

Target-specific cytotoxic activity of recombinant immunotoxin scFv(MUC1)-ETA on breast carcinoma cells and primary breast tumors

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Abstract

MUC1 is a mucin family protein, overexpressed in more than 90% of breast cancers in an underglycosylated form, exposing the core peptides of the extracellular domain that act as a potential target for antibody-mediated therapy. We have developed an anti-MUC1 scFv antibody from a phage library of mice immunized with synthetic peptide MUC1-variable number of tandem repeats. MUC1 binding phages were affinity selected through biopanning using a biotin-streptavidin pull-down method. The selected phage clones showed target-specific binding to MUC1-expressing cells. Fusion of truncated *Pseudomonas aeruginosa* exotoxin A (ETA) to a high binder, phage-derived scFv clone and bacterial expression and purification of recombinant scFv(MUC1)-ETA immunotoxin were done with good yield and purity. *In vitro* target-specific cytotoxic activity and target-specific binding of immunotoxin were shown on MUC1-expressing cells and primary breast tumor samples. A truncated ETA fusion protein expressed from the same vector but lacking scFv did not show cytotoxic effects, confirming target specificity. Our results suggest that the scFv(MUC1)-ETA immunotoxin has therapeutic potential and deserves further development and characterization for MUC1-specific breast cancers treatment. [Mol Cancer Ther 2007;6(2):562–9]

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Introduction

Target-specific delivery of therapeutic agents holds promise to improve the efficacy of cancer treatment by reducing damage to normal tissues and posttreatment complications. Differentially regulated proteins on the surface of malignant cells, such as the tumor-associated antigens, are effective targets for the development of alternative anticancer therapeutics. MUC1 mucin, a type I transmembrane glycoprotein, is one such target that is localized on the apical region of normal epithelial cells but gets aberrantly overexpressed and underglycosylated in many cancers (1). MUC1 overexpression enhances tumor invasiveness and promotes metastasis in breast cancers (2). Recently, MUC1 overexpression has been shown to be associated with tumorigenesis and resistance to genotoxic anticancer drugs (3).

The extracellular domain of the human MUC1 protein represents the longest rod-shaped molecule on the cell membrane. It contains a 20-amino-acid sequence (VTSAPDTRPAGSTAPPAHG) that is tandemly repeated 20 to 125 times (4, 5). In carcinoma of the breast, the core peptides become exposed due to underglycosylation (6) and act as a tumor antigen that can induce the formation of anti-MUC1 antibodies in patients' sera. The APDTRPA sequence within the tandem repeats has been found to be the most immunogenic sequence stretch of MUC1 reacting with almost all anti-MUC1 monoclonal antibodies (mAb; refs. 7, 8) and CTLs. Consequently, this immunogenic peptide sequence is well suited as a target for cancer immunotherapy (9–11). In addition, aberrant glycosylation of MUC1 also plays an important role in enhancing the internalization of MUC1 into the cytoplasm (12, 13), making MUC1 a very attractive therapeutic target for immunotherapeutic reagents, including those that require delivery.

Although mAbs offer a very specific means for targeted delivery of radionuclides or other therapeutic agents to cancer cells, their relatively bulky size and the presence of Fc regions can restrict tumor penetration (14). Single-chain Fv (scFv) fragments produced by cloning of V_H-V_L sequences from the hypervariable regions of these antibodies seem to be superior targeting agents over the whole antibodies because of better tumor penetration and more rapid clearance (15). Fusion of truncated toxins (bacterial or plant) with such single-chain antibodies results in recombinant immunotoxins, which have become a new modality for cancer treatment (16–18). These molecules bind to surface antigens that in turn get internalized and subsequently kill cancer cells by catalytic inhibition of protein synthesis within the target cell cytosol. Recombinant antibody and growth factor toxin have been successfully used in clinical trials of hematologic malignancies (19) and solid tumors (20).

Considering its unique biochemical features, positional advantage, and overexpression on various cancers, we chose MUC1 as a target for developing an anti-MUC1 scFv library from splenocytes of mice immunized with a 31-mer MUC1 peptide. Here we report the construction of the scFv library, selection of anti-MUC1 high binder phages from the library, fusion of a selected anti-MUC1 scFv with truncated *Pseudomonas* exotoxin A (ETA), and target-specific binding and cytotoxic activity against MUC1-expressing cancer cell lines and primary breast tumor samples. The results show that these molecules have therapeutic potential and deserve further development and characterization for treatment.

Materials and Methods

Cell Culture

Cell lines of diverse cancer origin, T47D, MCF7, SKBR3 (human breast carcinoma), SW480 (colon carcinoma), and PC-3 (prostate carcinoma), which were obtained from American Type Culture Collection (Manassas, VA), were grown and maintained in respective medium containing 10% FCS and penicillin-streptomycin at 37°C with 5% CO₂.

