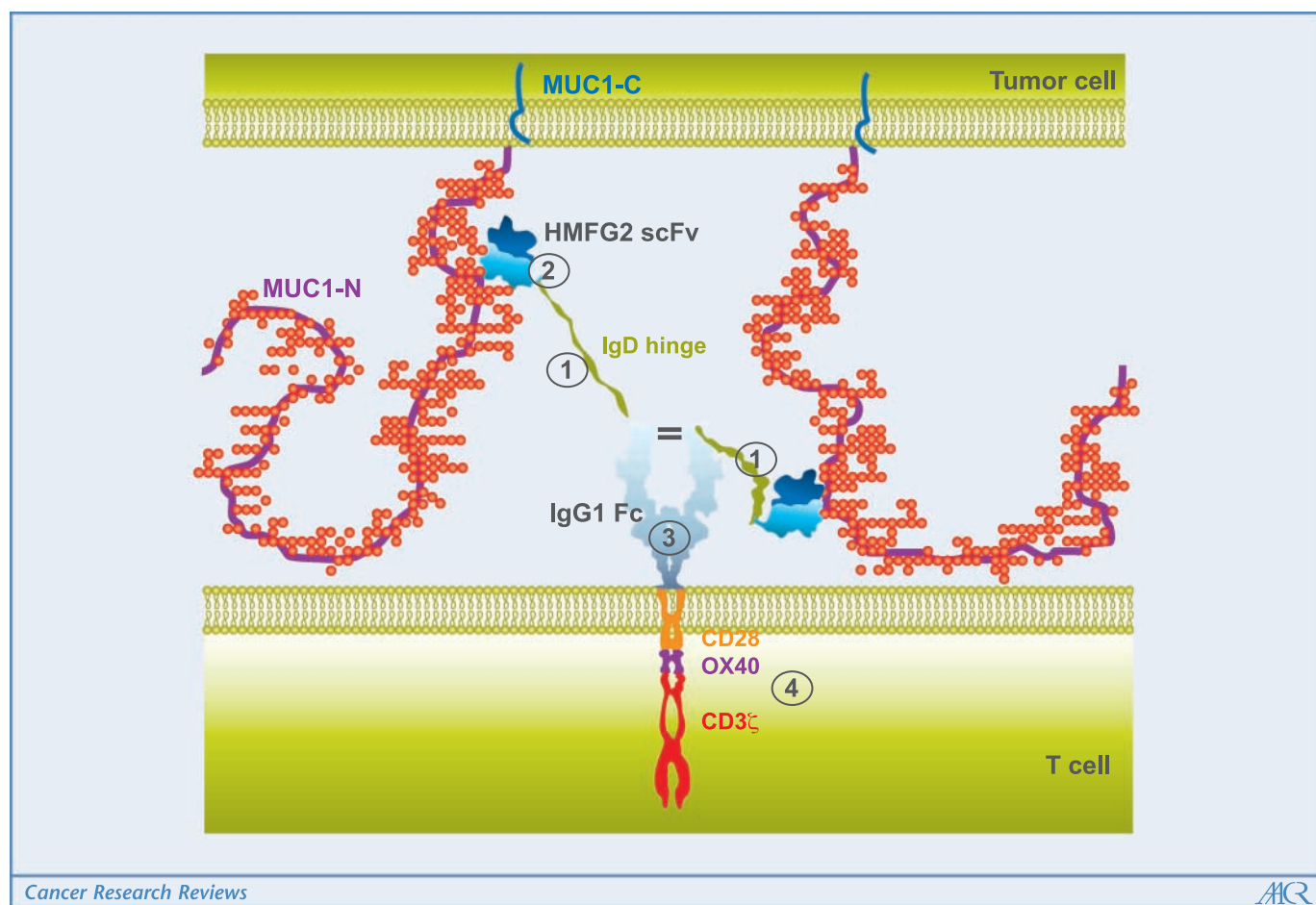


Figure 1. HOX: a chimeric antigen receptor specific for tumor-associated MUC1. The four key attributes of the HOX fusion receptor are (1) an IgD hinge, conferring flexibility and reach; (2) an HMFG2 scFv, which binds strongly to all tumor-associated glycoforms of MUC1 tested; (3) IgG1 Fc, providing stability; and (4) a tripartite endodomain, to optimize signaling upon MUC1 engagement. Glycans are depicted schematically as red circles and account for up to 80% of MUC1 mass.

