Development of a Hyperimmune Anti-MUC-1 Single Chain Antibody Fragments Phage Display Library for Targeting Breast Cancer

Michelle D. Winthrop, Sally J. DeNardo, Gerald L. DeNardo

Section of Radiodiagnosis and Therapy, University of California at Davis, School of Medicine, Sacramento, California 95816

Abstract

Radioimmunotherapy (RIT) has demonstrated potential for improving clinical cancer therapy. Optimizing the approach has proven difficult thus far. Antibody phage display libraries provide unique molecules that could improve RIT. A phage display library of single chain antibody fragments (scFv) against the MUC-1 mucin molecule, which is expressed on 90% of human breast cancers, was produced from the spleen cells of MUC-1 hyperimmunized BALB/c mice. Increased serum IgG levels, 15 times baseline, were detected following the third immunization. RNA from the spleen cells was isolated, cDNA was made, and variable heavy and variable light immunoglobulin chain gene regions were amplified using PCR technology. The variable heavy and variable light chain gene regions were combined with a flexible linker, ligated into the pCANTAB 5E phagemid vector, and electrooporated into TG1 Escherichia coli cells. A library of 10^7 initial colonies was compiled. Forty-six of 288 colonies screened for reactivity demonstrated binding to MUC-1-expressing MCF-7 breast cancer cell membrane fragments. Anti-MUC-1 library diversity evaluated by BstNI digest demonstrated that 52% of the anti-MUC-1 scFv binding MCF-7 possessed individual banding patterns representative of approximately 5 x 10^6 colonies likely able to recognize distinct epitopes present on MUC-1 positive human breast cancers. In summary, the anti-MUC-1 scFv antibody phage library contains diverse scFv molecules, which should provide unique characteristics and epitope recognition. These molecules will be used in the development of pretargeting RIT strategies designed to improve the clinical outcome of patients with breast cancer.

Introduction

RIT has shown efficacy in the treatment of advanced metastatic human breast cancer (1-3). However, the advantageous characteristics of tumor specificity attributed to the use of intact MoAbs is often diminished due to the increased radiation dose delivered to normal tissues during the extended time that the large antibody molecule (150 kDa) remains in circulation (4, 5). In addition, mouse or human-mouse chimeric MoAbs have frequently produced human antiglobulin response, which usually prevents subsequent RIT (6-10).

To improve RIT, various versions of genetically engineered multivalent antibodies, including diabodies (50 kDa) and minibodies (65 kDa), have been investigated (9, 10). Radiolabeled, these small molecules have the potential to retain the most useful characteristic of MoAbs, specific tumor targeting, while decreasing the radiation dose delivered to normal tissue during blood clearance. Their smaller size also leads to better tumor penetration and less immunogenicity (11-13).

scFv fragments, which are linked V\textsubscript{H} and V\textsubscript{L} chain regions of an antibody, can be connected to construct new molecules for RIT therapy. Although scFv can be produced from the numerous MoAbs that have previously been generated by hybridoma technology, antibody phage display library technology, using a diverse pool of antibody genes, can exponentially expand that repertoire while at the same time allowing for more efficient screening and retrieval (14-16). The filamentous phage, surface-displayed scFv can be simultaneously selected along with the scFv gene, making this process uniquely efficient (14, 17-19). Whereas only 10^2-10^3 colonies are usually examined from multiple fusion in hybridoma technology, evaluation of colonies chosen from antibody phage display technology is limited only by the selection process and the size of the library (10^7-10^12 colonies; Refs. 16, 19, and 20).

Anti-MUC-1 scFv engineered against the MUC-1 mucin molecule abundantly expressed on breast cancer, as well as other epithelial cancers (prostate, lung, and ovary) can be developed for use in RIT strategies. Although the MUC-1 protein is expressed by both normal and cancer cells, modifications in the degree of glycosylation by cancer cells produce atypical sugar structures and expose normally sequestered epitopes on the polypeptide core. Overexpression of deglycosylated MUC-1 by epithelial cancers has prompted clinical studies to evaluate anti-MUC-1 MoAbs for tumor detection, staging, and therapy (21-26).

Antibody phage display technology produces an array of anti-MUC-1 scFv with a wide range of epitope recognition sites and potentially increased tumor affinity, providing the means to explore various options for engineered tumor-targeted therapy. This technology creates opportunities for selecting and genetically modified antibody fragments; RPAS, recombinant phage antibody system; V\textsubscript{H}, variable heavy; V\textsubscript{L}, variable light.

