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Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library

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Abstract

The first step in developing a targeted cancer therapeutic is generating a ligand that binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. For this work, we generated human monoclonal antibodies to the EGF receptor (EGFR), an antigen overexpressed on many solid tumors. Single chain Fv (scFv) antibody fragments were directly selected by panning a phage display library on tumor cells (A431) overexpressing EGFR or Chinese hamster ovary cells (CHO/EGFR cells) transfected with the EGFR gene and recovering endocytosed phage from within the cell. Three unique scFvs were isolated, two from selections on A431 cells and two from selections on CHO/EGFR cells. All three scFv bound native receptor as expressed on a panel of tumor cells and did not bind EGFR negative cells. Phage antibodies and multivalent immunoliposomes constructed from scFv were endocytosed by EGFR expressing cells as shown by confocal microscopy. Native scFv primarily stained the cell surface, with less staining intracellularly. The results demonstrate how phage antibodies binding native cell surface receptors can be directly selected on overexpressing cell lines or transfected cells. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences. Depending upon the format used, the antibodies can be used to deliver molecules to the cell surface or intracellularly.

Keywords: Receptor mediated endocytosis; Epidermal growth factor receptor; Phage antibody library; Single chain Fv; scFv; Tumor targeting

1. Introduction

Traditional cancer therapies have relied on the differential toxicity of chemotheraputic agents on tumor cells compared to normal cells. Recently, improved understanding of the molecular basis of cancer makes possible the development of therapies with increased efficacy and reduced toxicity. Studies of tumorigenesis have identified cell surface receptors which are either tumor or lineage specific, such as CD20 (Einfeld et al., 1988) and mutant forms of epidermal growth factor receptor (EGFR) (Garcia de Palazzo et al., 1993) or receptors which are over-

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expressed in tumors, such as ErbB2 (Slamon et al., 1989). Such receptors can be targeted with antibodies to allow specific drug interaction with only the tumor cell. In some instances, binding of ''naked'' antibody to the tumor cell can cause growth inhibition (Carter et al., 1992) or apoptosis (Ghetie et al., 1997; Taji et al., 1998). Alternatively, the antibody can be used to deliver a ''toxic payload'' to the cell. Toxic mechanisms include activation of the immune system, e.g., with bispecific antibodies, fusions to co-stimulatory molecules, or fusion with a toxic payload including radioisotopes, chemotherapeutics, toxins, or genes. For some strategies, it is necessary for the antibody to remain on the cell surface (e.g., bispecific therapies). For other approaches, it is necessary that the antibody deliver its payload into the cytosol (e.g., immunotoxins and gene therapy). In both cases, antibody recognition of the native receptor as expressed on the cell surface is required.

Phage antibody libraries have become an important source for the development of completely human therapeutic antibodies (Marks and Marks, 1996; Marks et al., 1991) to a wide range of antigens including tumor growth factor receptors (Schier et al., 1995). Antibodies generated from phage libraries have typically been selected using purified antigens or peptides immobilized on artificial surfaces. This approach may select antibodies that do not recognize the native protein in a physiologic context, as on the surface of cells. Attempts have been made to select on antigen in native conformation using cell lysates (Parren et al., 1996; Sanna et al., 1995; Sawyer et al., 1997) fixed cells (Van Ewijk et al., 1997) or living cells (Andersen et al., 1996; Cai and Garen, 1995; de Kruif et al., 1995; Marks et al., 1993; Siegel et al., 1997). The few successful selections performed on such heterogeneous material were generally done using small libraries from immunized sources. The use of immunized libraries limits the spectrum of antigen specificities that can be potentially obtained from the same library and typically yields murine antibodies. Selection of binders from large naïve libraries by cell panning is greatly limited by high background binding of non-specific phage and relatively low binding of specific phage (Pereira et al., 1997; Watters et al., 1997; Becerril et al., 1999).