Tumor Sample Collection and Processing

Primary breast tumor samples were collected immediately after surgery, with prior approval from the Tata Memorial Hospital Ethics Committee and patient informed consent, in complete medium containing penicillin-streptomycin and were processed immediately. Single-cell suspensions were prepared from the biopsy samples in DMEM (10% FCS, 5 µg/mL aprotinin, 5 µg/mL leupeptin, and 2 mmol/L phenylmethylsulfonyl fluoride) through mincing followed by homogenization under sterile conditions and filtering through a 0.30-µm nylon mesh and were used for the indicated experiments.

Construction and Screening of the Anti-MUC1 Phage Display Library

A 31-mer peptide consisting of one full and a half variable number of tandem repeats (VTSAPDTRPAPG-STAPPAHG) from the extracellular domain was synthesized (MWG, Ebersberg, Germany) with a cysteine at the NH₂ terminus and conjugated with maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL). Six BALB/c mice (6–8 weeks old) and one New Zealand rabbit were immunized with MUC1 peptide-keyhole limpet hemocyanin conjugates. One week after the second boost, the mice were sacrificed and mouse spleen cells were isolated and used for cDNA synthesis using the polytract kit (Promega, Madison, WI). Degenerate PCR primers (Amersham Biosciences, Uppsala, Sweden) were used for amplification of V_H and V_L sequences, scFv assembly, and ligation of scFv into the pHEN-2 vector using the RPAS mouse scFv module (Amersham Biosciences). pHEN2-scFv constructs were electroporated into competent *E. coli* TG1 cells and then the bacteria were grown at 37°C for 30 min followed by growth overnight at 30°C. Phages from the anti-MUC1 scFv library were rescued using K13KO7

helper phage (Amersham Biosciences) following the procedure of Marks et al. (21). Before affinity selection, nonspecific phages from the library were removed by preincubating a phage with streptavidin-coated paramagnetic particles (MagnaBind streptavidin, Pierce). The supernatant containing the remaining phage pool was affinity selected using biotinylated MUC1 peptide at amounts decreasing 10-fold for each round of biopanning. Complexes were pulled down on a magnet stand and were eluted in 0.2 mol/L glycine (pH 2.2) followed by neutralization with 1 mol/L Tris-HCl (pH 9.1). Fresh *E. coli* TG1 cells were infected with the eluted phage clones and phages were rescued and used for the next round of biopanning.

Characterizations of Selected scFv Clones

HB2151 nonsuppressive *E. coli* cells were transformed with selected phagemid clones and grown in 2YT-AG medium (100 µg/mL ampicillin and 1% glucose). After induction with 1 mmol/L isopropyl-L-thio-B-D-galactopyranoside for 6 h at 30°C, the culture was centrifuged and individual cell pellets were resuspended in 1× TES buffer [20 mmol/L Tris-HCl (pH 6.8), 0.5 mmol/L EDTA, 500 mmol/L sucrose]. The mixture was diluted with 0.25× TES buffer (hypotonic shock) and incubated on ice for 30 min. The supernatants containing periplasmic scFv proteins were run on SDS-PAGE gel and were analyzed by ELISA and flow cytometry.

DNA Fingerprinting Analysis for Clonal Diversity

The MUC1 selected phages clones were isolated and the scFv fragments were PCR amplified using 460R-F-G (LMB3) and 460R-R-G (Fdseq1) primers (21). The scFv fragments were purified and digested with *Mva*I at 37°C. Samples were run on polyacrylamide gel and visualized by silver staining.

Construction, Expression, and Purification of scFv(MUC1)-ETA

A modified pFLAG-1 vector pSW202 (22), which contains a truncated *Pseudomonas* ETA gene lacking the cell binding domain *la*, was digested with *Hind*III and *Xba*I. The selected scFv(MUC1) fragment from the corresponding derivative pHEN2 was amplified by PCR using VH1BACK*Hind*III/VKWFOR*Xba*I primers (23) and digested with *Hind*III and *Xba*I (New England BioLabs, Ipswich, MA). The purified *Hind*III- and *Xba*I-digested scFv(MUC1) fragment was ligated to a linearized pSW202 vector. To express a cell binding domain *la*-truncated ETA, the *Hind*III- and *Xba*I-linearized pSW202 vector was ligated in-frame using *Hind*III and *Xba*I adaptors. The resulting constructs, pSW202-scFv(MUC1)-ETA and pSW202-*la*-truncated-ETA, were transformed into *E. coli* CC118 cells and expressed under the control of 1 mmol/L isopropyl-L-thio-B-D-galactopyranoside for 5 h at 30°C. The cell pellet was resuspended in 20 mmol/L phosphate buffer at pH 7.4 (500 mmol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, 100 µg/mL lysozyme, 20 mmol/L imidazole) and lysed with a One-Shot cell disruptor. After centrifugation at 25,000 × g for 30 min at 4°C, the lysate was filtered (0.45 µm) and the polyhistidine-tagged proteins were

