Development and in vitro validation of anti-mesothelin biobodies that prevent CA125/Mesothelin-dependent cell attachment

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Abstract

Preventing peritoneal implantation of carcinoma cells could prolong ovarian cancer patient remission and survival. Peritoneal cells constitutively express mesothelin, a ligand for CA125 that is expressed by tumor cells. Thus blocking CA125/mesothelin-dependent cell attachment may prevent or delay peritoneal metastatic recurrence. We developed a high-throughput screening system for reagents able to block CA125/mesothelin-dependent cell attachment with a sensitive quantitative readout. Using a novel yeast-expression system to produce secreted, in vivo biotinylated proteins and biobodies (Bbs), we generated anti-mesothelin Bbs. Anti-mesothelin Bbs derived from high affinity yeast-display scFv detected both membrane-bound and soluble mesothelins and inhibited CA125/mesothelin-dependent cell attachment.

Keywords: Mesothelin; CA125; Biobody; Tumor cell attachment; Ovarian cancer; Peritoneal metastasis

1. Introduction

Ovarian cancer represents one-fourth of the malignancies of the female genital tract and is the most common cause of death among women with gynecologic cancer. The American Cancer Society estimates that 22,430 women will be diagnosed with and 15,280 women will die of ovarian cancer in 2007 [1]. The 5-year relative survival rates correlate strongly with stage at diagnosis: 93% for early stage when the cancer is confined to the ovary, 69% for regional stage and 30% for advanced stage. However, only 19% of all ovarian cancers are found at an early stage [2]. Cytoreduction and chemotherapy induce remission in 80% of the cases, but recurrence can be expected in nearly all women with advanced disease at the time of diagnosis. A means to prevent or delay metastatic recurrence is needed.

The interaction between mesothelin and CA125 proteins could play an important role in the peritoneal implantation of ovarian tumor cells by promoting cell attachment between CA125-expressing
tumor cells and the peritoneal lining that constitu-

tively expresses a membrane-bound form of meso-
thelin [3–5]. Mesothelin is a 40 kDa protein expressed

at the surface of normal mesothelial cells and cancer
cells from diverse origins including ovary, pancreas

and mesothelium [6–8]. Soluble mesothelin is found

in ovarian cancer patients’ sera and ascites, and in

mesothelioma patients’ sera and pleural effusions

[9–13]. Soluble mesothelins arise through cleavage

of a hydrophobic glycosylphosphatidylinositol

(GPI) anchor at the C-terminal domain [6] (variants

1 and 2 encoded by MSLN 1 and 2, respectively), or

or less frequently, a reading frame shift that suppresses

1 and 2 encoded by MSLN 1 and 2, respectively), or

transformed into secreted scFv and Bbs, and vali-
dated by analyzing their binding to soluble and

membrane-bound forms of mesothelin by ELISA

and flow cytometry. We demonstrated that the

anti-mesothelin Bbs encoded by the consensus

sequence from the yeast-display scFv pool of high-
est affinity (P4) could recognize both membrane-

bound and soluble mesothelins, and inhibit

CA125/mesothelin-dependent cell adhesion.

2. Materials and methods

2.1. Antibodies, antigens and cell lines

Anti-flag mouse monoclonal antibody (mAb) was pur-

chased from Sigma–Aldrich (St. Louis, MO); anti-HIS

mAb was purchased from Roche (Alameda, CA); non-
labeled and horseradish peroxidase (HRP)-labeled anti-

V5 mAbs were purchased from Serotec (Raleigh, NC);

anti-c-myc mAb 9E10 was purchased from Research Diagnostics, Inc

(Fisher Biotech, Fair Lawn NJ) according to the manu-
facturer’s instructions. Anti-mesothelin mAbs (4H3 and

OV569) were obtained from In vit ogen (Carlsbad, CA); HRP-labeled

AffiniPure F(ab’2 fragment goat anti-mouse and anti-

human IgG (HRP anti-mlg and HRP anti-hlg, respec-

ively) were purchased from Serotec (Raleigh, NC).