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2 To whom requests for reprints should be addressed, at Molecular Cancer Institute, 1508 Alhambra Boulevard, Suite 3100, Sacramento, CA 95816.
3 The abbreviations used are: RIT, radioimmunotherapy; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; MoAb, monoclonal antibody; scFv, single chain antibody fragments; RPAS, recombinant phage antibody system; V\textsubscript{H}, variable heavy; V\textsubscript{L}, variable light.
cally engineering scFv, diabodies, and multivalent antibody fragments with characteristics not previously available, including improved affinity and specificity (11, 27–29). Here, we describe the construction and initial characterization of an anti-MUC-1 scFv antibody phage display library produced from hyperimmunized mice.

Materials and Methods  
**MCF-7 and HBT 3477 Cell Lines.** MUC-1 antigen positive human breast adenocarcinoma cell lines MCF-7 (American Type Culture Collection, Manassas, VA) and HBT 3477 (OncoGene, Seattle WA; Ref. 30) were grown to confluence, counted, and processed separately for ELISAs or combined in a 10:1 ratio of MCF-7 to HBT 3477 cells for immunization. Membrane fragments were isolated based upon a previously described protocol (31). Protein concentration of the cell membrane preparation was determined using the Micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL).

**MUC-1 Synthetic Peptide.** The MUC-1 synthetic peptide (80-mer) used to immunize the mice and to perform selection from the phage library was obtained from Olivera Finn (University of Pittsburgh, PA; Ref. 24).

**MUC-1 Conjugation.** Conjugation of MUC-1 synthetic peptide to either KLH or biotinylated BSA was carried out using the Immunogen 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride conjugation kit (Pierce).

**Mouse Immunizations.** BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were maintained according to University of California animal care guidelines on a normal diet ad libitum and under specific pathogen-free conditions. Five mice (6–8 weeks of age) weighing 19–21 g received an initial i.p. injection of MCF-7/HBT 3477 (10:1) cell membrane lysate (31), followed by three immunizations of KLH-MUC-1 at 3-week intervals. Ten days after each immunization, serum was obtained, and total serum IgG reactive against the MUC-1 positive MCF-7 cell line was determined by ELISA (24).

**RNA and mRNA.** The mice were sacrificed 4 days after the final immunization. Under sterile conditions, spleens were removed, and RNA was extracted using the RNAspin kit (Promega Corp., Madison, WI). A mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used to purify the mRNA.

**Anti-MUC-1 scFv.** cDNA synthesis, VgH and VgL gene amplification, scFv assembly, and ligation into the pCANTAB 5E vector were carried out using RPAS mouse ScFv module (Amersham Pharmacia Biotech). Briefly, VgH and VgL genes were amplified by PCR, isolated, and purified from an agarose gel using a GFX DNA and gel band purification kit (Amersham Pharmacia Biotech; Ref. 32). The VgH and VgL chain-encoding regions were linked together using primers that introduce specific restriction enzyme sites at the 5' (SfiI) and 3' (NotI) ends, respectively. Restriction digestion with the SfiI and NotI endonucleases and agarose gel purification preceded ligation of the anti-MUC-1 scFv into the SfiI- and NotI-digested pCANTAB 5E phagemid vector (33, 34).

**Electroporation.** The pCANTAB 5E vector containing the anti-MUC-1 scFv fragments was electroporated into competent TG1 *Escherichia coli* cells using the Bio-Rad gene pulser II (Bio-Rad Laboratories, Hercules, CA; Ref. 14). Fifty µl of competent TG1 cells were placed into each prechilled 0.2-cm electroporation cuvette (Bio-Rad), and 2 µl of salt-free pCANTAB 5E vector containing anti-MUC-1 scFv were added. The Bio-Rad gene pulser II was set at 25 µF, 2.5 kV at 200 ohms to yield a pulse with a time constant of 4.5–5 ms (35). The samples were transferred to 6 ml of 2× YT (17 g of Bacto-tryptone, 10 g of Bacto-yeast extract, and 5 g of NaCl per liter) containing 2% glucose and incubated at 37°C for 30 min. Serial dilutions were plated on SOBAG plates (0.4 g of Bacto-tryptone, 0.1 g of Bacto-yeast extract, 0.01 g of NaCl, 55.6 ml of 2× m glucose, 5 ml of 20 mg/ml ampicillin, and 15 g of Bacto-agar per liter) and grown overnight at 30°C to determine initial colony number. Colony counts from the serial dilutions were determined, and all clones were then harvested, transferred to 2× YT media containing 30% glycerol, and stored at −70°C. Electroporations were carried out until the anti-MUC-1 antibody phage display library initial colony number exceeded 10^7.

**Anti-MUC-1 Phage Rescue.** Phage aliquots were produced prior to initial selection using the RPAS Expression Module (Amersham Pharmacia Biotech). Phage were concentrated by precipitation with polyethylene glycol (20% polyethylene glycol 8000, 2.5 µl NaCl) followed by centrifugation. The phage were resuspended in TE (10 mM Tris, pH 8.0, and 0.1 mM EDTA), filtered (0.45 µm), and stored at 4°C.

