Antibody Targeting of Cell-Bound MUC1 SEA Domain Kills Tumor Cells

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Abstract
The cell-surface glycoprotein MUC1 is a particularly appealing target for antibody targeting, being selectively overexpressed in many types of cancers and a high proportion of cancer stem–like cells. However, the occurrence of MUC1 cleavage, which leads to the release of the extracellular α subunit into the circulation where it can sequester many anti-MUC1 antibodies, renders the target problematic to some degree. To address this issue, we generated a set of unique MUC1 monoclonal antibodies that target a region termed the SEA domain that remains tethered to the cell surface after MUC1 cleavage. In breast cancer cell populations, these antibodies bound the cancer cells with high picomolar affinity. Starting with a partially humanized antibody, DMB5F3, we created a recombinant chimeric antibody that bound a panel of MUC1\(^+\) cancer cells with higher affinities relative to cetuximab (anti-EGFR\(^1\)) or trastuzumab (anti-erbB2) control antibodies. DMB5F3 internalization from the cell surface occurred in an efficient temperature-dependent manner. Linkage to toxin rendered these DMB5F3 antibodies to be cytotoxic against MUC1\(^+\) cancer cells at low picomolar concentrations. Our findings show that high-affinity antibodies to cell-bound MUC1 SEA domain exert specific cytotoxicity against cancer cells, and they point to the SEA domain as a potential immunogen to generate MUC1 vaccines.

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Introduction
MUC1 is a mucin-like glycoprotein that can generate a variety of differing isoforms (1). Of these, the most intensely studied has been a polymorphic type I high molecular weight transmembrane protein (MUC-TM) consisting of an extracellular domain containing 20 to 125 tandem repeats of 20 amino acids, followed by a transmembrane domain and a short cytoplasmic tail (2–4). MUC1 is a heterodimer that is cleaved soon after synthesis within the SEA module, a highly conserved domain of 120 amino acids (4–6). Cleavage of MUC1 yields 2 unequal chains: a large extracellular α subunit containing the tandem repeat array specifically bound in a strong noncovalent interaction to a smaller β subunit containing the transmembrane and cytoplasmic domains of the molecule (4, 7).

MUC1 is highly expressed on a range of malignancies, including breast, pancreas, ovarian, prostate, and colon carcinomas, as well as on the malignant plasma cell of myeloma (8–13). Because of this overexpression, MUC1 has been the subject of a great amount of attention, primarily for its potential as a target for tumor-specific therapies. In fact, based on a number of ranked criteria required of an optimal cancer vaccine candidate, the MUC1 protein was listed by the National Cancer Institute Pilot Project the second best target from a list of 75 potential tumor-associated antigens (14). Moreover, within the categories of expression level, stem cell expression and number of patients with antigen-positive cancers, the MUC1 protein received perfect scores (14).

Most anti-MUC1 antibodies reported to date target the highly immunogenic tandem repeat array of the MUC1 α chain (for e.g., refs. 15–17). Because the MUC1 α chain is not directly tethered to the cell surface, it is also found in the peripheral circulation. There it sequesters circulating anti-tandem repeat antibodies, limiting their ability to reach the MUC1\(^+\) tumor cells (15, 16). In addition, deposition of immune complexes of antitandem α chain antibodies and circulating α chain potentially may result in end-organ damage (16).

Targeting MUC1 epitopes bound to the cell surface would avoid antibody sequestration by circulating α chain. The MUC1 SEA domain formed by the interaction of the α subunit with the extracellular portion of β subunit is an intricate structure, which remains fixed to the cell surface (4, 6) and significantly, results in a stable target structure. In previous initial studies, we showed the proof-of-principle of generating antibodies that specifically recognize the MUC1 SEA domain (18). The prototype DMC209 mAb described (18) has 2 disadvantages that compromise its potential clinical use: It is an IgM and it has relatively low affinity for its target.
As a result of these considerations, we sought highly specific, anti-MUC1 antibodies directed against the α/β junction that were also affinity matured and not of the IgM subclass. We report here the generation of 7 novel monoclonal antibodies (mAbs), 5 IgG1-γ1, and 2 IgA that bind the MUC1 protein within its SEA domain with picomolar affinity. Our findings suggest that the IgG1 and IgA mAbs targeting the MUC1 SEA domain may prove beneficial in the therapy of MUC1+ malignancies when either administered alone or in conjunction with other antitumor antibodies and chemocytotoxic agents.

Materials and Methods

Materials and antibodies

Reagents and chemicals were obtained from Sigma, unless otherwise specified. Anti-MUC1 SEA-module mAbs were generated as described below. The anti-MUC1 tandem repeat antibodies used here were as previously described (19, 20).

Cell lines

Human breast carcinoma cell lines T47D, MCF7, and ZR75, human epidermoid carcinoma A431 and KB cell lines, human gastric carcinoma N87 cell line, and human pancreatic carcinoma Colo357 cell line were maintained as previously described (18).

Cell culture

Cells were grown at 37°C and 5% CO2 in culture media supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L l-glutamine, 100 IU/mL penicillin, and 25 mg/mL streptomycin. DA3 (mouse mammary tumor cells) and CHO-K1 (Chinese Hamster Ovary cells) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) or DMEM: F12 nutrient mixture (1:1), respectively.