Anti-mesothelin mAbs (4H3 and OV569) were obtained

from the Hellström Laboratory [10]; OV569 mAb was

biotinylated using the EZ-Link-sulfo-NHS LC biotin kit

(Fisher Biotech, Fair Lawn NJ) according to the manu-
facturer’s instructions. Anti-CA125 mAbs (X52 and

X306) were purchased from Research Diagnostics, Inc

(RDI, Flanders, NJ). Mesothelin recombinant protein

fused to an immunoglobulin tail (meso-Ig [5]) was pro-

duced in mammalian cells, OvCar-3 cells (ATCC, Manas-
sas, VA), HEK293F cells, wild type (WT) or transfected

sas, VA), HEK293F cells, wild type (WT) or transfected

with full-length mesothelin (MSLN-tf), and culture condi-
tions were previously described in [5].

2.2. Yeast-expression of soluble, in vivo biotinylated

recombinant proteins

Mesothelin cDNA was amplified from cDNA clone

MGC:10273 IMAGE:3957372 (ATCC) using the forward

5’-gattataaagatgcagtaaatggttgtggtccctcagc gaagtgg
gaagacagccgtg-3' and reverse 5'-gggttagggaatggcttacctgg tgcctaaatgcg agtgctaggtggacaac-3' primers to add the cloning sequences necessary for gap repair [34]. MUC16 repeat sequence was reverse-transcribed and amplified from OvCar3 mRNA using the forward 5'-gat tataaagctagcataaggggtgtttgtcctgagc ggtcacacagagc ctggc-3' and reverse 5'-gggttagggaatggcttacctgg tgcctaaatgcg agtgctaggtggacaac-3' primers.

PCR products were purified on a Qiaquick gel extraction column according to manufacturer instructions (Qiagen, Valencia, CA) and cotransformed with linearized pTOR2 vector [34] into the yeast strain YVH10 [35]. Yeast colonies prototrophic for uracil secreted mesothelin (meso) or MUC16 repeat domain (muc) recombinant proteins and were mates with yeast carrying pTOR-BIR. Resulting uracil/tryptophan-prototrophic diploid colonies secreted HIS-, V5- and flag-tagged, in vivo biotinylated mesothelin (b-meso) or MUC16 repeat domain (b-MUC16) recombinant proteins. The recombinant protein b-meso was isolated to isolate anti-mesothelin Bbs and b-MUC16 was used as control.

2.3. Generation of anti-mesothelin yeast-display scFv pool and conversion into yeast-secreted scFv

A yeast-display scFv library was first enriched for scFv that bound to b-meso by two rounds of magnetic enrichment and three rounds of fluorescent cell sortings [34,35]. The anti-mesothelin yeast-display scFv were then transformed into yeast-secreted scFv as previously described [34]. Briefly, scFv recognition sequences from a sorted yeast pool were PCR-amplified with the primers forward (5'-gattataaagatgacgataaaggtggtggttctgctagc ggtcacacagagc ctggc-3') and reverse (3'-gggttagggaatggcttacctgg tgcctaaatgcg agtgctaggtggacaac-3') for cloning by gap repair. The purified PCR products were then cotransformed with 100 ng of linearized pTOR2 vector into YVH10 cells. Resulting uracil-prototrophic yeast colonies were inoculated in growth medium in deep well 96-well plates (Fisher Scientific, Pittsburgh PA), grown until saturation and then induced to secrete scFv in presence of galactose for 72 h.

