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 alpha-subunit into the circulation where it can sequester many anti-MUC1 antibodies, renders the target to some degree problematic. 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-EGFR1) 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.

Introduction
MUC1 is a mucin-like glycoprotein which 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 20-125 tandem repeats of 20 amino acids, followed by a transmembrane domain and a short cytoplasmic tail (2-4). MUC1 is a heterodimer which is cleaved soon after synthesis within the SEA module, a highly-conserved domain of 120 amino acids (4-6). Cleavage of MUC1 yields two unequal chains: a large extracellular alpha subunit containing the tandem repeat array specifically bound in a strong non-covalent interaction to a smaller beta 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 alpha chain, [for example (15-17)]. Since the MUC1 alpha 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 anti-tandem alpha chain antibodies and circulating alpha chain potentially may result in end-organ damage (16).

Targeting MUC1 epitopes bound to the cell surface would avoid antibody sequestration by circulating alpha chain. The MUC1 SEA domain formed by the interaction of the alpha-subunit with the extracellular portion of beta-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 demonstrated the proof-of-principle of generating antibodies that specifically recognize the MUC1 SEA domain (18). The prototype DMC209 mAb described (18) has two 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 alpha/beta junction, that were also affinity-maturated and not of the IgM subclass. We report here the generation of seven novel monoclonal antibodies, five Ig-gamma1 and two IgA, that bind the MUC1 protein within its SEA domain with picomolar affinities. 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 anti-tumor antibodies and chemo-cytotoxic agents.

MATERIALS AND METHODS

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

Cell lines Human breast carcinoma lines T47D, MCF7 and ZR-75, 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, 2mM L-glutamine, 100 IU/ml penicillin and 25mg/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 co-transfected 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 six histidine residues at their N-terminus, followed by the extracellular domain of the MUC1-X/SEA/SEA-4G proteins, respectively.
**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 alpha and beta subunits that strongly, yet non-covalently, 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 non-secreting 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** ElisaImmunoAssay plates (Costar, Corning, NY) 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 performed with HRP-conjugated anti-mouse antibody.

**Two-tiered screening for selection of anti-MUC1 monoclonal antibodies** The primary screen of the hybridomas was performed 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** A 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 (SP) 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 is comprised of 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-Polyacrylamide protein gel electrophoresis (SDS-PAGE) Proteins separated on SDS PAGE were electro-transferred for 2 hours at 0.5Amp 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 horseradish peroxidase (Chemicon International, Temecula, CA) 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 ZR-75 breast cancer cells, Colo357 pancreatic cancer cells, as well as additional cancer cells as detailed in Results), were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. These cells (10,000 cells per well in 100 microliters of medium) were seeded in 96 well cell culture plates (Corning; Corning, New York) and grown at 37°C in 5%CO2 in culture media. Five hours after seeding, 50 microliters of the chDMB5F3 was diluted by decimal dilutions starting from 1600pm and mixed with 50 microliters ZZ-PE38 toxin. The immunotoxin mixture was directly applied to the cells (final ZZ-PE38 toxin concentration was 250ng/ml). Negative controls included adding to the target cells...
the following (instead of the chDMB5F3:ZZ-PE38 toxin immunoconjugates) - (i) concentrated medium from parental CHO-K1 cells that is devoid of the anti-MUC1 chDMB5F3 monoclonal antibody, (ii) ZZ-PE38 alone, (iii) chDMB5F3 monoclonal antibodies 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 CHO (Chinese Hamster Ovary) cells of recombinant DMB-5F3-constant region of human IgG1 [C-hIgG1] 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 gamma1 heavy and human kappa light chains, respectively.