**Affinity Selection.** Phage were subjected to three rounds of affinity selection in a 3% nonfat milk PBS solution containing 0.2% Tween 20 with decreasing amounts (100, 50, and 10 mM) of MUC-1 conjugated to biotinylated BSA and magnetic streptavidin beads (Dynal, Inc. Lake Success, NY). The beads were washed three times with 0.1% Tween in PBS, resuspended in PBS, and mixed with MUC-1 conjugated to biotinylated BSA. The bound MUC-1-reactive phage were dissociated from...
the beads using 100 mM triethylamine followed by the addition of 1 M Tris buffer, pH 7.4. The phage were allowed to reinfect TG1 cells and the anti-MUC-1 clones were grown at 37°C overnight on SOBAG plates. A 96-well "master plate" was made from isolated clones, and an aliquot was taken for the next round of selection. The remaining clones were frozen in glycerol.

**ELISA for Detection of MUC-1, KLH, and HSA Reactivity.** Using a replica of the master plate, anti-MUC-1 scFv was induced by the removal of glucose and the addition of isopropyl-β-D-thiogalactopyranoside (34). Reactivity of the scFv against MCF-7 cell lysate, KLH, or HSA was determined by ELISA (24). Positive reactivity with the MCF-7 cell lysate was defined as greater than 2.5 over background, which corresponded to an absorbance of greater than 3.0 at A490 nm.

**Analysis of MUC-1 scFv Diversity.** The anti-MUC-1 scFv DNA was amplified from the pCANTAB 5E vector by PCR using the protocol and reagents contained within the RPAS detection module (Amersham Pharmacia Biotech). The PCR-amplified scFv DNA was digested with the BstN1 restriction enzyme to indicate clonal diversity of the affinity selected anti-MUC-1 phage. Briefly, 30 µL of PCR product were digested for 2 h at 60°C with 5 units of BstN1 in NEBuffer2 [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT (pH 7.9 at 25°C); New England Biolabs, Beverley, MA] containing 1% BSA.

**Results**

**Anti-MUC-1 Serum Levels.** Mice were immunized with MCF-7 cell membrane initially followed by MUC-1 conjugated to KLH. Following the third immunization, a substantial increase in serum levels of IgG binding MCF-7 cell membrane fragments was detected in all of the mice (n = 5; Fig. 1). The average preimmune serum level was 0.111 µg/ml. Following the third immunization with KLH-conjugated MUC-1, the average IgG serum level was 1.704 µg/ml, an increase of 15 times over the baseline level.

**Anti-MUC-1 scFv Gene Assembly and Expression.** VH and VL immunoglobulin chain fragments were identified following amplification using PCR. A 340-bp band corresponding to the VH and a 325-bp band corresponding to the VL immunoglobulin chain fragment were visualized with ethidium bromide using 1% agarose gel electrophoresis.

**Affinity selection of the library with MUC-1 magnetic streptavidin beads**

- 50 nM (Rd 2) 96 clones
- 10 nM (Rd 3) 192 clones

**Phage ELISA**

- (Rd 2) 96 clones
- (Rd 3) 192 clones

**"Master Plates" 288 clones**

**Positive scFv ELISA for anti-MUC-1 (MCF-7 cell membrane)**

- 46 reactive clones identified
- 18 "most" reactive clones identified
- 1 cross-reactive with KLH
- 0 cross-reactive with HSA

**Anti-MUC-1 BstN1 DNA digest**

- 46 clones → "18 patterns"
- 18 clones → "9 patterns"

Fig. 3 Schematic for selection and evaluation of anti-MUC-1 scFv library.
Affinity Selection. The first round affinity selection of anti-MUC-1 phage against 100 nM of MUC-1-conjugated biotinylated BSA yielded $3.2 \times 10^8$ MUC-1-reactive anti-MUC-1 scFv. Second round selection of anti-MUC-1 phage with 50 nM of MUC-1-conjugated biotinylated BSA resulted in the isolation of $1.1 \times 10^8$ clones, whereas third round selection with 10 nM of MUC-1-conjugated biotinylated BSA resulted in $5.0 \times 10^4$ MUC-1-reactive clones.

Anti-MUC-1 Reactivity. Reactivity of the anti-MUC-1 scFv was determined by ELISA against the MCF-7 cell membrane fragments, KLH, and HSA. Based upon initial ELISA screening, 46 of 288 clones reactive against the MCF-7 cell membrane fragments from the second and third round of anti-MUC-1 affinity selection were chosen for further evaluation. Of the 46 clones, 39% (18 of 46) bound MCF-7 cell membrane fragments at least 5 times greater than KLH binding and 10 times greater than HSA binding (Figs. 3 and 4). Only one of the 18 selected clones (D11) cross-reacted with KLH, whereas none of the 18 clones cross-reacted with HSA.