Generation of stable DA3 mouse mammary tumor cell transfectants expressing MUC1-TM

DA3 cells were cotransfected with the eukaryotic expression plasmids pCL-MUC1-TM and with pSV2neo (which codes for neomycin resistance). Expression constructs were transfected into cells using the calcium phosphate procedure. Stable transfectants were selected with neomycin.

Generation of bacterial recombinant MUC1-X, SEA, SEA-4G proteins

Bacterial MUC1-X, SEA, SEA-4G proteins were prepared as the (His)6-MUC1-Xex, (His)6-SEA, and (His)6-SEA-4G fusion proteins as previously described (21) and comprise 6 histidine residues at their N-terminus, followed by the extracellular domain, dark blue and cytoplasmic domain, pink. The SEA domain repeat array, light blue; N-terminal part of SEA domain, red; and the cleaved MUC1-X protein. The domains are color schemed as follows: signal peptide, purple; N-terminal 30 amino acids (N30), yellow; sequences flanking the tandem repeat array, green; central tandem repeat repeat array, light blue; N-terminal part of SEA domain, red; C-terminal part of SEA domain, hatched red and white; transmembrane domain, dark blue and cytoplasmic domain, pink. The SEA domain comprising the interacting α and β subunits was indicated by the dashed black circles. Screening of hybridomas was carried out with horseradish peroxidase (HRP)-conjugated anti-mouse antibody.

Immunization of mice and generation of hybridomas

Mice were initially immunized with 5 consecutive intradermal DNA immunizations spaced at 21-day intervals. The immunizing DNA consisted of the pCL-MUC1-TM expression vector plasmid that codes for the MUC1-TM protein (21). The extracellular domain of MUC1-X protein (recombinant bacterial MUC1-Xex, see below) together with incomplete Freund’s adjuvant was then used to boost the mice. Bacterially synthesized recombinant MUC1-Xex protein used for these immunizations spontaneously self-cleaves as previously described (4), generating the MUC1-X C- and β subunits that strongly, yet noncovalently, interact with each other forming the very stable heterodimeric cleaved MUC1-Xex protein (depicted in Figs. 1 and 4). Hybridomas were prepared by fusion of nonscreting myeloma cells with immune splenocytes and screened by ELISA assay (see below).

ELISA for determining binding of anti-MUC1 polyclonal and monoclonal antibodies to the extracellular domain of the MUC1-X protein

Elisa Immunoassay plates (CoStar) were coated with recombinant MUC1 proteins followed by blocking. Spent culture media from the initial hybridomas was then applied to the wells. Following incubation, samples were removed and the wells were washed with PBS/Tween. Detection of bound antibodies was done with horseradish peroxidase (HRP)-conjugated anti-mouse antibody.

Two-tiered screening for selection of anti-MUC1 monoclonal antibodies

The primary screen of the hybridomas was carried out by assessing antibody binding to the extracellular domain of
MUC1-X (MUC1-Xex) as described in the ELISA assay (above). To select hybridomas secreting antibody that recognize not only MUC1-Xex but also the complete cell surface MUC1-TM protein, those hybridomas presenting a positive signal in the first screen were subjected to a second-tier screen. This consisted of flow cytometric analysis using mouse cell transfectants (DA3-TM) expressing human MUC1-TM and, in parallel, to the same parental cells (DA3-PAR) that do not express human MUC1. This procedure ensured selection of antibody that not only bound MUC1 moieties common to both the MUC1-X and MUC1-TM but also recognized cell surface human MUC1-TM as expressed by mammalian cells.

Isolation of MCF7 breast cancer side population of cancer cells

An MCF7 breast cancer cell subpopulation that has been reported to show some characteristics of cancer stem cells (22) were isolated as described by Finn (22). Briefly, MCF7 cells were stained with the fluorescent dye Hoechst 33342. Due to their increased efflux of the dye a side population of MCF7 cells was isolated by flow cytometry. The resultant cells were then confirmed as showing some characteristics of stem cells using a battery of stem cell markers.

Preparation of ZZ-Pseudomonas exotoxin and chDMB5F3/ZZ-PE38 immunotoxin

The ZZ-PE38 fusion protein comprises the Pseudomonas exotoxin PE38 and the ZZ domain derived from protein A. The ZZ portion binds to the Fc region of IgG to form immunotoxin conjugates. Plasmid pET22-NN-ZZ-PE38 was used for expression of soluble ZZ-Pseudomonas exotoxin A (PE38) fusion protein secreted to the periplasm of BL-21 (DE3) Escherichia coli cells, which was then purified as previously described (23).

SDS-PAGE/Western blot

Proteins separated on SDS-PAGE were electrotransferred for 2 hours at 0.5 Amp on to nitrocellulose filters in transfer buffer. Blots were blocked with 5% skimmed milk followed by incubation with the primary antibody (anti-MUC1 antibodies, see Results). Bound primary antibody was detected with secondary anti-mouse antibody conjugated to HRP (Chemicon International) followed by enhanced chemiluminescence.