Surface Plasmon Resonance (SPR) binding assay Recombinant MUC1-X protein was immobilized on a CM5 chip. As control, anti-CD20 IgG (Rituxan®) was treated in a like manner. Remaining active groups were saturated with 1mM ethanolamine. SPR was performed using Biacore®3000 according to manufacturer's specifications (GE Healthcare, Piscataway NJ 08855). Serial dilutions of antibody DMB5F3 (0.4-7nM) were measured for 4 minutes-association and 30 minutes -dissociation. The chip was regenerated with 5mM NaOH. Data fitting was performed using algorithm of the Biacalevalution 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 M. The resultant mixture was dialyzed against PBS pH8, 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 performed as follows: 5 x 10^5 cells were used in each experiment. After trypsinization, cells were washed and monoclonal antibodies at varying dilutions were added to the cell tubes for 1 hour at 4°C. After washing with FACS buffer, FITC-labeled goat anti-human Fc antibody was added to the tubes for 45 min at 4 °C. Detection of bound IgG was performed by means of flow cytometry on a FACS-Calibur (Becton Dickinson, CA), and results analyzed with theCELLQuest program (Becton–Dickinson).
Results

**Generation of monoclonal antibodies to the cell-bound MUC1 alpha/beta 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 alpha chain and the cell bound beta chain. This provided the advantage of incorporating the cell bound region consisting of the alpha/beta 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 non-covalently interacting MUC1 alpha and beta subunits.

Using our immunization protocol (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 two-tiered screen consisting of (a) binding to MUC1-X protein, and (b) a more stringent second tier assessing binding to MUC1-expressing cancer cells as assayed by flow cytometry. Hybridoma formation resulted in seven monoclonal antibodies, five of the Ig-gamma1 isotype, designated DMB-4B4, DMB-4F4, DMB-5F3, DMB7F3 and DMB10F10. Surprisingly two monoclonals of the IgA subclass were also isolated, DMB10B7 and DMB13D11.

**Cytometric analyses of monoclonal anti-junctional antibodies** Flow cytometric analyses demonstrated that all seven monoclonal antibodies bound strongly to DA3-TM cells that express the full length MUC1. In contrast, untransfected DA3-PAR cells which 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, 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 non-competitive with moieties present in the shed alpha chain of the MUC1-TM protein. In addition to the flow cytometric studies, initial immunohistochemical analyses were performed with one of these monoclonal antibodies (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 anti-junctional antibodies to MCF7 side population cells that show some characteristics of breast cancer stem cells** In order 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 MCF-7 side population (SP). 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 (22) (Fig. 3, orange, middle and right panels) and mature MCF7 cancer cells (Fig. 3, green, middle and right panels). The SP cells bound to the anti-MUC1 antibodies were demonstrated to have multiple characteristics of stem/progenitor cells (22). These include- [1] preferential efflux of the fluorescent DNA-binding dye Hoechst 3342, [2] phenotypic characterization as CD44+/CD24-/low, luminal and epithelial markers CK15 and EpCAM, and the stem cell/progenitor marker CK19, [3] preferential formation of mammospheres in suspension culture, and [4] 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 indicate 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 DMB-4B4 and DMB4F4 no less effectively than MCF7 'mature' MUC1+ tumor cells.

Despite our characterization of the SP as described above, the MCF7 SP is itself a non-homogeneous population of cells (22) and the phenotypic identification of true stem cells
especially in ER+ cancer cells (such as MCF7) remains a challenge (24, 25). Indeed the caveat should be stressed that it is not clear whether this MCF7 side population represent 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 α−β bound by a 4 glycine peptide (6). These proteins were generated in bacteria, and as previously reported (4), the MUC1-Xex and SEA domain proteins spontaneously self-cleave in bacteria, at the cleavage site FRPG|SVVV, where | indicates cleavage, generating the interacting alpha and beta subunits (depicted in Figure 4, bottom schema). The mutant SEA-4G protein (depicted in Figure 4, bottom schema) comprises the amino acid sequence FRPGGGGSVVV (instead of the above wild type sequence), resulting in a non-cleaved protein as previously described (6). Results showed that all seven mAbs bound the three 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 monoclonal antibodies to these three target proteins demonstrated distinct binding patterns for mAbs DMB5F3, DMB4F4 and DMB7F3 to each of these proteins (Figure 4, top panel), 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 that all five Ig-gamma1 monoclonal antibodies 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 alpha and the beta 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. 4, bottom panel, A) shows its cleaved constituent alpha and beta chains, as well as protein SEA-4G, a mutant construct consisting of uncleaved alpha-beta bound by a 4
glycine peptide(6). As expected, probing western blots with the previously described anti-beta subunit specific mAb BOS10D2 (26) (linear epitope indicated in bottom schema, Fig. 4) demonstrates reactivity with the beta subunit but none at the position of the alpha-subunit (lane 1). It further demonstrates strong binding to the alpha-4G-beta construct (lane 2) as well as to small amounts of uncleaved alpha/beta protein (4) (lane 1). Antibodies DMB5F3 and DMB4F4 in contrast react strongly with uncleaved alpha-4G-beta but are non-reactive with both the alpha and beta subunits (Fig. 5, Panels C and D, respectively). Removal of SDS during the blotting procedure likely allows uncleaved alpha-4G-beta protein to adopt a structure similar to that of the SEA module, previously shown to form an unusually stable three-dimensional structure (6). In contrast, Panel E demonstrates that antibody DMB4B4 reacts with none of the proteins, not with alpha, not with beta subunits in isolation, nor with the alpha-4G-beta mutant isoforms, suggesting that its binding site requires the presence of both the alpha and beta chains in an epitope resulting from conformationally-determined cleavage.