BstNI Fingerprinting. BstNI fingerprinting of the 46 clones reactive against the MCF-7 cell membrane fragments identified 52% of the clones possessing their own fingerprint.

bromide under UV light following agarose gel electrophoresis (Fig. 2). The $V_h$ and $V_l$ bands were isolated and combined in equal amounts with the (Gly$_3$Ser)$_3$ flexible linker to produce anti-MUC-1 scFv. Following agarose gel electrophoresis, a 750-bp band corresponding to the correct size of anti-MUC-1 scFv gene was visualized (Fig. 2).

**Fig. 4** ELISA analysis of chosen anti-MUC-1 scFv clones. A, MUC-1 scFv from the second round of affinity selection using 50 nM of MUC-1 conjugated to biotinylated BSA. B, MUC-1 scFv from the third round of affinity selection using 10 nM of MUC-1 conjugated to biotinylated BSA. Forty-six clones demonstrated reactivity with the MUC-1 antigen positive MCF-7 cell membrane fragments. Eighteen of these clones demonstrated reactivity with the MCF-7 cell membrane fragments (2.5 times background): 1 of 18 (D11) was cross-reactive with KLH, and 0 of 18 were cross-reactive with HSA.
pattern, suggesting at least 24 different DNA sequences (Fig. 5). The 18 clones that reacted most strongly with the MCF-7 cell membrane fragments (at least 2.5 times background) also represent nine unique BstNI patterns.

Discussion
Breast cancer causes the death of over 44,000 women in the United States yearly (36). Therefore, the development of effective new therapies for breast cancer are needed. Although RIT has shown some efficacy for human breast cancer, the promise of this therapeutic approach has remained unfulfilled because tumor targeting and normal tissue clearance have not been optimized (11). One approach to improving the therapeutic index is pretargeting. In pretargeting, the labeled MoAb is allowed to localize to the tumor prior to administration of the radiolabeled hapten. One method of pretargeting, capitalizing on the high avidity of avidin for biotin, demonstrated 2-3-fold increased tumor to blood ratios compared to use of radiolabeled MoAbs, and an added clearing agent further increased the tumor to blood ratio, allowing greater administered doses (37-46). However, this pretargeting approach may be limited by the immunogenicity of the streptavidin (37, 38). Pretargeting with genetically engineered bispecific constructs can effectively reduce the amount of radiation delivered to normal tissue without induction of an immune response, and the smaller size of engineered antibody constructs (60-70 kDa) should allow more effective diffusion of these constructs through the interstitial space, thus increasing radioactivity delivered to tumor (11, 37, 38).

The anti-MUC-1 scFv library is a foundation for the genetic engineering of bispecific antibody fragments for pretargeting RIT strategies. Novel MUC-1 epitopes unmasked by deglycosylation on malignant epithelial tissues are available for specific tumor targeting (21). Anti-MUC-1 MoAbs recognize epitopes within a highly immunogenic 20-amino acid tandem repeat of the polypeptide core. Although these MoAbs recognize overlapping epitopes within the same region, the epitopes recognized are different from each other, possibly due to differences in the conformation of native MUC-1 antigens. These differences will likely result in anti-MUC-1 scFv to a number of different epitopes expressed on epithelial cancers.

The library consists of MUC-1 clones from the individual phage that bound to the MCF-7 cell membrane fragments. From the library, 288 clones were randomly selected from an aliquot of the pooled library. Initial characterization of the anti-MUC-1 library showed that 46 of the 288 randomly selected anti-MUC-1 clones reacted with MCF-7 membrane fragments, and 18 consistently reacted at least 2.5 times greater than controls. None of the 18 MCF-7-reactive clones were cross-reactive with HSA, and only one was cross-reactive with KLH. The diversity of the anti-MUC-1 library evaluated by BstNI fingerprint enzyme DNA digest demonstrated that 52% of the 46 breast cancer binding clones possessed discrete “fingerprints” representative of unique gene sequences (Fig. 5). Therefore, the library contains at least $5.0 \times 10^6$ genetically different anti-MUC-1 scFv clones that likely recognize unique epitopes.

In summary, recent advances in antibody engineering, along with emerging information on the size, orientation, and flexibility of the targeting molecules, can be used to develop clinically relevant anti-MUC-1 bispecific antibody constructs to achieve optimal tumor localization and penetration with decreased accumulation of radioactivity in normal tissue (5, 10, 11, 29, 47).

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