2.4. High throughput purification of scFv

Induced yeast cultures were pelleted by centrifugation. Eight-hundred microliters of yeast supernatants were transferred into clean deep well 96-well plates and equilibrated with 80 µl of equilibration buffer [3M Sodium chloride (Sigma–Aldrich) and 0.5 M Tris pH 9 (Sigma)]. HIS-Select-Nickel Affinity Gel (Sigma) (10 µl per well) was washed once with water and once with wash buffer [50 mM sodium phosphate pH 8 (Sigma), 0.3 M sodium chloride and 10 mM imidazole (Fisher)], resuspended in 5 ml of wash buffer per plate and 50 µl were distributed into each well. Plates were sealed with plastic covers (Varian Inc., Palo Alto, CA) and rotated on a Labquake® rotator (Barnstead Thermolyne Dubuque, IA) for 45 min at 4°C. Supernatants incubated with HIS-Select-Nickel Affinity Gel were transferred to pre-wet MultiScreen®-HV filter plates (Millipore Corporation, Billerica, MA) and drained with a vacuum manifold (Millipore) so that only nickel-bound scFv were retained by the filters. Nickel-bound scFv were washed twice with 100 µl of wash buffer per well on a plate shaker at 300 rpm for 10 min at room temperature (RT). ScFv were eluted from the HIS-Select-Nickel Affinity Gel by competition with imidazole. Fifty microliters of elution buffer (50 mM sodium phosphate pH 8, 0.3 M sodium chloride and 250 mM imidazole) were distributed in each well; a plate adaptor and a polypropylene 96-well round bottom plate (Fisher) were placed under the filter plate and wrapped together with the filter plate in foil. Plates were shaken at 300 rpm for 15 min at RT and the eluates were vacuumed into polypropylene plates. The elution process was repeated once and the eluates were stored at 4°C for up to one week before testing by immunoassays.

2.5. Sequencing

Fifty microliters of yeast from frozen stocks were inoculated in 4 ml of growth medium supplemented with tryptophan and grown to saturation at 30°C. DNA from yeast pellets was isolated [36] and amplified with the primers Gal1 forward (aatatacctctatactttaacgtc) and Cyc1 reverse (gggtagggataggcttaccctgt) and Cycl reverse (ggtgatgctaggacggtgagaac-3) primers by the Fred Hutchinson Cancer Research Center sequencing facility and analyzed using Vector NTI® software (Invitrogen).

2.6. Immunoassays

ELISA immunoassays were performed in Nunc Amino™ or Streptavidin Immobilizer™ plates (Nunc, Rochester, NY) with gentle agitation at RT. All washes and incubations were done in phosphate buffer saline (PBS) (Invitrogen) supplemented with 0.05% Tween (Fisher) (PBST) except for Nunc Amino™ plate coatings which were performed in bicarbonate buffer (carbonate–bicarbonate buffer capsules, Sigma–Aldrich). Colorimetric signals were generated with TMB One Solution (Promega, Madison, WI), stopped with 1 N H2SO4 (Acros Organics USA, Morris Plains, NJ) and read at 450 nm on a Spectra Max 250 (Molecular Devices, Sunnyvale, CA).

When used as capture reagents, 25 µl of high-throughput Ni-purified scFv or 5 µg/ml of Bbs were coated in a final volume of 50 µl of coating buffer on a Nunc Amino™ plate or of PBST on a Streptavidin Immobilizer™ plate, respectively, for 1 h at RT. When used as detection reagents, scFv (1 µg/ml) were preincubated with HRP-anti-V5 mAb (1 µg/ml) and Bbs (1 µg/ml) were
preincubated with poly-HRP80 streptavidin (SA-poly-HRP80) (Research Diagnostics, Inc, Concord, MA) diluted 1/1000 in PBS, for 30 min on ice. To detect the presence of recombinant proteins in yeast supernatants, double determinant “sandwich” ELISA assays were performed on Nunc Amino™ plates, using anti-flag mAb diluted to 2 µg/ml in bicarbonate buffer as the capture antibody and HRP-anti-V5 mAb diluted at 1/1000 in PBST as the detection antibody.