**Affinity of anti-junctional antibodies** Having demonstrated 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 ZR-75 MUC1+ breast cancer cells in flow cytometry (Methods). ZR-75 was reacted with the antibodies at an initial concentration of 4 microgram/ml. As seen in Figure 5 (upper panel, left), all three antibodies, DMB-4B4, DMB-4F4, and DMB5F3 showed binding at that concentration. At 1 microgram/ml binding of DMB-4B4 and DMB-4F4 was diminished, whereas DMB5F3 clearly showed binding (Fig. 5, middle panel). DMB5F3 continued to bind the ZR-75 cells to a concentration as low as 62ng/ml (Fig. 5, right panel).

**Binding affinity of DMB5F3 by Surface Plasmon Resonance (SPR)**

As DMB5F3 showed the highest binding affinity, we concentrated further studies on this antibody. An SPR binding assay was performed with serial dilutions of DMB5F3 ranging from 7nM to 0.4nM as described in *Methods*. Over a period of 2250 seconds, the degree of dissociation is almost imperceptible in each of the seven curves shown (Fig. 5). These studies were extended to
a partially humanized chimeric version of DMB5F3 (chDMB5F3, see below), and demonstrated that for chDMB5F3 an association rate (Ka 1/Ms) of $1.25 \times 10^6$ and a dissociation rate (Kd (1/s) of $7.37 \times 10^{-6}$ were obtained, and an extraordinary low dissociation constant (K_D) of $5.89 \times 10^{-12}$M was calculated (Panel A). To determine the binding kinetics of the Fab fragment, DMB5F3 Fab was similarly subjected to the SPR binding assay using Biacore®3000 with serial dilutions. Analysis of chDMB5F3 Fab (purified as in Methods) by the SPR binding assay showed an association rate (Ka 1/Ms) of $2.37 \times 10^6$, a dissociation rate (Kd (1/s) of $1.26 \times 10^{-3}$, and a dissociation constant (K_D) of $4.84 \times 10^{-10}$M (Fig. 5, Panel B). Given the increased avidity associated with a bivalent IgG, the 100-fold K_D enhancement seen in the full immunoglobulin molecule as compared to the Fab is as expected.

**Generation of humanized DMB5F3 in CHO (Chinese hamster ovary)** As an initial demonstration of its clinical application, the picomolar-affinity DMB5F3 antibody was partially humanized. In order 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. Based on 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, while 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 kappa light chain, containing 5 a.a. mutations compared to its closest germline sequence 23-48. Of the 5 mutations, 3 are present in the CDR1 and CDR3 regions.