Cell killing assay

Mouse mammary tumor cells transfected and stably expressing human MUC1-TM (DA3-TM), the parental cells that do not express human MUC1 (DA3-PAR wild type), and MUC1+ human cell lines (T47D and ZR75 breast cancer cells, Colo357 pancreatic cancer cells, as well as additional cancer cells as detailed in Results), were grown in DMEM supplemented with 10% FCS. These cells (10,000 cells per well in 100 microliters of medium) were seeded in 96-well cell culture plates (Corning) and grown at 37°C in 5% CO2 in culture media. Five hours after seeding, 50 microliters of the chDMB5F3 was diluted by 1:600 pm and mixed with 50 microliters ZZ-PE38 toxin. The immunotoxin mixture was directly applied to the cells (final ZZ-PE38 toxin concentration was 250 ng/mL). Negative controls included adding to the target cells the following (instead of the chDMB5F3/ZZ-PE38 toxin immunotoxjugates)—(i) concentrated medium from parental CHO-K1 cells that is devoid of the anti-MUC1 chDMB5F3 mAb, (ii) ZZ-PE38 alone, (iii) chDMB5F3 mAb alone, devoid of the ZZ-PE38 toxin. Cell viability was assessed by measuring alkaline phosphatase activity per well.

Construction of vectors for mammalian expression in Chinese hamster ovary cells of recombinant DMB-5F3–constant region of human IgG1 [C-hlgG1]

Methods used to generate these vectors essentially followed those previously reported (23). The mammalian vectors pMAZ-IgH and pMAZ-IgL (19) were used as backbones for the expression of the VH and VL regions of DMB5F3 fused to human γ1 heavy and human κ light chains, respectively.

Surface plasmon resonance binding assay

Recombinant MUC1 protein was immobilized on a CMS chip. As control, anti-CD20 IgG (Rituxan) was treated in a like manner. Remaining active groups were saturated with 1 mmol/L ethanolamine. Surface plasmon resonance (SPR) was done using Biacore3000 according to manufacturer’s specifications (GE Healthcare). Serial dilutions of antibody DMB5F3 (0.4–7 nmol/L) were measured for 4 minutes association and 30 minutes dissociation. The chip was regenerated with 5 mmol/L NaOH. Data fitting was carried out using algorithm of the BIAevaluation software.

Preparation of DMB5F3 Fab

DMB5F3 IgG1 was reacted with papain to give a papain-to-antibody ratio of 1:20 (ww) followed by incubation at 37°C for 5 hours. The reaction was stopped with crystalline iodoacetamide at a final concentration of 0.03 mol/L. The resultant mixture was dialyzed against PBS pH 8.5, passed on a protein G column, and Fab fractions collected.

Analysis of binding to MUC1/Her2/EGFR1-expressing tumor cells by flow cytometry

Evaluation of binding by flow cytometry was carried out as follows: 5 × 105 cells were used in each experiment. After trypsinization, cells were washed and mAbs at varying dilutions were added to the cell tubes for 1 hour at 4°C. After washing with fluorescence-activated cell sorting (FACS) buffer, fluorescein isothiocyanate-labeled goat anti-human Fc antibody was added to the tubes for 45 minutes at 4°C. Detection of bound IgG was done by means of flow cytometry on a FACS-Calibur (Becton Dickinson), and results analyzed with the CELLQuest program (Becton Dickinson).

Results

Generation of monoclonal antibodies to the cell-bound MUC1 α/β junction

To avoid generating antibodies to the circulating α chain, the MUC1 immunogen should ideally be cell bound at all times. To comply with these criteria, we used the extracellular domain of the naturally occurring MUC1-X isoform that incorporates elements derived from both the extracellular α
chain and the cell bound β chain. This provided the advantage of incorporating the cell-bound region consisting of the α/β junction present in the full naturally occurring MUC1 protein (Fig. 1), yet at the same time is devoid of the highly immunogenic tandem repeat array (Fig. 1, MUC1-X). The MUC1 protein used for these immunizations is cleaved MUC1-Xex protein synthesized in bacteria. Within the bacteria, MUC1-Xex undergoes spontaneous self-cleavage and comprises the noncovalently interacting MUC1 α and β subunits.

Using our immunization protocol (Materials and Methods), high titer anti-MUC1-X polyclonal antibody sera up to 1:100,000 dilutions were obtained. Spleens of such mice were used for hybridoma formation and hybridoma supernatants subjected to a 2-tiered screen consisting of (i) binding to MUC1-X protein, and (ii) a more stringent second tier assessing binding to MUC1-expressing cancer cells as assayed by flow cytometry. Hybridoma formation resulted in 7 mAbs, 5 of the Ig-γ1 isotype, designated DMB4B4, DMB4F4, DMB5F3, DMB7F3, and DMB10F10. Surprisingly 2 monoclonals of the IgA subclass were also isolated, DMB10B7 and DMB13D11.

**Cytometric analyses of monoclonal anti-junctional antibodies**

Flow cytometric analyses showed that all 7 mAbs bound strongly to DA3-TM cells that express the full-length MUC1. In contrast, untransfected DA3-PAR cells that do not express MUC1 were consistently negative with all antibodies (Fig. 2). Similarly, Colo357, a MUC1+ pancreatic cancer cell line showed unequivocal reactivity with all anti-MUC1 monoclonals (Fig. 2) as did several breast cancer cell lines, such as T47D.