Cell immunoassays were performed on tissue culture-treated 96-well plates (BD Falcon, San Jose, CA). Plates were coated with 50 µl of 0.01% poly-L-lysine (Sigma) and incubated at 37 °C for 1 h. Excess liquid was then removed and the plates were air dried. After one wash with RPMI-1640 (Invitrogen), 200 µl of 2 x 10^5 OvCar-3 cells/ml were plated to obtain about 90% confluence and incubated at 37 °C overnight. The day of the experiment, adherent cells were gently washed one time with pre-warmed media and 50 µl of fresh media was added to each well without disturbing the cell monolayer. At the same time 10 µg/ml of Bbs were preincubated with 1/1000 SA-polyHRP80 for 30 min in PBS before being added to each well (50 µl/well, final concentration of 5 µg/ml of Bbs). After an incubation of 30 min with gentle agitation at RT, wells were washed three times with PBS supplemented with 0.5% of bovine serum albumin (BSA) (Sigma–Aldrich). Colorimetric signals were detected as described above.

2.7. Flow cytometry analysis

Five micrograms per milliliter of Bbs were premixed with 1.25 µg/ml of streptavidin-PE (SA-PE) (Becton Dickinson (BD) Pharmingen, San Diego, CA) in PBS for 15 min at RT [34]. MSLN-tf cells were lifted with Versene (Invitrogen) and washed twice with PBS supplemented with 1% BSA (PBS–BSA). Cells were pelleted and 10^6 cells were resuspended with 100 µl of SA–PE/ Bbs complexes. After 30 min of incubation on ice, labeled cells were washed twice in cold PBS–BSA, resuspended in PBS–BSA, and analyzed on a BD FACScan Cytometer. As a positive control for mesothelin expression, cells were incubated with the anti-mesothelin mAb 4H3 at 5 µg/ml followed by PE-labeled anti-mlg mAb diluted one hundred fold.

2.8. Cell adhesion assays

Cell adhesion assays were performed as previously described [5]. Briefly, WT or MSLN-tf cells were labeled with Calcein AM (5 µM) from the Vybrant® Cell Adhesion Assay Kit (Invitrogen-GIBCO) for 30 min at 37 °C. During blocking assays, labeled cells were preincubated for 30 min with 10 µg/ml anti-mesothelin mAb 4H3 or anti-CA125 mAb X52, and for various incubation times (5, 10, 15, 30 and 60 min) with 10 µg/ml of scFv or of Bb premixed with 2.5 µg/ml of streptavidin (Molecular Probes Inc, Eugene OR) in DMEM (Invitrogen) for 15 min at RT. Antibody-coated cells were then washed and resuspended in DMEM and incubated in quadruplicate with adherent OvCar3 cells for various periods of time (5, 10, 15, 30 and 60 min) at 37 °C. Cell fluorescence was measured before washing and after every two washes with FLx800™ Multi-Detection Microplate Reader (BioTek Instruments, Inc. Winooski, VT). Normalized values (NV) after four washes were calculated as ratios of fluorescence percentages before (FBW) and after wash (FAW) between MSLN-tf cells incubated with mAb, scFv or Bb (sample) versus cells incubated in medium only (medium), thus NV = (FAW/FBW_sample)/(FAW/ FBW_medium).