Using a model system and an ErbB2 phage antibody we recently demonstrated that phage antibodies binding internalizing surface receptors can be endocytosed by mammalian cells and recovered in infectious form from within the cell (Becerril et al., 1999). Enrichment of ErbB2 phage over non-specific phage was 10–30 times higher when phage were recovered from within the cell compared to recovery from the cell surface, suggesting that cell selection specificity could be increased by recovering internalized phage. We confirmed this by applying this methodology to generate a panel of anti-tumor antibodies which were endocytosed into the breast tumor cell line SKBR3 as well as other tumor cells (Poul et al., 2000). Two of the specificities isolated included ErbB2 and transferrin receptor antibodies.

For this work, we applied the methodology to generate EGFR antibodies which recognized the native receptor on cells and could be used for tumor targeting. EGFR is overexpressed in many carcinomas (Baselga and Mendelsohn, 1994; Chrysogelos and Dickson, 1994; De Jong et al., 1998; Harris, 1994; LeMaistre et al., 1994) and can be exploited to differentiate and target cancer cells from normal cells. For selections, two cell lines were used as the source of antigen: a transfected Chinese hamster ovary cell (CHO/EGFR) and EGFR-overexpressing cancer cell line A431. The results indicate the generality of the approach and its usefulness in generating antibodies to known receptors in the absence of purified recombinant protein.

2. Materials and methods

2.1. Cell culture

CHO cells stably transfected with EGFR full length receptor (Morrison et al., 1993) (CHO/EGFR) were grown in F12 selective media (G418, Mediatech, 0.8 g/l) supplemented with 10% fetal calf serum (FCS). The parent cell line (CHO) was grown in non-selective F12 complete media supplemented with 10% FCS. A431 cells were grown in DMEM supplemented with 10% FCS. MDA-MB-453 and MDA-MB-468 cells were grown in Leibovitz media supplemented with 10% FCS in the absence of CO_2. All other cell lines were grown at 37°C in the presence of 5% CO_2.

2.2. Phage antibody selections

2.2.1. Selections on CHO/EGFR cell monolayer

CHO/EGFR cells grown on a 10-cm plate at 80–90% confluence were incubated with 1 ml of phage antibody library (5×10^{12} cfu/ml) (Sheets et al., 1998) in the presence of 2×10^{6} CHO cells in complete media (3 ml) for 1.5 h at 4°C. CHO cells were used to deplete the library of non-specific clones. The supernatant was aspirated and cells were washed six times in cold complete media for 10 min per wash. Receptor internalization was induced by addition of pre-warmed (37°C) complete media and incubation at 37°C, 5% CO_2 for 15 min. This time period has been shown to be appropriate for the observation of EGFR internalization (Vieira et al., 1996). After internalization, non-internalized cell-membrane bound phage were eluted by washing cells on the plate with cold glycine buffer (50 mM glycine, 150 mM NaCl, 200 mM urea, 2 mg/ml polyvinylpyrrolydone, pH 2.8) three times for 10 min per wash at 4°C. Immobilized cells were washed 1× in complete media. The internalized phage were recovered by removing cells in trypsin and washing in complete media. Cells were pelleted by centrifugation at 1000 rpm, lysed in 0.5 ml 100 mM triethylamine (TEA) for 10 min and neutralized in 1 ml 1 M Tris, pH 7.

2.2.2. Selections on A431 cells in suspension

A431 cells growing on a 15-cm culture dish (90% confluence) were removed in 2 mM EDTA–phosphate-buffered saline (PBS) and washed twice in cold PBS (25 ml). To deplete the library of non-specific phage, 5×10^{6} fibroblast cells (ATCC, CRL1634) were incubated with 1 ml of phage antibody library in 3 ml complete media (DMEM–10% FCS) for 1 h rocking at 4°C. Fibroblast cells were pelleted by centrifugation at 1000 rpm and the supernatant was recovered. A431 cells were incubated in a 15-ml culture tube with the depleted phage antibody library (supernatant from the previous step) for 1.5 h rocking at 4°C. Cells were subsequently washed 10 times in cold complete media. Cells were incubated for 30 min at 37°C in pre-warmed complete media to allow receptor internalization. Non-internalized phage were removed from the cell surface by 10 washes in cold PBS and a final wash in glycine buffer. Cells were lysed immediately, following a single glycine wash, in 0.5 ml 100 mM TEA and neutralized in 1 ml 1 M Tris, pH 7.