In order to demonstrate 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. 6, panels C and D, respectively). Both antibodies bind the MUC1-Xex protein at similar low picomolar concentrations (Fig. 6, panel D). 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 DyLight649-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 in order 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, non-transfected cells that do not express human MUC1 are unaffected by chDMB5F3-ZZ-PE:38 immunotoxin (Fig.6I, diamond symbols) whereas DA3 transfectants expressing human MUC1-TM (DA3-TM) are exquisitely sensitive to chDMB5F3:ZZ-PE38 conjugate with cytocidal activity seen at picoMolar concentrations (Fig. 6I square symbols and see inset). These findings were extended to T47D human breast cancer cells which natively express the MUC1 protein. The chDMB5F3:ZZ-PE38 immunotoxin conjugate was potently cytocidal 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 cytocidal 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 demonstrated almost complete relocalization of the labeled antibody to within the cell, reflecting highly efficient internalization into the cell interior (Fig. 7A'). Having demonstrated 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 demonstrated similar cell-membrane reactivity with both chDMB5F3 and Erbitux (Fig. 7, B and B', respectively), and to 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 16pM (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). In order to demonstrate 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 ZR75 breast cancer cells showed highly effective killing with an IC50 of 3pM whereas addition of MUC1 Xex competitor abolished cell killing (Fig. 7D, compare continuous and dotted orange lines). While reaction of Herceptin:ZZ-PE38 to ZR75 cells resulted in cell killing, addition of MUC1 Xex did nothing to abrogate cell death (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 immunoconjugate (data not shown).

Taken together these results demonstrate the specificity of cell killing mediated by the chDMB5F3:ZZ-PE38 immunotoxin, and furthermore demonstrate that in certain cancer lines, anti-MUC1-SEA module DMB5F3 immunotoxin is the most potent of the toxin conjugates tested.
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 20-125 tandem repeats of a 20 amino acid sequence present in the MUC1 alpha chain [for example, (17) (28-30)]. As the alpha chain is bound non-covalently with the cell-bound beta subunit, it is often shed from the surface of MUC1+ cells and freely circulates peripherally. It is here that the shed alpha-subunits sequester anti-tandem repeat array antibodies thereby precluding their ability to reach MUC1+ tumor cells (15, 16).

We previously described mechanisms whereby the cleaved junction composed of the MUC1 alpha and beta chains is formed (4). Specifically, we analyzed the potential for cleavage of an MUC1-X isoform that contains the same intra-cellular and membrane domains as the full MUC1-TM molecule (4). While 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 alpha/beta 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 alpha and beta subunits(4).

All seven anti-MUC1 monoclonal antibodies described here are directed against the cell-bound MUC1 SEA domain that embraces the MUC1 alpha/beta junction. Since the membrane-bound alpha/beta junction involves an intricate structure of alpha helices and intertwined beta strands (6), antibodies directed against the native structure would likely recognize epitopes composed of elements contributed by both the alpha- and beta- subunits. As shown (Fig. 4), this is in fact the case: binding by all antibodies requires intact conformation involving elements contributed by
both the alpha and the beta subunits. The antibodies not only conform to the expectation of conformational binding, but they also bind the intact, native glycosylated MUC1 molecule on MUC1+ breast cancer cells (Fig. 5) and MUC1+ pancreatic cancer cells (Fig. 2), with very high affinity— a Kd of approximately 6 picoMolar was calculated for DMB5F3 IgG binding to MUC1 protein. Considering the large number of tandem repeat epitopes in the MUC1 alpha-subunit as compared to the unique epitopes present in the SEA domain, the high affinity of the anti-SEA compared to anti-alpha antibodies is all the more impressive. The DMB5F3 Kd is 3-4 logs higher than the Kds reported for either Cetuximab(31) or Trastuzumab(32), both humanized IgGs derived from murine hybridomas, and 3 logs higher than an 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 cytocidal activity as an immunotoxin. Expression of chDMB5F3 in CHO cells demonstrated that it retains the same specificity and picomolar binding affinity as antibody synthesized by the original hybridoma cells. The results demonstrate 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 cytocidal activity of PE38-immunconjugates against pancreatic cancer cells and against hairy cell leukemia (33, 34). In addition, the cytotoxic activity of chDMB5F3 immunonoconjugate was compared to that of PE38 immunotoxins formed with Cetuximab (Erbitux®) (anti-EGFR)(35) and with Trastuzumab (Herceptin®, anti-ErbB2 or Her2)(36). In human cancer cell lines assessed for their sensitivity to the three 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 anti-tumor activity of the chDMB5F3:ZZ-PE38 immunoconjugate within the intact organism, we have recently initiated a study that utilizes 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 cytocidal 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+ Hodgkins disease(37), where 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 (ADC).