3. Results

3.1. Yeast diploids secrete in vivo biotinylated mesothelin and CA125 repeat domain recombinant proteins

In order to isolate anti-mesothelin yeast-display scFv, biotinylated mesothelin and control proteins were needed. We produced b-meso and b-MUC16 recombinant proteins with the yeast expression system which was also used for Bb production. B-meso was used to select yeast-display scFv that bound to mesothelin while b-MUC16 was used to eliminate the non-specific and/or cross-reactive scFv. The presence of recombinant proteins in supernatants was demonstrated by sandwich ELISAs. Supernatants from eight yeast colonies cotransformed with mesothelin cDNA and 16 yeast colonies cotransformed with MUC16 repeat sequence were incubated in plastic wells coated with anti-flag mAb, and captured recombinant proteins were detected with HRP anti-V5 mAb. Seven out of eight yeast colonies cotransformed with mesothelin cDNA and nine out of 16 cotransformed with MUC16 repeat sequence secreted tagged proteins (Fig. 1a and data not shown). Recombinant proteins secreted by meso #4, #7, #8 and muc #4, #9, #14 cotransformed yeast (Fig. 1a) were Ni-purified from yeast supernatants and immobilized on Nunc Amino™ plates to assess their specificity. Fig. 1b shows that all Ni-purified proteins were detected with anti-tag mAbs, that proteins secreted by mesothelin cotransformed yeast were detected with anti-mesothelin mAb 4H3 and that proteins secreted by MUC16 repeat sequence cotransformed yeast were detected with anti-CA125 mAb X52. The same validation procedures were repeated on the purified in vivo biotinylated proteins secreted by diploid yeast. Results shown in Fig. 1c demonstrate that diploid-secreted proteins were in vivo biotinylated as they bound to streptavidin-coated plastic, and tagged with V5 and HIS since they were detected with anti-tags antibodies. Similar to their non-biotinylated counterparts, in vivo biotinylated mesothelin and
MUC16 repeat proteins were specifically detected by anti-mesothelin mAb 4H3 or anti-CA125 mAb X52. In addition, b-meso proteins were also weakly detected by anti-mesothelin mAb OV569 and b-MUC16 proteins by anti-CA125 mAb X306. Altogether, this suggests that diploid yeast were able to secrete in vivo biotinylated recombinant proteins with posttranslational modifications comparable to native proteins. Because b-meso-7
and b-MUC16-9 were best detected by both specific mAbs (Fig. 1c), they were used for all subsequent experiments.

3.2. Generation of anti-mesothelin scFv and Bbs

A yeast-display scFv library was enriched for scFv that bind to b-meso-7 by two magnetic and three fluorescent cell sortings, and antigen-binding of each yeast-display scFv pool was analyzed after each sorting by flow cytometry (Fig. 2). Antigen binding to b-meso-7 (Y axis) was correlated with scFv expression as measured by anti-c-myc mAb (X axis). Of note, scFv are not expressed by all yeast due to the particular growth cycle of Saccharomyces cerevisiae. S. cerevisiae grow by budding and all newly synthesized proteins are transported from mother yeast to buds, thus the buds are the only yeast able to display scFv on their surface. During galactose induction, yeast divide 2 or 3 times and mother yeast that do not express scFv constitute an unavoidable fraction of the induced population. Consequently, about half of the yeast displays scFv (Fig. 2b). Yet, the percentage of yeast-display scFv that specifically bound to b-meso-7 increased after each fluorescent cell sorting, from less than 0.05% in the initial library (Fig. 2c) to 0.16% after two magnetic sortings (Fig. 2d) and >90% after three fluorescent cell sortings (Fig. 2f). During the 3rd fluorescent cell sorting, three distinct populations of yeast-display scFv (P2, P3 and P4) were gated and sorted separately as shown in Fig. 2f. P2, P3 and P4 Y-means were 157, 222 and 420, respectively (data not shown), corresponding to three distinct populations of low, medium and high affinity [37]. Yeast-display scFv from each of the P2, P3 and P4 pools were then converted into yeast-secreted scFv and screened for specific binding to mesothelin by capture ELISA assays.

ScFv in supernatants from 282 secreting yeast colonies (94 from each of the P2, P3 and P4 populations) were high-throughput Ni-purified and immobilized on Nunc Amino™ plates. ScFv binding specifically to b-meso-7 was contrasted with non-specific binding to b-MUC16-9 and results were expressed as a ratio between the optical densities at 450 nm (OD) of b-meso-7 binding versus b-MUC16-9 binding (Fig. 3a). All the scFv derived from the P2 and P4 groups and most of the scFv derived from the P3 group (84 out of 94 clones) bound to b-meso-7. However, a third of P4 scFv bound specifically to b-meso-7 (ratio >2) and 5 P4 scFv bound specifically and strongly to b-meso-7 (ratio>5), while only a sixth of P2 scFv and one P3 scFv specifically bound to b-meso-7. Twenty yeast-secreted scFv were sequenced, eight from P2 group, one from P3 group and 11 from P4 group. Four scFv in P2 group and eight in P4 group shared identical sequences and were referred as P2 or P4 consensus sequences in the rest of the study. P2 sequence was 64% homologous to P4 sequence (Fig. 3b); P3 sequence was truncated and did not include the linker. Within a same group scFv sequences differed from each other only by