affinity purified on HiTrap chelating HP (5 mL) column using AKTA EXPLORER-100 (Amersham Biosciences) in a linear gradient of 40 to 500 mmol/L imidazole [20 mmol/L phosphate buffer (pH 7.4) and 500 mmol/L NaCl]. Fractions were pooled, desalted in 50 mmol/L phosphate buffer (pH 7.4) with 150 mmol/L NaCl, and concentrated through Amicon Ultra-15 centrifugal filter unit (Millipore, Billerica, MA) before further purification on a HiLoad 16/60 Superdex 75 prep grade gel filtration calibrated column. Finally, the purified proteins were analyzed by SDS-PAGE followed by Western blotting with anti-His, anti-FLAG, or anti-ETA antibody.

Flow Cytometric Analysis

A total of 5×10^5 cells were incubated with 1 to 2 μg of purified scFv or scFv(MUC1)-ETA or the respective antibodies on ice and then washed with PBS. Cells were incubated with the respective secondary antibodies; bound antibody was detected by flow cytometry on a FACSCalibur (Becton Dickinson, Franklin lakes, NJ); and the results were analyzed with the CELLQuest program. For dual staining, primary breast tumor single-cell suspensions were fixed with 4% paraformaldehyde and blocked with 1% fetal bovine serum in PBS. Cells were incubated simultaneously with anti-MUC1 and CK-19

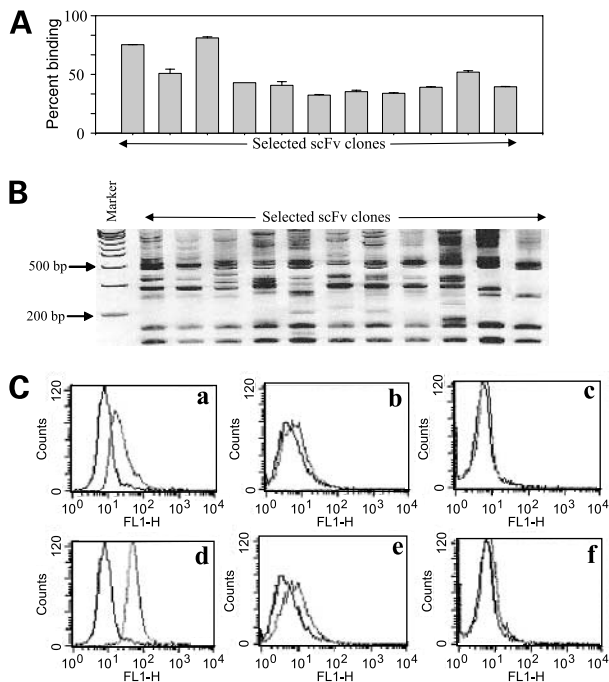


Figure 1. Molecular characterization and target-specific binding of selected MUC1 scFv clones. **A**, percent binding of selected scFv(MUC1) protein on T47D cells by flow cytometry. **B**, DNA fingerprints produced by *Mva*I digestion of PCR-amplified scFv inserts from respective clones showing molecular diversity. **C**, binding profile of SM3 (anti-MUC1) on T47D (a), MCF7 (b), and PC-3 (c) cell lines and of selected scFv(MUC1) clone on T47D (d), MCF7 (e), and PC-3 (f) cell lines.

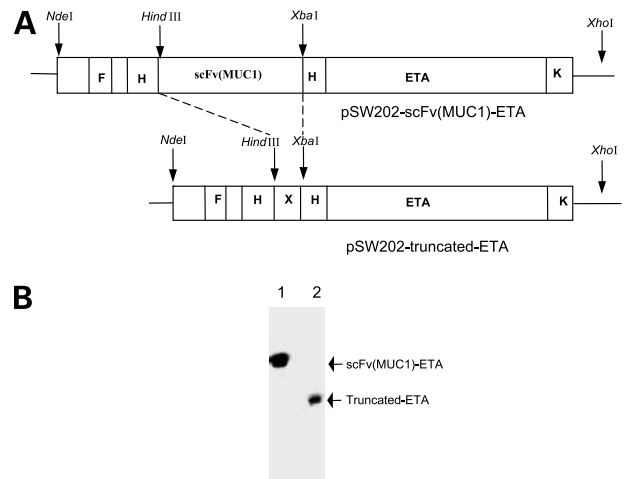


Figure 2. **A**, schematic representation of constructs for bacterial expression of scFv(MUC1) conjugated to truncated *Pseudomonas* ETA and truncated ETA control protein lacking the scFv domain. **B**, Western blot analysis of purified scFv(MUC1)-ETA and truncated ETA control protein probed with anti-ETA polyclonal antibody. X, six nucleotide adaptor in-frame; F, Flag tag; H, hexahistidine tag; K, endogenous KDEL-like endoplasmic reticulum retention signal of *Pseudomonas* exotoxin A; ETA, domain Ia-truncated exotoxin A (ETA amino acids 252–613).

antibodies followed by FITC- and propidium iodide-conjugated secondary antibodies. Detection of bound antibodies was determined by flow cytometry and the results were analyzed with the CELLQuest program.