tumor-associated antigen. However, a recent study provides evidence that this does not impede immunotherapy with CD28-containing fusions, owing to the sustained survival of CAR⁺ T cells (13).

Implications: As Clear As MUC?

The MUC1 mucin forms an antiadhesive shield around the tumor cell membrane that protects against specific and nonspecific T-cell attack (14). Nonetheless, we have found that T cells can be effectively targeted against MUC1 by using an iteratively optimized CAR. The implications of this work for rational CAR design are necessarily speculative, in particular owing to the heterogeneous nature of MUC1 as a target. Nonetheless, some inferences may be drawn from precedents established by using other CARs. When CARs bind antigen at a membrane-proximal location, potent T-cell activation is generally the result. By contrast, weak activation is commonly observed when membrane-distal epitopes are targeted (15–17), although function may be enhanced by removal of the CAR spacer domain (15). Intriguingly, however, if epitope is coexpressed at membrane-distal and membrane-proximal locations in separate molecules, potent T-cell activation ensues (17). The importance of epitope location in predicting CAR function is consistent with a kinetic segregation model of how the T-cell receptor/CD3 complex mediates physiological T-cell activation. This model proposes the formation of subregions of very close contact between T lymphocytes and activating partner cells, leading to exclusion of larger inhibitory phosphatases, such as CD45 (18). Taken together, this strongly suggests that HOX must engage with membrane-proximal VNTR epitopes in order to mediate activation of retargeted T cells.

At first sight, this may seem implausible because HOX would be expected to preferentially engage with the outermost, more accessible VNTR within MUC1. To reconcile this, we propose that a small number of HOX molecules initially access membrane-proximal epitopes within MUC1, whereas the majority dock on membrane-distal VNTRs. Rapidly thereafter, the binding interaction between HOX and MUC1 may evolve to preferentially involve more proximally located epitopes. Such a progression would be favored by the following factors. First, the intermediate

affinity of each HMFG2 scFv for MUC1 would be expected to promote frequent disassociation of individual binding arms, particularly from the least favored and abundant MUC1-ST. In agreement with this, biophysical studies with other scFvs targeted to the MUC1 VNTR have estimated an average single-bond lifetime of less than 5 min (19). Second, the flexibility and reach of the IgD hinge would increase the number of epitopes available for reengagement. Progressive selection of membrane-proximal epitopes would be thermodynamically favored because the resultant closer approximation of T-cell and tumor cell membranes would allow for greater interactions between other adhesion molecule pairs. Furthermore, the ability of HOX to achieve local (antibody-like) capping of MUC1 (20) may reduce the steric inhibition of other adhesive interactions at the margins of the contact zone between the T cell and tumor. Finally, flexibility built into the CAR (in the hinge region) would also help to favor a close adhesive interaction between the T cell and the target.

Our study may also have implications for antibody targeting of MUC1. Owing to increased epitope availability, the tumor selectivity of many MUC1 antibodies may conceivably reflect a shift to increased bivalent binding, with its resultant avidity effect. Previously, the HMFG2 antibody has proven difficult to produce in bulk owing to instability of the hybridoma. However, this antibody may warrant further study now that its sequence has been cloned. Careful hinge engineering might be expected to enhance bivalent epitope engagement even further, although such efforts would need to carefully preserve antibody effector activities, such as Fc receptor engagement.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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