![Fig. 2. Enrichment for yeast-display scFv that bind to in vivo biotinylated mesothelin recombinant protein secreted by yeast. A yeast-display scFv library was progressively enriched for scFv that bind to b-meso-7. (a–b) Controls representative of all experiments are depicted in this figure; (a) as a negative control, yeast were labeled only with PE and FITC-labeled secondary reagents; (b) to control for the scFv-expression, yeast were labeled with anti-c-myc mAb followed by secondary reagents. (c) The initial yeast-display scFv library was labeled with anti-c-myc mAb and biotinylated mesothelin antigen followed by secondary reagents. (d–f) Yeast were labeled with anti-c-myc mAb and biotinylated mesothelin antigen followed by secondary reagents after two magnetic sortings followed by one (d), two (e) or three (f) fluorescent cell sortings; during the last sorting, yeast were gated in regions P2, P3 and P4 as shown and were sorted separately.](image-url)
point mutations, as follows: P4a had a substitution (G to A) in 324 with change from Ser to Gly in the linker; P4b: (T to C) in 471 without AA change; P4c: (A to G) in 519 without AA change; P2a: (A to G) in 137 with change from Asp to Ser in vH; P2b: (T to C) in 417 with change from Ala to Val in vL; P2c: (A to G) in 499 with change from Ala to Ser in vH.

Fig. 3. Characterization of yeast-secreted scFv derived from 3 yeast-display scFv pools enriched for mesothelin-binders. (a) Functional screening of scFv by Capture ELISA. High-throughput Ni-purified scFv were immobilized in two 96-well Nunc Amino™ plates. One plate was incubated with b-meso-7 and the other one with b-MUC16-9. Captured proteins were then detected with SA-HRP followed by TMB. ODs were measured at 450 nm and plotted as a ratio as indicated. (b) Comparison between P2 and P4 scFv consensus sequences.

Fig. 4. Characterization of anti-mesothelin Bbs affinity binding to soluble recombinant mesothelin protein. (a) Capture ELISA: Bbs were immobilized in duplicate in a 96-well Streptavidin Immobilizer™ plate and incubated with 100, 10 or 0 ng/ml of meso-Ig protein. Captured meso-Ig proteins were detected with HRP-anti-hIg followed by TMB. (b–c) Detection ELISA: serial dilutions of meso-Ig protein were immobilized on amino™ plate and detected with duplicates of (b) scFv preincubated with HRP-labeled anti-V5 mAb or (c) biobodies preincubated with SA-polyHRP80. Each experiment included four P2 Bbs and eight P4 Bbs.
from Thr to Ala in vL; P2d: (A to G) in 212 with change from Ser to Gly in vH. All 20 sequenced clones were mated with yeast carrying pTOR-BIR to generate Bbs.

3.3. Validation of anti-mesothelin Bbs

Bbs were Ni-purified and validated for their specificity to mesothelin by immunoassays and flow cytometry analysis. Capture ELISA showed that all Bbs but two (P3 and P2b) bound to meso-Ig, but the limit of detection of P4 Bbs appeared lower than that of P2 Bbs (Fig. 4a). By detection ELISA none of the P2 scFv or Bbs bound to meso-Ig (Figs. 4b and c) while P4 Bbs lower detection limits were in the ng/ml range (Fig. 4c). Consistent with previous findings [34], P4 Bb affinities were 5- to 10-fold greater than the corresponding P4 scFv (compare Fig. 4b–c). Of note, the affinity of Bb P4a (mutated in the linker) appeared 4-fold lower than the other P4 Bbs (Fig. 4c). Finally, all P4 Bbs and P2c Bb recognized constitutively expressed membrane-bound mesothelin as measured by cell ELISA (Fig. 5a) and bound to MSLN-tf cells by flow cytometry analysis (Fig. 5b).