![Figure 2](image_url)

**Figure 2.** Flow cytometric analyses of DMB anti-MUC1 SEA module α/β junction mAbs with MUC1-TM expressing cells. Parental nontransfected mouse mammary tumor DA3 cells and the same cells transfected with cDNA coding for and expressing full-length MUC1-TM. Columns designated DA3-PAR and DA3-MUC1 were reacted with the DMB series of anti-MUC1 SEA module α/β junction mAbs. Flow cytometry with the fluorescently labeled secondary antibody alone (red tracing) and with anti-MUC1 antibody followed by secondary antibody (green tracing) are as shown. Colo357 cells were similarly reacted with the 7 mAbs, either in the absence or presence of competing soluble extracellular domain of the MUC1-Xex protein (column Colo357 and column Colo357 + MUC1-Xex).
MCF7, and ZR75 (data not shown). Further confirmation of binding specificity was provided by addition of soluble exogenous MUC1-Xex protein, the extracellular domain of the MUC1-X protein, which abolished all reactivity (Fig. 2). These studies underscore the fact that the DMB antibodies bind a cell-bound domain noncompetitive with moieties present in the shed α chain of the MUC1-TM protein. In addition to the flow cytometric studies, initial immunohistochemical analyses were carried out with one of these mAbs (DMB5F3) using sections of breast cancer tissue. These stainings showed significant DMB5F3 immunoreactivity with the breast cancer cells (data not shown) results clearly, in line with the well-known overexpression of MUC1 by breast (and other) adenocarcinoma cells comprehensively documented in numerous studies.

**Binding of antijunctional antibodies to MCF7 side population cells that show some characteristics of breast cancer stem cells**

To determine whether the DMB series of anti-MUC1 junctional antibodies bind not only to differentiated MUC1+ tumor cells but also to MCF7 side population cells that show some characteristics of cancer stem cells (22), the antibodies were reacted with the MCF7 side population. Both antibodies DMB4B4 and DMB4F4 anti-MUC1 yielded sharp shifts in the gated cell population of both the MCF7 side population previously shown to bear some characteristics of cancer stem cells (ref. 22; Fig. 3, orange, middle and right panels) and mature MCF7 cancer cells (Fig. 3, green, middle and right panels). The side population cells bound to the anti-MUC1 antibodies were shown to have multiple characteristics of stem/progenitor cells (22). These include (i) preferential efflux of the fluorescent DNA-binding dye Hoechst 33342, (ii) phenotypic characterization as CD44+/CD24−/low, luminal and epithelial markers CK15 and EpCAM, and the stem cell/progenitor marker CK19, (iii) preferential formation of mammospheres in suspension culture, and (iv) gene array including preferential expression of a variety of protein products including Wiskott–Aldrich syndrome interacting protein and insulin-like growth factor–binding protein. These findings indicated that the side population of MCF7 cells representing cells that show some characteristics of cancer stem cells (22) can be targeted by anti-MUC1 junctional antibodies DMB4B4 and DMB4F4 no less effectively than MCF7 "mature" MUC1+ tumor cells.
Despite our characterization of the side population as described above, the MCF7 side population is itself a nonhomogeneous population of cells, and the phenotypic identification of true stem cells especially in ER\(^+\) cancer cells such as MCF7 remains a challenge. Indeed the caveat should be stressed that it is not clear whether this MCF7 side population represents stem cells as appearing in ER\(^+\) cancers. Thus to what degree the anti-MUC1 junctional antibodies target true stem cells will require a more precise characterization of SC in ER\(^+\) tumor cells.

**The DMB mAbs all recognize the SEA domain yet are different from one another**

The DMB mAbs were assessed by ELISA for binding to the MUC1-Xex protein, the SEA domain itself, and the SEA-4G protein, a mutant construct consisting of uncleaved \(\alpha\)–\(\beta\) bound by a 4-glycine peptide. These proteins were generated in bacteria, and as previously reported, the MUC1-Xex and SEA domain proteins spontaneously self-cleave in bacteria at the cleavage site FRP\(_G\)SVVV, in which \(\_\) indicates cleavage, generating the interacting \(\alpha\) and \(\beta\) subunits (depicted in Fig. 4B, bottom schema). The mutant SEA-4G protein (depicted in Fig. 4, bottom schema) comprises the amino acid sequence FRP\(_G\)GGG\(_G\)SVVV (instead of the above wild-type sequence), resulting in a noncleaved protein as previously described. Results showed that all 7 mAbs bound the 3 proteins (each bound to wells of an ELISA plate) at picomolar antibody concentrations and confirmed that the primary target of these antibodies is the SEA domain. Inspection of the binding curves of the Ig\(\_\gamma1\) mAbs to these 3 target proteins showed distinct binding patterns for mAbs DMB5F3, DMB4F4, and DMB7F3 to each of these proteins (Fig. 4A), indicating that the precise epitope within the SEA domain is different for each of these antibodies. Additional analyses including flow cytometry with a battery of MUC1-expressing cancer cells (data not shown) as well as Western blotting analyses (see below) further showed

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![Figure 4](image_url)
that all 5 IgG1 mAbs differ one from the other in their binding characteristics.