Immunofluorescence

Cells were grown on coverslips and, after fixing with 4% paraformaldehyde, cells were blocked with 3% bovine serum albumin (PBS) and incubated with purified scFv(MUC1)-ETA or the respective antibodies. For scFv(MUC1)-ETA, cells were subsequently incubated with anti-FLAG M2 mAb followed by FITC-conjugated goat anti-mouse secondary antibody. All incubations were carried out at room temperature in a humid chamber with intermediate washing with chilled PBS between each step. The slides were visualized under a Carl Zeiss Axioskop fluorescence microscope.

Immunohistochemistry

Thin sections (5 μm) from paraffin-embedded breast tumors were deparaffinized and rehydrated before treatment with 3% H_2O_2 -methanol and blocking with 3% bovine serum albumin. Sections were incubated with scFv(MUC1)-ETA and/or the respective antibodies. The sections were incubated with biotin-conjugated multilink swine anti-goat, anti-mouse, and anti-rabbit immunoglobulin antibody followed by horseradish peroxidase (HRP)-conjugated streptavidin and treated with 1 mg/mL 3,3-diaminobenzidine in PBS. The scFv(MUC1)-ETA-treated sections were incubated with anti-FLAG M2 mAb followed by HRP-conjugated goat anti-mouse secondary antibody. Finally, sections were counterstained for 30 to 60 s with Harris's hematoxylin.

Cytotoxicity Assay

The cytotoxic activity of scFv(MUC1)-ETA was measured both on tissue culture cells and on primary breast tumor single-cell suspensions *in vitro* by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the procedure of Wels et al. (24). A total of 1×10^4 cells were plated in triplicate in 96-well microtiter plates and incubated with various concentrations of scFv(MUC1)-ETA and *Ia*-truncated-ETA as a control for 48 h. Subsequently, 10 μ L (10 mg/mL in PBS) of MTT were added to each well and cells were incubated for another 4 h. Finally, cells were lysed for 16 h by the addition of 90 μ L of lysis buffer (20% SDS in 50% dimethylformamide, pH 4.7). Plates were read at 590 and 670 nm on a microplate reader.

Immunoblotting

The T47D, MCF7, SKBR3 SW480, and PC-3 cells were lysed in radioimmunoprecipitation assay buffer. Equal amounts of cell lysates (100 μ g) were loaded onto 7% SDS-PAGE gel. The protein was transferred on the membrane and incubated with anti-MUC1 polyclonal antibody (made in house) followed by HRP-conjugated antibody. All incubations were carried out at room temperature followed by washing at each step. An enhanced chemiluminescence detection kit was used and the membrane was exposed to X-ray (Hyperfilm) film for 1 to 15 min.

Results

Construction, Screening, and Characterization of the scFv Phage Display Library

The constructed scFv(MUC1) phage library contained 1×10^{12} colony-forming units/mL as determined by

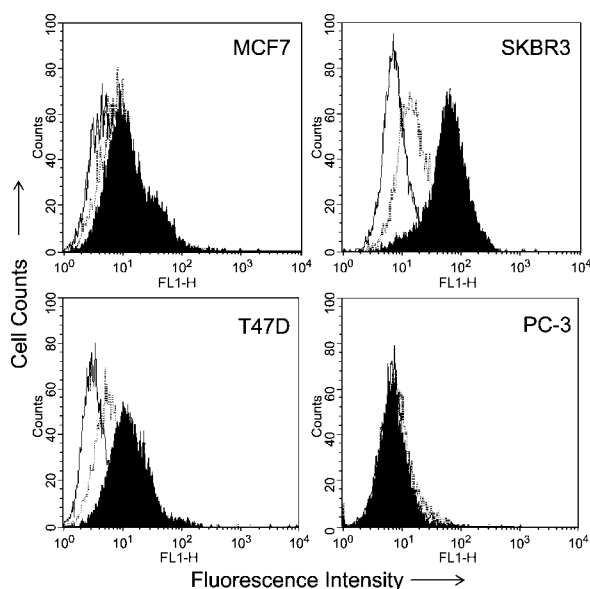


Figure 3. Flow cytometry analysis of target-specific binding of recombinant scFv(MUC1)-ETA proteins to human cancer cells. *Filled areas*, scFv(MUC1)-ETA followed by respective FITC-conjugated secondary antibodies; *open areas*, FITC-conjugated secondary antibodies only (negative control); *dotted line*, scFv(MUC1)-ETA preadsorbed with a 100-fold molar excess of MUC1 peptide.