2.3. Phage rescue, preparation and titration

Phage were titered by infection of eluted phage into *Escherichia coli* TG1 (Marks et al., 1991). Phage were prepared for the next round of selection by infection of *E. coli* TG1 with eluted phage and rescue with VCS-M13 (Stratagene) helper phage as previously described (Marks et al., 1991). After overnight growth at 30°C, phage were purified and concentrated from bacterial supernatant with polyethylene glycol 8000 (PEG8000) (Marks et al., 1991) and resuspended in 1.5 ml PBS for use in the next round of selection or for use in flow cytometry. For each cell type, a total of three rounds of selection were performed.

2.4. Polyclonal phage enzyme-linked immunosorbent assay (ELISA)

EGFR-ECD was expressed in CHO cells and purified by concavalin A agarose (Vector Laboratories) affinity chromatography. Ninety-six-well microtiter plates (Falcon, 353912) were coated overnight at 4°C with 10 μg/ml of EGFR-ECD in PBS. Plates were washed three times with PBS and 50 μl of 1.0×10^{11} cfu/ml of polyclonal phage in PBS buffer (prepared as described in Section 2.3) was added to each well and incubated for 1 h. Wells were washed three times with PBS containing 0.1% Tween 20 (TPBS) and three times with PBS. Binding of phage antibodies was detected with peroxidase-conjugated anti-M13 antibody (Amersham-Pharmacia) diluted 1:1000 in PBS and ABTS (Sigma) as substrate.

2.5. Evaluation of polyclonal phage mixtures by flow cytometry

Polyclonal phage were screened for binding to whole cells by fluorescence activated cell sorting (FACS) analysis. EGFR expressing cell lines MDA-MB-468 and CHO/EGFR were used to identify EGFR binding antibodies and EGFR negative cell lines MDA-MB-453 and CHO were used to de-

termine specificity. Cells were grown to 80–90% confluence and removed in 2 mM EDTA–PBS. Cells were counted and washed once in cold PBS and twice in FACS buffer [cold 0.5% bovine serum albumin (BSA) (fraction V, Sigma)–PBS]. Cells were placed in FACS tubes (100 000 cells/well) and incubated with polyclonal phage (50 μl/10^{12} cfu/ml) for an hour on ice. Cells were washed twice in FACS buffer and incubated with α-M13-biotin (Amersham-Pharmacia, 1:5000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and incubated with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and then analyzed by FACS on the PE channel. Fluorescence was measured in a FACSort™ (Beckton Dickinson) and mean fluorescence was calculated using the Cellquest™ software.

2.6. Isolation and characterization of monoclonal EGFR antibodies

To facilitate subsequent purification of soluble single chain Fv (scFv), the polyclonal scFv gene population from the third round of selection was subcloned in batch into the expression vector pUC119mycHis (Schier et al., 1995) resulting in the addition of a c-myc epitope tag and hexahistidine tag at the C-terminus of the scFv. Briefly, phagemid DNA was prepared from the third round of selection, the scFv genes excised using the restriction enzymes *Sfi*1 and *Not*1, and the gene repertoire gel purified and ligated into pUC119mycHis digested with *Sfi*1 and *Not*1. After transformation of *E. coli* TG1, single ampicillin resistant colonies were packed into 96-well microtiter plates and scFv expression induced by the addition of IPTG as previously described (Schier et al., 1996). Bacterial supernatant containing scFv was used directly for ELISA. For EGFR-ECD ELISA using unpurified scFv, microtiter plates (Falcon) were coated with 10 μg/ml EGFR-ECD in PBS overnight at 4°C. Plates were incubated with bacterial supernatants at room temperature for 1 h and then washed once in TPBS and twice in PBS. Protein binding was detected with anti-myc tag antibody 9E10 followed by incubation in secondary antibody anti-mouse-horseradish peroxidase (HRP) (Sigma) for 30 min as previously described (Schier et al., 1996). Following a final set of washes, binding was detected with ABTS substrate. For further studies of monoclonal scFv, expression from pUC119 mycHis was scaled up into 500-ml cultures in 2 L culture flasks. Cultures were grown and scFv expressed (De Bellis and Schwartz, 1990) as previously described (Schier et al., 1996). scFv was harvested from the bacterial periplasm by osmotic shock (Breitling et al., 1991) and purified by immobilized metal affinity chromatography (Hochuli, 1988) using a Ni-NTA column (Qiagen) and gel filtration, as previously described (Schier et al., 1996).