**The epitopes of anti-SEA module monoclonal antibodies DMB5F3, DMB4F4, and DMB4B4 are largely conformational and involve elements contributed by both the α and the β subunits**

To define the nature of the epitopes bound by the anti-SEA antibodies, they were assessed by Western blotting with a series of SEA module constructs. Staining of SDS-PAGE gels of the SEA module (Fig. 4B, a) shows its cleaved constituent α and β chains, as well as protein SEA-4G, a mutant construct consisting of uncleaved α/β bound by a 4-glycine peptide (6). As expected, probing Western blots with the previously described anti-β subunit-specific mAb BOS10D2 (ref. 26; linear epitope indicated in bottom schema, Fig. 4B) shows reactivity with the β subunit but none at the position of the α subunit (Fig. 4B, lane 1). It further shows strong binding to the α-4G-β construct (Fig. 4B, lane 2) as well as to small amounts of uncleaved α/β protein (ref. 4; Fig. 4B, lane 1). Antibodies DMB5F3 and DMB4F4 in contrast react strongly with uncleaved α-4G-β but are nonreactive with both the α and β subunits (Fig. 5C and D, respectively). Removal of SDS during the blotting procedure likely allows uncleaved α-4G-β protein to adopt a structure similar to that of the SEA module, previously shown to form an unusually stable 3-dimensional structure (6). In contrast, panel E shows that antibody DMB4B4 reacts with none of the proteins, not with α, not with β subunits in isolation, nor with the α-4G-β mutant isoforms, suggesting that its binding site requires the presence of both the α and β chains in an epitope resulting from conformationally determined cleavage.

**Affinity of antijunctional antibodies**

Having shown the specificity of the DMB monoclonals to a MUC1 cell-bound domain, the antibodies were examined by serial dilutions to evaluate their affinity for intact in situ cell-surface MUC1. To do so, the antibodies were reacted with ZR75 MUC1+ breast cancer cells in flow cytometry.

**Figure 5. Titration analysis of DMB monoclonal anti-MUC1 SEA module α/β junction antibodies to human breast cancer cells expressing MUC1 and affinity analysis.** MUC1+ human ZR75 breast cancer cells were reacted with antibodies DMB4B4, DMB4F4, and DMB5F3 at final concentrations of 4 and 1 μg/mL (left and center, respectively), and antibody binding was assessed by flow cytometry. Antibody DMB5F3 was shown to have the highest affinity. Its reactivity with ZR75 cells was assessed at decreasing concentrations (as indicated, right), down to 62 ng/mL, equivalent to a 0.4 nmol/L concentration of DMB5F3 (right). SPR binding assays for the determination of DMB5F3 full mAb and Fab fragments (bottom A and B, respectively) were carried out as described in Materials and Methods.
Dissociation rate (binding assay using Biacore3000 with serial dilutions. Analysis of the Fab fragment, DMB5F3 was similarly subjected to the SPR assay showed an association rate (the CDR1 and CDR3 regions. Analysis of the heavy chain amino acid sequences revealed it to be a member of the germline gene region sequences. Analysis of the heavy chain amino acid sequence, nucleotide primers were generated from known human Ig H and L constant primers generated for use in PCR of DMB5F3 cDNA together with over a period of 2.250 seconds, the dissociation rate is almost imperceptible in each of the 7 curves shown (Fig. 5). These studies were extended to a partially humanized chimeric version of DMB5F3 (chDMB5F3, see below), and showed that for chDMB5F3 an association rate ($K_a$) of $7.37 \times 10^{10}$ and a dissociation rate ($K_d$) of $5.89 \times 10^{-12}$ mol/L was calculated (panel A). To determine the binding kinetics of the Fab fragment, DMB5F3 Fab was similarly subjected to the SPR binding assay using Biacore3000 with serial dilutions. Analysis of chDMB5F3 Fab (purified as in Methods) by the SPR binding assay showed an association rate ($K_a$) of $2.37 \times 10^9$, a dissociation rate ($K_d$) of $1.26 \times 10^{-8}$, and an extraordinary low dissociation constant ($K_d$) of $2.89 \times 10^{-10}$ mol/L (Fig. 5B). Given the increased avidity associated with a bivalent IgG, the 100-fold enhancement seen in the full immunoglobulin molecule as compared with the Fab is as expected.

**Binding affinity of DMB5F3 by surface plasmon resonance**

As DMB5F3 showed the highest binding affinity, we concentrated further studies on this antibody. An SPR binding assay was carried out with serial dilutions of DMB5F3 ranging from 7 to 0.4 nmol/L, as described in Methods. Over a period of 2.250 seconds, the dissociation rate is almost imperceptible in each of the 7 curves shown (Fig. 5). These studies were extended to a partially humanized chimeric version of DMB5F3 (chDMB5F3, see below), and showed that for chDMB5F3 an association rate ($K_a$) of $7.37 \times 10^{10}$ and a dissociation rate ($K_d$) of $5.89 \times 10^{-12}$ mol/L was calculated (panel A). To determine the binding kinetics of the Fab fragment, DMB5F3 Fab was similarly subjected to the SPR binding assay using Biacore3000 with serial dilutions. Analysis of chDMB5F3 Fab (purified as in Methods) by the SPR binding assay showed an association rate ($K_a$) of $2.37 \times 10^9$, a dissociation rate ($K_d$) of $1.26 \times 10^{-8}$, and an extraordinary low dissociation constant ($K_d$) of $2.89 \times 10^{-10}$ mol/L (Fig. 5B). Given the increased avidity associated with a bivalent IgG, the 100-fold enhancement seen in the full immunoglobulin molecule as compared with the Fab is as expected.