titration of the library and plating on TYE-agar plates. Affinity selection of the scFv(MUC1) phage display library by repeated biopanning with MUC1 peptide resulted in a linear increase of MUC1 reactive phages measured by phage ELISA. Of 480 phages from the fourth biopanning pool, 479 (99.6%) appeared positive in phage ELISA with MUC1 peptide. However, a differential reactivity was observed when phages were blocked with 25-fold molar excess of MUC1 peptide before phage ELISA. Of 479 clones, 282 (58.7%) remained unchanged whereas the remaining 198 (41.3%) clones showed substantial reduction in their binding to immobilized MUC1 peptide when pretreated with soluble MUC1 peptide as competitor.⁴ The 11 clones were selected as high MUC1 binders and were chosen for further studies. The periplasmic fractions of selected scFv clones were analyzed for their binding study by flow cytometry on T47D cells. The result showed the differential binding of selected clones on T47D by flow cytometry (Fig. 1A).

To study clonal diversity of the selected clones, the ~750-bp scFv(MUC1) PCR fragments were digested with *Mva*I restriction endonuclease. The results presented in Fig. 1B show differential DNA fingerprint patterns for all selected clones, confirming wide clonal diversity.

The expression and purification of selected scFv clones were analyzed by immunoblot and ELISA. The binding of selected scFv clones on MUC1-positive T47D and MCF7 breast cancer cells were much more pronounced as compared with the SM3 anti-MUC1 mAb, whereas there was no binding on MUC1-negative PC-3 cells (Fig. 1C).

Construction, Expression, and Purification of scFv(MUC1)-ETA

From a selected high binding scFv(MUC1) clone, the scFv sequence was reamplified by PCR, to provide it with *Hind*III and *Xba*I restriction sites, and inserted in-frame at 5' of a truncated *Pseudomonas ETA* gene in the bacterial expression vector pSW202. Likewise, for bacterial expression of an ETA control protein lacking the scFv(MUC1) domain, a pSW202 derivative containing an open reading frame of domain *Ia*-truncated ETA was created (Fig. 2A).

The scFv(MUC1)-ETA immunotoxin and the truncated ETA control protein were expressed and purified via His tag. After a second purification step on a Superdex 75 gel filtration column, 5 to 8 mg of immunotoxin were obtained from 1 liter of culture cell pellets. A 90% to 95% pure immunotoxin was obtained as a single band of the expected molecular weight of 66 kDa (Fig. 2B).

Selective Binding of scFv(MUC1)-ETA

The binding of purified scFv(MUC1)-ETA proteins to MUC1-expressing cells was investigated by flow cytometric analysis. Differential binding of immunotoxin was observed on MCF7, SKBR3, and T47D cells whereas there was no binding on PC-3 cells. The binding intensity was much more pronounced and strong on SKBR3 cells as

⁴ R. Singh and D. Bandyopadhyay, unpublished data.

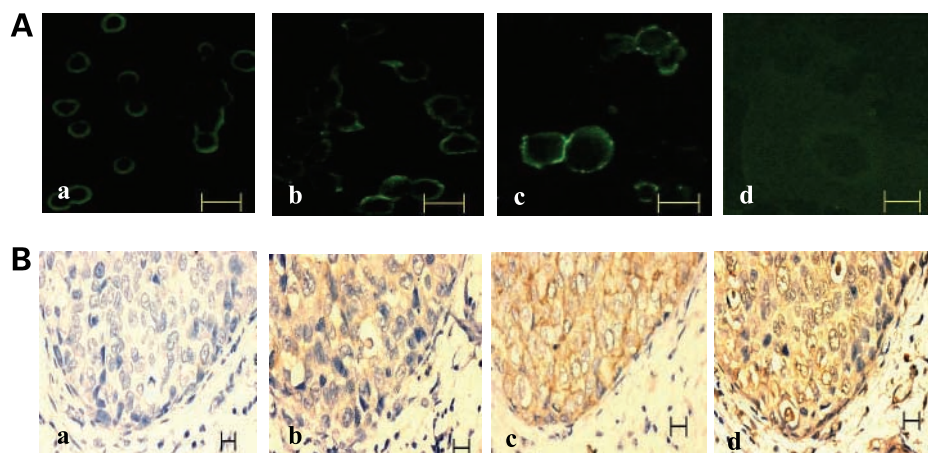


Figure 4. **A**, immunofluorescence microscopic analysis of scFv(MUC1)-ETA binding on T47D (a), MCF7 (b), human primary breast tumor cells (c), and PC-3 cells (d). Bound scFv(MUC1)-ETA immunotoxin was detected with anti-FLAG M2 mAb followed by antimouse FITC-conjugated antibody. **B**, immunohistochemical staining of paraffin-embedded breast tumor sections. **a**, without addition of primary antibody (negative control); **b**, VU-4H5 anti-MUC1 mAb; **c**, anti-MUC1 polyclonal antibody; **d**, scFv(MUC1)-ETA immunotoxin (followed by anti-FLAG M2 mAb). Binding was detected with HRP-conjugated antibody (bar, 50 μ m).