Cetuximab-induced tumor cytotoxicity has not been found to directly correlate with the degree of tumor expression of 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)(36). However ErbB2 is overexpressed in only a minority subset of patients, approximately 20% of breast cancer(36) and 22% of gastric cancers (41). Since MUC1 is overexpressed in some 70-80% 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 demonstrated here augurs well for use in those tumors not amenable or non-responsive to therapy with other monoclonal antibodies.

Additional investigations, beyond the scope of the present report, will be required to see whether the anti-MUC1-SEA-domain DMB monoclonal antibodies reported here possess intrinsic anti-tumor 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 are comprised of a cell subpopulation capable of self-regeneration and are thought to result in tumor cell repopulation following ablation of all detectable malignant disease by anti-tumor 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 alpha and beta chains on differentiated tumor cells are also present on these cells.

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

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References


Figure 1 Scheme of MUC1 proteins used for immunizing mice and subsequent screening for anti-MUC1 SEA module alpha/beta junction monoclonal antibodies

Schema of isoforms

The cleaved full-length MUC-TM protein containing both the beta-chain and the alpha chain with its tandem repeat array 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 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 comprised of the interacting alpha and beta subunits is indicated by the dashed black circles. Screening of hybridoma supernatants for antibodies that selectively recognize the SEA module alpha/beta junction was carried out with an ELISA screen using wells precoated with MUC1-Xex protein (see Methods). The series of seven anti-MUC1 antibodies (the DMB series, red lettering), as well as the previously described (18) IgM DMC209, bind the SEA module.

Figure 2 Flow cytometric analyses of DMB anti-MUC1 SEA module alpha/beta junction monoclonal antibodies 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 alpha/beta junction monoclonal antibodies. 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].

Figure 3 Binding of anti-junctional antibodies to MCF7 side population cells

An MCF7 breast cancer cell population showing some characteristics of cancer stem cells was isolated as described by Finn (22). MCF7 cells were stained with the fluorescent dye Hoechst 33342. Due to their increased efflux of the dye, a cone-shaped side population (SP) of MCF7 stem cells (5.1% of the overall population) was isolated and characterized (top right plots). Reaction with IgG1 isotype control resulted in no cell shift (left lower panels). Reaction with 10μg/ml of either DMB-4B4 or DMB-4F4 resulted in near-total shifts in both the MCF7 side cell side population (orange color, middle and right lower panels) as well as in 'mature' MCF7 breast cancer cells (green color; middle and left lower panels).
Figure 4 Immunoreactivity of DMB mAbs with MUC1-Xex, SEA module and MUC1 4G-SEA module proteins [I] ELISA plates were coated with one of three recombinant bacterial MUC1 proteins- extracellular domain of the MUC1-X protein (dotted lines), MUC1 SEA module (dashed lines), or the uncleaved MUC1 4G-SEA module protein (dashed and dotted lines). The indicated monoclonal antibodies were applied and bound antibody was detected as described in Methods. The three monoclonal antibodies DMB-5F3, DMB-4F4 and DMB-7F3 show different patterns of binding for the three MUC1 proteins. [II] Both the MUC1 SEA module (SEA, lanes 1) and the mutant uncleaved MUC1 SEA module (SEA-4G, lanes 2) were resolved by SDS-PAGE and stained with Coomassie blue stain (panel A). This revealed the separate alpha and beta subunits of the SEA module (lanes 1, indicated by alpa and beta) or the alpha and beta subunits connected by the four- glycine residue linker (alpha-4G-beta). Identical gels were western blotted and probed with monoclonal antibodies BOS10D2, DMB5F3, DMB4F4 or DMB4B4 (panels B-E, respectively). The epitope recognized by the BOS10D2 antibodies has been previously reported (26) and is indicated in the lower panel. Color scheme of the protein segments is the same as in Fig. 1.

Figure 5 Titration analysis of DMB monoclonal anti-MUC1 SEA module alpha/beta 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 micrograms/ml (left and center panels, 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 panel), down to 62 nanograms/ml, equivalent to a 0.4nM concentration of DMB5F3 (right panel). Surface Plasmon Resonance (SPR) binding assays for the determination of DMB5F3 full mAb and Fab fragments (lower panels A and B, respectively) were performed as described in Methods.