**Generation of humanized DMB5F3 in Chinese hamster ovary cells**

As an initial demonstration of its clinical application, the picomolar affinity DMB5F3 antibody was partially humanized. To generate humanized DMB5F3 mAb, the H and L chains were isolated from SDS-PAGE gels, and their N-terminal sequences (10–15a.a.) determined. On the basis of that amino acid sequence, nucleotide primers were generated for use in PCR of DMB5F3 cDNA together with primers generated from known human Ig H and L constant region sequences. Analysis of the heavy chain amino acid sequence revealed it to be a member of the VH3 gene family, differing in a total of 9 amino acids from germline gene VH36-60/A1/85 (not shown). Of the 9 mutations, 5 are located in the CDR regions, whereas 2 are present near the usually highly conserved N terminal of the VH FR1 segment; one of those mutations (from L to V) occurs at a.a. 4 from the FR1 N terminal. The DMB5F3 VL chain is a k light chain, containing 5 a.a. mutations compared with its closest germ-line sequence 23 to 48. Of the 5 mutations, 3 are present in the CDR1 and CDR3 regions.

To show that chimeric DMB5F3 generated by mammalian cells retains its specific anti-MUC1 activity, recombinant chDMB5F3 was produced in CHO cells. To assess chDMB5F3 immunoreactivity, wells were coated with MUC1-Xex protein and reacted with dilutions of either recombinant chDMB5F3 or mouse hybridoma DMB5F3 mAb (Fig. 6C and D, respectively). Both antibodies bind the MUC1-Xex protein at similar low picomolar concentrations (Fig. 6D). Flow cytometry analyses confirmed both the specificity and remarkably strong affinity of the recombinant chDMB5F3 antibody to the MUC1 protein. No binding was observed to MUC1-negative parental non-transfected DA3 cells, whereas robust reactivity was seen with mouse transfectants expressing human MUC1-TM (DA3-TM: Fig. 6E and F, respectively). In fact, binding was still clearly observed at a concentration of chDMB5F3 as low as 20 picoMolar.

**Internalization of the DMB5F3 mAb**

To assess degree of internalization of the DMB5F3 mAb and thereby its ability to transport bound drugs into the cell, MUC1-expressing cells were incubated with DyLight488-labeled DMB5F3 either at 4°C or at 37°C and fluorescence monitored by confocal laser microscopy (Fig. 6G and H, respectively). At 4°C, labeled DMB5F3 was restricted to the cell membrane, indicating binding to the cell surface only. Subsequent incubation at 37°C showed relocalization of the labeled antibody to within the cell, reflecting highly efficient internalization to the cell interior.

**Linkage of chDMB5F3 generated by CHO to Pseudomonas ZZ-PE38 toxin and cytotoxicity of the immunoconjugate**

Having seen that the DMB5F3 antibody undergoes efficient intracellular internalization, we then assessed its ability to ferry cytotoxic moieties into the cell. The ZZ-PE38 fusion Pseudomonas toxin is devoid of a cell-binding domain, and to effect its cell killing activity, it must be linked to an agent capable of internalizing it into the cell. Chimeric DMB5F3 (chDMB5F3) contains human Fc to which the ZZ domain (derived from protein A) of the ZZ-PE38 toxin binds well. Parental, nontransfected cells that do not express human MUC1 are unaffected by chDMB5F3-PE38 immunotoxin (Fig. 6I, diamond symbols), whereas DA3 transfectants expressing human MUC1-TM (DA3-TM) are exquisitely sensitive to chDMB5F3-PE38 conjugate with cytoidal activity seen at picoMolar concentrations (Fig. 6I, square symbols and see inset). These findings were extended to T47D human breast cancer cells that natively express the MUC1 protein. The chDMB5F3-PE38 immunotoxin conjugate was potently cytoidal to these cells as well, with extensive cell killing observed at as low as picoMolar concentrations (Fig. 6I).

**Efficacy and specificity of cell killing by chDMB5F3:ZZ-PE38 immunotoxin conjugates and comparison of cytoidal activity with cetuximab (Erbitux) and trastuzumab (Herceptin)**

As seen with DA3 cells transfected with human MUC1 (Fig. 6G and H), human adenocarcinoma cells showed that following incubation at 4°C labeled DMB5F3 was limited to the cell membrane, indicating binding only to the cell surface (Fig. 7A). Subsequent incubation at 37°C showed almost complete relocalization of the labeled antibody to within the cell, reflecting...
highly efficient internalization into the cell interior (Fig. 7A). Having shown internalization of chDMB5F3 to MUC1-positive human adenocarcinoma cells, we next proceeded to assess the cytotoxicity of immunotoxin formed with chDMB5F3 and to compare its activity with that of Erbitux and Herceptin immunotoxins. Fixed cells showed similar cell-membrane reactivity with both chDMB5F3 and Erbitux (Fig. 7B and B’, respectively), and with Herceptin (data not shown). Recombinant chDMB5F3:ZZ-PE38 reacted with the MUC1+ human pancreatic cancer cell line Colo357 resulted in cell killing, and serial dilutions of the antibody revealed an IC50 of approximately 16 pmol/L (Fig. 7C). However, when chDMB5F3:ZZ-PE38 was reacted with Colo357 simultaneously with soluble recombinant extracellular domain of MUC1-X (MUC1-Xex), competition was seen with a resultant marked reduction in cell killing (Fig. 7C, orange dotted curve). To show the specificity of that competition, soluble MUC1 Xex was added to Erbitux:ZZ-PE38 at an equal concentration. MUC1 Xex failed to abrogate Erbitux immunotoxin activity (Fig. 7C, blue dotted curve). In a similar way, serial dilutions of chDMB5F3:ZZ-PE38 reacted with 2R75 breast cancer cells showed highly effective killing with an IC50 of 3 pmol/L, whereas addition of MUC1 Xex competitor abolished cell killing (Fig. 7D, compare continuous and dotted green lines). Cell lines such as A431 (epidermoid carcinoma) and N87 (gastric carcinoma) that were found to be only very slightly positive for MUC1, as assessed by flow cytometry with DMB5F3 mAbs, were not affected by the chDMB5F3:ZZ-PE38 immunotoxin conjugate (data not shown).