compared with MCF7 and T47D cells. To confirm binding specificity further, scFv(MUC1)-ETA was preadsorbed with a 100-fold molar excess of MUC1 peptide before binding study. This resulted in a marked reduction of cell binding when compared with scFv(MUC1)-ETA in the absence of competitor (Fig. 3).

Binding of anti-MUC1 mAb and scFv(MUC1)-ETA to MUC1-expressing cells was also investigated by immunofluorescence microscopic analysis of paraformaldehyde-fixed MCF7, T47D, and PC-3 cells and primary (single-cell suspension from human primary breast tumor) cancer cells. As shown in Fig. 4A, specific membrane staining with scFv(MUC1)-ETA was detected on MUC1-expressing MCF7 and T47D cells and primary breast cancer cells but not on MUC1-negative PC-3 cells.

Binding of scFv(MUC1)-ETA to primary breast cancer cells in paraffin-embedded breast tumor sections was investigated along with anti-MUC1 (31-mer peptide) polyclonal antibody and VU-4H5 anti-MUC1 mAb. Bound antibodies were detected with HRP-conjugated secondary antibody. Strong membrane and cytoplasmic staining was detected with anti-MUC1 polyclonal antibody and VU-4H5 anti-MUC1 mAb. Staining with scFv(MUC1)-ETA was predominantly seen on the membrane. Staining of adjoining tissue sections allows a clear comparison of the binding intensities of the antibodies and of the scFv(MUC1)-ETA immunotoxin and show the cellular localization of MUC1. As expected, no signal was detected in the negative control stained without a primary antibody (Fig. 4B).

***In vitro* Cytotoxic Effects of scFv(MUC1)-ETA**

In vitro cytotoxic effects of immunotoxin were determined by MTT assays on MCF7, SKBR3, T47D, and PC-3 cell lines. Strong cytotoxic activity of immunotoxin was observed against MCF7 and SKBR3 cells, resulting in ~80% cell killing at an immunotoxin concentration of 100 ng/mL (IC₅₀, 35 ng/mL for MCF7 cells and 44 ng/mL for SKBR3 cells). T47D cells were less sensitive, with only 23% cell killing with scFv(MUC1)-ETA concentration of

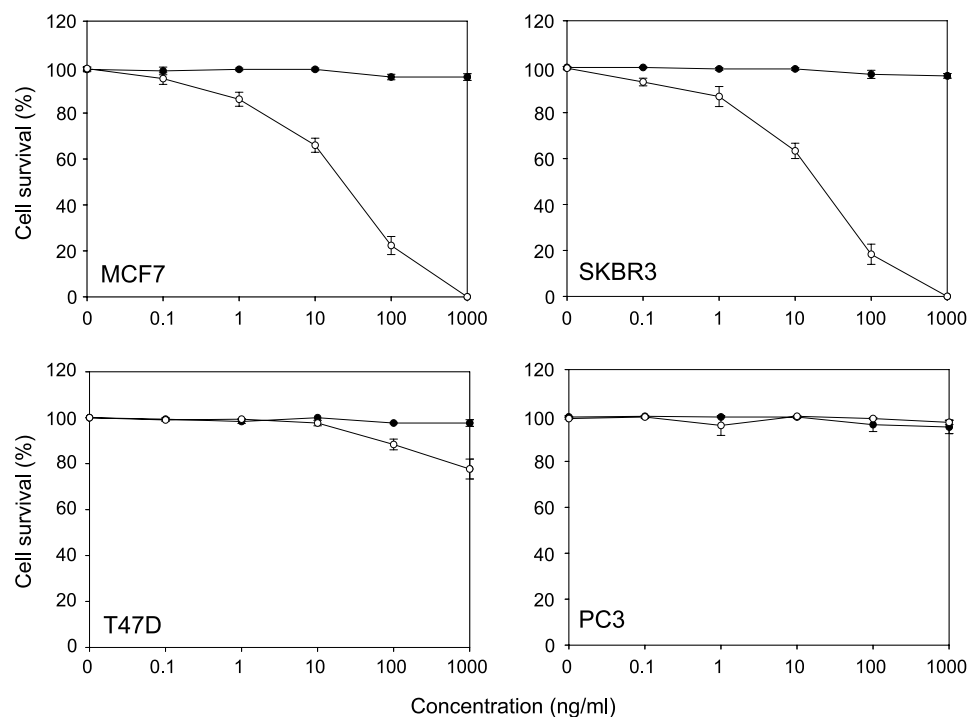
1 μ g/mL. To show specificity of the effects, a recombinant truncated ETA molecule was included in the experiments that lack the scFv domain for binding to MUC1. This control protein did not show cytotoxic activity even at concentrations of 1 μ g/mL. ScFv(MUC1)-ETA was at least 100 times more potent to MCF7 and SKBR3 cells than against T47D cells. In contrast, MUC1-negative PC-3 cells were not affected by the immunotoxin. There was no effect on cell viability at concentrations up to 1 μ g/mL in PC-3 cells (Fig. 5).