Altogether, these results demonstrate that yeast co-transformation of pools of yeast-display scFv recognition sequences with pTOR2 followed by high-throughput screening by immunoassays is an efficient platform to time- and cost-effectively generate antigen-specific recombinant antibodies. These results also underline a positive association between high affinity mesothelin-specific yeast-display scFv (P4) and Bb affinity for native and

Fig. 5. P4 Bbs bind to membrane-bound recombinant mesothelin. (a) Cell ELISA: OvCar3 cells immobilized on poly-L-lysine coated wells were detected with duplicates of Bbs (P2 in gray, P3 with horizontal stripes, P4 in black) premixed with SA-polyHRP80 followed by TMB. ODs generated by the binding of each Bb to mesothelin-expressing cells were plotted as shown. (b) Flow cytometry analysis. MSLN-tf cells were incubated with Bbs premixed with SA–PE and mean fluorescence generated by the binding of each Bb to mesothelin-expressing cells were plotted as shown. As a positive control, cells were incubated with 4H3 mAb followed by PE anti-mIg mAb (vertical stripes); as a negative control, cells were incubated with SA–PE only (white). P2 Bbs are shown in gray, P3 with horizontal stripes, and P4 in black. Results are representative of two independent experiments; each experiment included four P2 Bbs and eight P4 Bbs.
recombinant mesothelins (Figs. 4 and 5). Finally, some protein substitutions had a functional effect regarding Bb antigen binding properties. In particular, substitution of Thr by Ala in P2c vL increased Bb affinity, while substitutions of Ala by Val in P2b vL and of Ser by Gly in P4a linker decreased it. Bbs that failed validation tests (P2b and P3) were not used for the rest of the study.

3.4. Cell adhesion assay

Anti-mesothelin yeast-secreted scFv and Bbs were tested for their ability to block CA125/mesothelin-dependent cell attachment. CA125/mesothelin-dependent cell attachment was observed only when incubation times were of at least 30 min, and blocking of cell attachment was significant only when fluorescently labeled MSLN-tf were preincubated for at least 30 min with antibodies (data not shown). Yeast-secreted scFv or Bb/streptavidin complexes were incubated with fluorescently labeled MSLN-tf cells or with WT cells as controls. As previously described [5], anti-CA125 mAb X52 blocked CA125/mesothelin-dependent cell attachment but anti-mesothelin mAb 4H3 did not (Fig. 6). Fig. 6 shows that complexing Bbs with streptavidin modified their blocking abilities as compared to scFv, as it consistently decreased CA125/mesothelin-dependent cell attachment in the presence of P4 Bbs. These results suggest that P4 Bbs may be able to decrease CA125/mesothelin-dependent cell attachment in vivo and thus could reduce the peritoneal implantation of tumor cells.

4. Discussion

Mesothelin is a non-essential protein of the peritoneal lining [38] that is constitutively expressed at the mesothelial cell surface and that binds to CA125, an ovarian cancer biomarker. In contrast with other cancers, peritoneal metastases are often present when epithelial ovarian cancer is first diagnosed. Combining early detection through a panel of serum biomarkers with therapies to prevent metastatic recurrence may reduce ovarian cancer’s toll. Such therapies could include blocking CA125/mesothelin-dependent cell attachment.

We previously developed an in vitro cell adhesion assay to high-throughput screen for reagents able to block CA125/mesothelin-dependent cell attachment with a sensitive quantitative readout. We also developed a new system of protein expression in yeast for the secretion of tagged and in vivo biotinylated...
recombinant proteins and antibodies. Using yeast-secreted mesothelin recombinant proteins we isolated anti-mesothelin yeast-display scFv and we transformed them into soluble scFv. Two main groups of recognition sequences (P2 and P4) were identified by screening the soluble scFv by capture ELISA, and were further transformed into anti-mesothelin Bbs P2 and P4 by yeast mating. Anti-mesothelin Bb P4 could consistently inhibit CA125/mesothelin-dependent cell attachment, but a single mutation in the linker between vH and vL (Bb P4a) was sufficient to abolish Bb P4 activity.