Taken together, these results showed the specificity of cell killing mediated by the chDMB5F3:ZZ-PE38 immunotoxin, and furthermore, showed that in certain cancer lines, anti-
MUC1-X is comprised solely of the 120-amino acid SEA module immunogenic tandem repeat array, the extracellular domain of membrane domains as the full MUC1-TM molecule (4). Where-MUC1-X isoform that contains the same intracellular and Speci

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sequester antitandem repeat array antibodies, thereby pre-

junction composed of the MUC1

subunit, it is often shed from the surface of MUC1 þ cells and freely circulates peripherally. It is here that the shed α subunits sequester antitandem repeat array antibodies, thereby precluding their ability to reach MUC1 þ tumor cells (15, 16).

We previously described mechanisms whereby the cleaved junction composed of the MUC1 α and β chains is formed (4). Specifically, we analyzed the potential for cleavage of an MUC1-X isoform that contains the same intracellular and membrane domains as the full MUC1-TM molecule (4). Where-as the extracellular domain of MUC1-TM contains the highly immunogenic tandem repeat array, the extracellular domain of MUC1-X is comprised solely of the 120-amino acid SEA module fused to a 30 N-terminal amino acid segment of MUC1, resulting in a less complex structure. Significantly, not only is the MUC1-X α/β junctional isoform cleaved, it is cleaved at an identical site as is the full-length MUC1-TM molecule and therefore results in the same noncovalent interaction of the α and β subunits (4).

All 7 anti-MUC1 mAbs described here are directed against the cell-bound MUC1 SEA domain that embraces the MUC1 α/β junction. Because the membrane-bound α/β junction involves an intricate structure of α helices and intertwined β strands (6), antibodies directed against the native structure would likely recognize epitopes composed of elements contributed by both the α and β subunits. As shown (Fig. 4), this is in fact the case: binding by all antibodies requires intact conformation involving elements contributed by both the α and β subunits. The antibodies not only conform to the expectation of conformational binding, but they also bind the intact, native glycosylated MUC1 molecule on MUC1 þ breast cancer cells (Fig. 5) and MUC1 þ pancreatic cancer cells (Fig. 2), with very high affinity—a Kd of approximately 6 picoMolar was calculated for DMBSF3 IgG binding to MUC1 protein. Considering the large number of tandem repeat epitopes in the MUC1 α subunit as compared with the unique epitopes present in the SEA domain, the high affinity of the anti-SEA compared with anti-α antibodies is all the more impressive. The DMBSF3 Kd is 3 to 4 logs higher than the Kd values reported for either cetuximab (31) or trastuzumab (32), both humanized IgGs derived from murine hybridomas, and 3 logs higher than an

Discussion

Despite its potential to serve as an effective immunotherapeutic agent for a variety of high-expressing MUC1 malignancies, the MUC1 molecule has proved to be an elusive prey. This is in large measure due to the failure to identify and target MUC1 moieties that are permanently cell bound (27).

Almost all anti-MUC1 antibodies reported to date are directed against the highly immunogenic polymorphic array of 20 to 125 tandem repeats of a 20-amino acid sequence present in the MUC1 α chain (for example; refs. 17, 28–30). As the α chain is bound noncovalently with the cell-bound β subunit, it is often shed from the surface of MUC1 þ cells and therefore results in a less complex structure. Significantly, not only is the MUC1-X α/β junctional isoform cleaved, it is cleaved at an identical site as is the full-length MUC1-TM molecule and therefore results in the same noncovalent interaction of the α and β subunits (4).

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Figure 7. Specificity and affinity of chDMBSF3 immunotoxin (IT) for cell killing of cancer cells—comparison with Erbitux and Herceptin immunotoxins (IT). Human adenocarcinoma cells were incubated at 4 °C (A) followed by incubation at 37 °C (A′) with DyLight650-labeled DMBSF3 mAb—internalization of the antibody is clearly seen at the 37 °C incubation. Unambiguous membrane labeling was seen following incubation of fixed human adenocarcinoma cells with either chDMBSF3 (B, green label, detection with Alexa488-labeled goat anti-human Fc) or Erbitux (B′, red label, detection with R-phycoerythrin-labeled goat anti-human Fc). Human Colo357 pancreatic and ZR75 breast cancer cells (C and D, respectively) were incubated with chDMBSF3, Erbitux, or Herceptin ZZ-PE38 immunotoxin conjugates, and cell viability (y ordinate) was assessed using the alkaline phosphatase assay. Addition of 50 μg/mL of soluble MUC1 Xex to chDMBSF3 immunotoxin abrogated cell killing (dotted orange lines). In contrast, addition of MUC1-Xex competitor did not abrogate cell killing mediated by immunotoxin conjugates formed with either Erbitux (C, compare dotted blue and solid blue lines) or Herceptin (D, compare dotted green and solid green lines).
anti-MUC1 tandem repeat array IgG1 isolated from in vitro selection of an Fab phage library.