The cytotoxic effects of scFv(MUC1)-ETA were also observed on seven primary breast tumors cells by MTT assay (Table 1). The table presents the percent survival of tumor cells with 1 and 10 μ g/mL of scFv(MUC1)-ETA after 48 h and the percent MUC1- and CK-19-positive cells present in those tumor samples as determined by dual staining by flow cytometry. The high percent survival of primary tumor cells after treatment with immunotoxin was directly correlated with the percent MUC1-positive cells and percent epithelial (CK-19) positive cells present in the tumor samples. Most of the samples used in these studies had low MUC1- and CK-19-positive cells. The low cytotoxic effects might also correlate with the heterogeneity of MUC1-producing cells in the tumor population⁴ and/or the differential glycosylation or inefficient MUC1 internalization in these tumor samples.

MUC1 Expression in Human Cancer Cell Lines

The MCF7, T47D, SKBR3, SW480, and PC-3 cells were tested for MUC1 expression by reverse transcription-PCR and Western blotting. As shown in Fig. 6A, the PCR-amplified 287-bp MUC1 cDNA fragment was seen in all cell lines except PC-3 cells. MUC1 protein expression was confirmed by immunoblot analysis. The intensity of the anti-MUC1 polyclonal antibody-reactive ~220-kDa MUC1 protein band was stronger in MCF7 and T47D as compared with SKBR3 and SW480 cells (Fig. 6B). PC-3 prostate cancer cells did not show expression of MUC1 at the RNA level or protein level. PC-3 cells were thus included in this study as MUC1-negative control.

Figure 5. *In vitro* cytotoxicity of scFv(MUC1)-ETA (○) and truncated ETA (●) against human breast cancer MCF7, SKBR3, and T47D cell lines and prostate cancer PC-3 cell line by MTT assay. Points, mean of triplicates.



Discussion

The recent advances in conjugation of genetically modified toxins to recombinant antibodies have made it possible to achieve tumor-specific cell killing (17). A number of chemical and recombinant immunotoxins that use either plant or bacterial toxins as effector domains and that target distinct cell-surface antigens associated with tumor cells have been shown to be potent and effective anticancer agents in preclinical studies (17–20). The importance of MUC1 has been recognized for a long time and has been a potent target for immunotherapeutic applications, including vaccine development in various cancers (25). Here, we summarized our results obtained with a scFv-based immunotoxin specific for epithelial mucin MUC1.

We present here the report of conjugation of a single-chain antibody against MUC1 with *la* domain-truncated *Pseudomonas* ETA. The results presented here show the successful production of a functional scFv(MUC1)-ETA immunotoxin with potent cytotoxic activity against breast

cancer cells. A total of 11 scFv clones were selected from the fourth biopanning pool that showed high MUC1-specific binding in MUC1-peptide preadsorption phage ELISA assay. The cytotoxic activity of scFv(MUC1)-ETA has clearly shown target-specific cell killing.

Both MCF7 and SKBR3 cells showed high sensitivity to scFv(MUC1)-ETA with IC_{50} values of 35 and 44 ng/mL respectively, whereas truncated ETA was not reactive at even higher concentrations. Because the cytotoxic domain is preserved in truncated ETA, the only limiting factor that makes scFv(MUC1)-ETA a potent killer lies in its target-specific binding domain, scFv(MUC1), which interacts with MUC1 on the cell membrane. A differential cytotoxic effect of scFv(MUC1)-ETA was also observed among the MUC1-expressing cells studied. Although MCF7 and T47D cells express almost the same amount of MUC1, the cytotoxic effect of scFv(MUC1)-ETA was more pronounced in MCF7 cells as compared with T47D cells. It was also interesting to note that SKBR3 cells, having comparatively lower MUC1

Table 1. *In vitro* cytotoxicity of scFv(MUC1)-ETA on human primary breast tumor cells