The affinity of Bb P4 for mesothelin was higher than that of P2 as measured by detection ELISA, cell ELISA and flow cytometry analysis. Yet, by flow cytometry the fluorescent signals generated by Bb P4 were lower than the signal of the anti-mesothelin mAb 4H3, which might suggest a lower affinity of Bb P4 compared with 4H3 mAb. However, as demonstrated by ELISA, Bb P4 limits of detection were in the range of ng/ml, which is comparable to that of 4H3 mAb (data not shown) and thus hints at another possible explanation for this seeming discrepancy in affinity. The fact that biobodies form tetramers when combined with streptavidin [34] suggests that four biobodies are detected by only one fluorescent molecule, while each mAb is detected by a labeled mAb bearing several fluorescent molecules (one per lysine). Thus, with the secondary reagents used in this study, Bbs would generate lower fluorescent signals than mAbs even with similar antigen affinities.

Not all of the scFv that bound to mesothelin were able to block the CA125/mesothelin interaction. Anti-mesothelin P2 scFv and Bbs could not inhibit cell attachment, and unlike P4 Bbs, complexing P2 Bbs with streptavidin actually increased cell attachment. This might be due to a fast dissociation rate allowing some of the tetramerized P2 Bbs to bind to OvCar3-bound mesothelin while the other tetramer partners remained bound to MSLN-tf cells, thus creating additional links between cells. Likewise 4H3, a high affinity mAb which was derived against a native form of soluble mesothelin and which recognizes all three mesothelin variants as well as meso-Ig [5,10,39], could not block CA125/mesothelin-dependent cell attachment. These results suggest that 4H3 mAb and P4 Bbs are directed against different epitopes.

Sequencing showed that same-group scFvs were highly similar, suggesting that anti-mesothelin yeast-display scFvs were derived from only a few recognition sequences that were point-mutated. Screening for recognition sequences able to specifically bind to an antigen reduces sequence diversity but cannot explain the frequency of point mutations that we observed. Point mutations might have arisen during the construction of the yeast-display scFv library for which a cDNA reamplification step was mandatory to link heavy to light chains, or during the recognition sequence amplification for cotransformation with pTOR2 into yeast-secreted scFv, despite the use of a high fidelity polymerase.

Yeast-secreted mesothelin was detected by 4H3 mAb but was not detected by OV569 mAb, a mAb derived against ovarian cancer cells [10] that binds to native mesothelins but not to meso-Ig (data not shown). However, OV569 mAb bound weakly but significantly to in vivo biotinylated mesothelin, which implies that OV569 binding epitope became available after in vivo biotinylation. Altogether, this suggests that OV569 mAb binds to a conformational epitope that appears after mesothelin modifications in C-terminus such as in vivo biotinylation or GPI-linkage. It also suggests that antibodies specific for membrane-bound or soluble forms of mesothelin may be generated against conformational changes triggered by the GPI-anchor before and after cleavage [40]. Membrane-bound-specific anti-mesothelin affinity reagents would be particularly useful for targeted therapies and in vivo imaging.

In conclusion, our results confirm the notion that therapeutic agents such as recombinant antibodies against mesothelin may help prevent peritoneal implantation of ovarian carcinoma cells and thus recurrent metastatic disease. Such therapeutic agents would need to display not only fast association and slow dissociation rates with a membrane-bound form of mesothelin, but also must specifically bind to the mesothelin epitope(s) involved with CA125 binding. In addition, agents' in vivo stability would be essential, thus in vivo testing in an animal model of ovarian cancer peritoneal implantation such as described by Strobel and colleagues [41] will be necessary to determine whether Bbs could prevent or delay cancer cell attachment to the peritoneal lining. It is also conceivable that the presence of anti-mesothelin autoantibodies [42] might significantly modify the evolution of metastatic peritoneal implantation, and in order to address these questions it would be necessary to develop a model of peritoneal implantation in immuno-competent animals.
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