Naked antibodies against carcinoma cell surface tumor antigens are seldom curative by themselves and are usually administered in combination with chemotherapy. However, very potent cytotoxic agents are often, as stand-alone therapeutics, too toxic and should be ideally directed solely to the tumor antigen expressing cancer cell. This can be achieved via immunoconjugates that will selectively deliver potent therapeutics to a tumor, thereby reducing systemic toxicity.

As an initial demonstration of ultimate clinical application, the DMB5F3 anti-MUC1 was partially humanized and produced in CHO cells as chimeric DMB5F3 (chDMB5F3), and then tested for cytotoxic activity as an immunotoxin. Expression of chDMB5F3 in CHO cells showed that it retains the same specificity and picomolar binding affinity as antibody synthesized by the original hybridoma clones. The results show that chDMB5F3 not only binds MUC1⁺ malignant cells but that as an immunoconjugate, it also internalizes ZZ:P38 Pseudomonas toxin resulting in very potent cell death (Fig. 6). This is consistent with the previously reported cytoidal activity of PE38-immun conjugates against pancreatic cancer cells and against hairy cell leukemia (33, 34). In addition, the cytotoxic activity of chDMB5F3 immunoconjugate was compared with that of PE38 immunotoxins formed with cetuximab (anti-EGFR; ref. 35) and with trastuzumab (Herceptin, anti-ErbB2 or Her2; ref. 36). In human cancer cell lines assessed for their sensitivity to the 3 immunotoxin conjugates, chDMB5F3:ZZ-PE38 resulted in cytotoxicity comparable or superior to the cetuximab and trastuzumab conjugates in pancreatic and breast cancer cells (Fig. 7).

To assess the antitumor activity of the chDMB5F3:ZZ-PE38 immunoconjugate within the intact organism, we have recently initiated a study that uses a nude mouse model of pancreatic tumor growth using MUC1⁺ positive human pancreatic cancer cells. To date, our in vivo findings show abrogation of tumor growth corroborating the potent in vitro cytoidal activity of chDMB5F3:ZZ-PE38 (to be presented in a separate publication).

The anti-CD30 immunoconjugate, Brentuximab vedotin, has been recently approved for use in CD30⁺ Hodgkin disease (37), in which more than a third of patients with refractory Hodgkin lymphoma achieved complete remission and partial remissions were observed in another 40%. This is further indication of the effective targeting capabilities of immunoconjugates and highlights the clinical potential of such antibody–drug conjugates.

Cetuximab-induced tumor cytotoxicity has not been found to directly correlate with the degree of tumor expression of epidermal growth factor receptor (EGFR), but to be primarily effective in the subset of EGFR⁺ tumors having the KRAS mutation in colorectal adenocarcinoma and squamous cell carcinoma of the head and neck (38, 39). Furthermore, cetuximab has shown limited activity against other EGFR-expressing tumors such as breast cancer (40). The antitumor effect of trastuzumab, on the other hand, does in general correlate with tumor overexpression of its target protein, ErbB2 (Her2; ref. 36). However ErbB2 is overexpressed in only a minority subset of patients, approximately 20% of breast cancer (36) and 22% of gastric cancers (41). Because MUC1 is overexpressed in some 70% to 80% of adenocarcinomas including gastric, pancreatic, colorectal, prostate, and ovarian cancers, as well as on the malignant plasma cell of multiple myeloma (8–13), the high degree of chDMB5F3:immunotoxin conjugate–induced cytotoxicity showed here augurs well for use in those tumors not amenable or nonresponsive to therapy with other mAbs.

Additional investigations, beyond the scope of this article, will be required to see whether the anti-MUC1-SEA domain DMB mAbs reported here possess intrinsic antitumor activity within the organism when administered as naked antibodies. Such activity shown for cetuximab and trastuzumab has been attributed to immunologically based and Fc-dependent mechanisms (42), including ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity). We do not know as yet whether this will be the case for all or any of the DMB mAbs reported here. Antibodies against the tandem repeat part of MUC1 fail to induce complement lysis despite appreciable binding by flow cytometry. This has been attributed to the great distance from the cell surface that complement is activated (43). In this regard, it is pertinent to note that all DMB mAbs bind MUC1-SEA domain epitopes that are located close by to the cell membrane making CDC a distinct possibility for DMB mAbs.

Tumor stem cells comprise a cell subpopulation capable of self-regeneration and are thought to result in tumor cell repopulation following ablation of all detectable malignant disease by antitumor therapies (44). The present findings indicate that the side population of MCF7 cells previously shown to bear some characteristics of cancer stem cells (22) express MUC1⁺. The fact that antibodies DMB4B4 and DMB4F4 bind this side population of MCF7 cells suggests that the same conformationally determined MUC1 epitopes formed by the α and β chains on differentiated tumor cells are also present on these cells.

Targeting conformationally determined sites on the cell-bound α/β MUC1 junction may successfully overcome the difficulty encountered to date in targeting the MUC1 shed α chain. Whether the ultimate optimal clinical application are the antijunctional antibodies described here or development of anti-α/β MUC1 junction T-cell vaccines (44–48)—or both—will be answered by further studies.

Disclosure of Potential Conflicts of Interest

D.H. Wreschner has ownership interest in Biomodifying, Inc.

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