Figure 6 Activity of recombinant chimeric DMB5F3 (chDMB5F3) monoclonal antibody synthesized in CHO cells. Recombinant antibody (chDMB5F3, see Methods) secreted into CHO culture medium was assessed by dot-blotting dilutions (ten fold to eighty fold) followed by detection with HRP-conjugated anti-human Fc (Panel B). To assess absolute amounts of chDMB5F3, a parallel dot blot was performed with known amounts of human IgG (Panel A). The activity of the chDMB5F3 recombinant antibody was compared to that of mouse hybridoma DMB5F3 mAb by ELISA (panels C and D, respectively). Flow cytometry analyses were performed with the chDMB5F3 antibody at the indicated concentrations using MUC1-negative parental DA3 cells (DA3-PAR) and mouse cell transfectants (DA3-TM) expressing human MUC1-TM (E and F, respectively). MUC1+ cells were incubated with DyLight649-labeled DMB5F3 mAb at 4°C and at 37°C (panels G and H, respectively) and photographed by confocal laser microscopy. Whereas at 4°C antibody remained at the cell surface, at 37°C DMB5F3 was all but completely internalized into the cell. Panel I- Cytocidal activity of chDMB5F3:ZZ-PE38 immunotoxin conjugate was tested.
(Panel G) on DA3-PAR, DA3-TM and human breast cancer T47D cells by applying varying levels of chDMB5F3 antibody, as indicated on the x-axis, together with ZZ-PE38. Cell viability (y-ordinate) was assessed using the alkaline phosphatase assay (Methods).

**Figure 7 Specificity and affinity of chDMB5F3 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 DMB5F3 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 chDMB5F3 (B, green label, detection with Alex488-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 (panels C and D, respectively) were incubated with chDMB5F3, Erbitux or Herceptin ZZ-PE38 immunotoxin conjugates, and cell viability (y-ordinate) was assessed using the alkaline phosphatase assay. Addition of 50 microg/ml of soluble MUC1 Xex to chDMB5F3 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 (panel C, compare dotted blue and solid blue lines) or Herceptin (panel D, compare dotted green and solid green lines).
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Figure 2
Figure 3
Figure 4

DMB-5F3
DMB-4F4
DMB-7F3

Antibody concentration (nM)

A. BOS10D2
B. SEA
C. BOS10D2
D. SEA-4G

MUC1-Xex

SEA module

BOS10D2

β

α-4G-β

α

β

1

2

1

2
**Figure 5**

**ZR-75 breast cancer cells**

- **A**
  - DMB5F3 mAb
  - Reaction rates for different concentrations:
    - 4μg/ml
    - 1μg/ml
    - 250ng/ml
    - 62ng/ml
  - Time range: 0 to 2000 s
  - Kinetic parameters:
    - $K_a$ (M): $1.25 \times 10^6$
    - $K_d$ (s$^{-1}$): $7.39 \times 10^6$
    - $K_M$ (M): $5.89 \times 10^{-12}$

- **B**
  - DMB5F3 Fab
  - Reaction rates for different concentrations:
    - 4μg/ml
    - 1μg/ml
    - 250ng/ml
    - 62ng/ml
  - Time range: 0 to 2000 s
  - Kinetic parameters:
    - $K_a$ (M): $2.37 \times 10^6$
    - $K_d$ (s$^{-1}$): $1.26 \times 10^{-3}$
    - $K_M$ (M): $4.84 \times 10^{-10}$
**Figure 6**

A. hIgG standard

B. CHO recombinant hDMB5F3

C. hDMB5F3 CHO recombinant

D. mDMB5F3 hyb. SM

E. 1300 pM

F. 1300 pM

G. 4°C

H. 37°C

I. Cell viability (%) vs. antibody concentration (pM)

- DA3-PAR
- DA3-TM
- T47D
C. Colo357 pancreatic cancer

IC50 ~ 16pM

D. ZR75 breast cancer

IC50 ~ 3pM
Antibody targeting of cell-bound MUC1 SEA domain kills tumor cells
Edward Pichinuk, Itai Benhar, Oded Jacobi, et al.

Cancer Res  Published OnlineFirst April 16, 2012.

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