Primary tumor samples	% CK-19-positive cells	% MUC1-positive cells	% Survival [1 μ g/mL scFv(MUC1)-ETA]	% Survival [10 μ g/mL scFv(MUC1)-ETA]
1	36.6	25.4	88.3 \pm 6.94	60.2 \pm 4.31
2	10.6	97.4	87.5 \pm 8.3	62.2 \pm 3.72
3	5.0	13.1	95.1 \pm 4.18	71.4 \pm 1.96
4	22.6	30.2	85.8 \pm 7.51	64.4 \pm 4.16
5	45.8	48	90.7 \pm 1.32	66.2 \pm 0.90
6	12.1	13.4	85.2 \pm 5.08	63.4 \pm 2.30
7	31.7	21.3	87.2 \pm 5.68	61.9 \pm 2.02

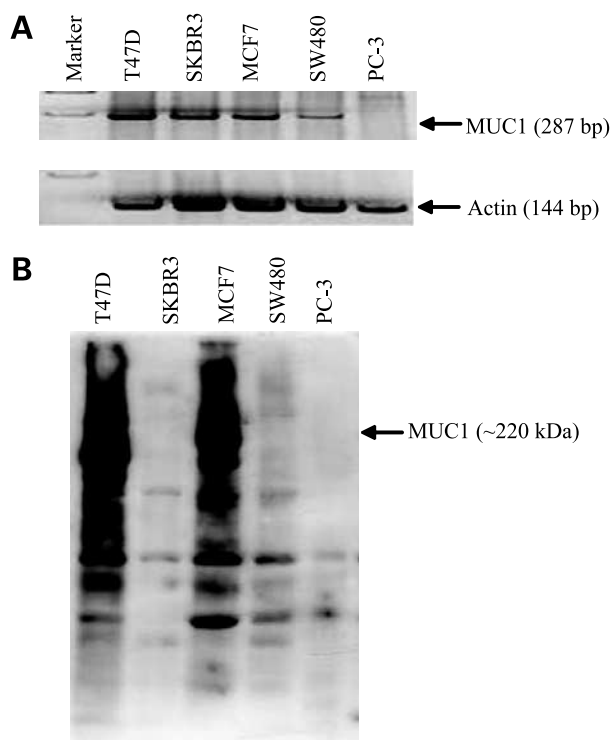


Figure 6. Expression of MUC1 mRNA and protein in human cancer cell lines. **A**, reverse transcription-PCR analysis detecting a PCR-amplified MUC1 cDNA fragment in different cell lines. **B**, immunoblotting of total cell extract probed with anti-MUC1 polyclonal antibody followed by HRP-conjugated secondary antibody.

expression than T47D cells, showed higher sensitivity to scFv(MUC1)-ETA, similar to MCF7 cells. Similar observations had been observed earlier by Kihara and Pastan (26). Wels et al. (22) also reported that cytotoxic killing of ErbB-2-specific scFv(FRP5)-ETA depended more on the mechanism of internalization and intracellular processing of the immunotoxin rather than the binding of scFv(FRP5)-ETA to ErbB-2 and epidermal growth factor receptor on the membrane (27).

The cytotoxic effects of scFv(MUC1)-ETA on primary breast tumors showed a partial sensitivity to immunotoxin, suggesting a possible heterogeneity of MUC1-positive cells presents in tumor samples (nonepithelial origin)⁴ and/or the differential underglycosylation patterns that might affect the rate of internalization. Because *Pseudomonas aeruginosa* requires efficient internalization and activation along a multistep pathway for cell killing, the intracellular routing of the receptor/toxin complex rather than the binding to the target receptor on the cell surface may be rate limiting (28). The underglycosylation in MUC1 alters its internalization and subcellular localization due to truncated O-glycans (13). As toxin requires internalization for its cytotoxic effects, the membrane trafficking rate of the underglycosylated MUC1 in cancer cells would limit the efficacy of the immunotoxin. Considering the existence of a rate-limiting and membrane trafficking mechanism for

the recycling of MUC1, differential immunotoxin sensitivity among MUC1-expressing epithelial cells is expected. We conclude that the MUC1-expressing breast cancer cells react to scFv(MUC1)-ETA immunotoxin in a manner that is possibly dependent on the rate-limiting membrane trafficking and subcellular localization of MUC1. A future study would be required to establish an association between the subcellular distribution of MUC1 and tumor grade and estrogen receptor status for improving the efficacy of the scFv(MUC1)-ETA. Target-specific cell killing in MCF7 xenografts with scFv(MUC1)-ETA but not with *ln*-truncated-ETA (data not shown) suggests that scFv(MUC1)-ETA has therapeutic potential and deserves further development and characterization for therapeutic purposes.

In conclusion, the ability of scFv(MUC1)-ETA immunotoxin to control the growth of Adriamycin-resistant and radiation-resistant MCF7 cells makes this recombinant immunotoxin a potential alternative for targeted therapy in breast and other MUC1-expressing cancers. As the expression of MUC1 and erbB2 in breast cancer among different patients and in tissues from individual patients is partially overlapping but discoordinate, therapy directed towards both antigens would increase the number of patients who might benefit compared with targeting either antigen.

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