2.7. Covalent labeling of α-EGFR scFv with fluoroisothiocyanate (FITC)

A 1-ml (1 mg) volume of E12 scFv was dialyzed against 50 mM carbonate buffer, pH 8.5 overnight. Fluoroisothiocyanate (FITC) labeling reagent, 6-(fluorescein-5-[and-6]-carboxamido)hexanoic acid, succinimidyl ester [5(6)-SFX, Molecular Probes] was dissolved in dimethylsulfoxide (DMSO) or dimethylformide (DMF) (5–10 mg/ml) and added to the scFv at a volume:volume ratio of 1:20. The reaction was conducted for 1 h at room temperature. Free labeling reagent was separated from labeled antibody on a S-25 gel filtration column (Sephadex). E12 scFv was incubated with CHO cells and CHO/EGFR cells and no background binding due to free label could be detected. No fluorescence shift was detected for CHO cells stained with the labeled antibody. The fluorescence shift detected on CHO/EGFR cells was therefore wholly attributed to the antibody–receptor interaction.

2.8. Microscopy

2.8.1. Fixed cell microscopy

Confirmation of α-EGFR phage antibody internalization was obtained by confocal microscopy. Cells were grown on coverslips in 24-well plates and at 80% confluence were incubated with phage antibody (10^{10} cfu/ml, in fresh complete media) for 2 h at 37°C. Plates were placed on ice to halt receptor internalization and the coverslips washed 10 times in cold PBS (1 ml per wash). Cells were then washed three times for 10 min in cold glycine buffer, pH 2.8

(50 mM glycine, 150 mM NaCl) to remove surface bound antibody. Cells were washed twice in cold PBS, fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized in cold methanol for 10 min at room temperature. Coverslips were incubated with α-M13-biotin (5′–3′, 1:2000 dilution in 0.5% BSA–PBS) for 30 min followed by two washes in complete media. Phage were detected by incubation with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min while followed by two washes in complete media. Coverslips were mounted on microscope slides and 2–3 μl Vectorshield (Vector Laboratories) was applied to each coverslip to preserve fluorescence upon irradiation.

2.8.2. Live cells

scFv-mediated internalization of FITC-labeled soluble-native scFv (100 μg/ml) or fluorescent immunoliposomes was measured on live cells grown on coverslips. E12 scFv immunoliposomes were constructed as previously described and contained on average 25 scFv/liposome (Park et al., 1998). Cells were plated and grown overnight on coverslips to 80% confluency. Cells were incubated with 100 μg/ml FITC-labeled (0.5 ml volume) or unlabeled scFv or with 10 μM immunoliposomes for 2 h. Cells were washed with PBS, coverslips removed and mounted onto microscope slides for imaging. Images were collected immediately using a Leica TCS NT confocal laser fluorescence microscope with digital camera (Leica, Deerfield, IL, USA).

2.9. Affinity measurement on whole cells by FACS

Cells (A431, MDA-MB-468, MDA-MB-453) were grown to 90% confluence in DMEM (A431) and Leibovitz (MDA-MB) media supplemented with 10% FCS. Cells were harvested in 2 mM EDTA–PBS. scFv was incubated with 2.5×10^5 cells for an hour at varying concentrations (50 nM–2 μM). Cell binding was performed on ice in PBS containing 0.25% BSA in a total volume of 100 μl. After two washes in PBS–BSA (250 μl), cells were incubated with saturating amounts of anti-myc 9E10 for 30 min followed by two washes in PBS–BSA. Bound scFv was detected by staining with saturating amounts of anti-mouse FITC, Fc specific (1:200 dilution, Sigma). After a 30 min incubation, cells were washed twice and resuspended in PBS containing 1% paraformaldehyde. Determination of the binding affinity was determined using a flow cytometry based assay as previously described (Benedict et al., 1997).

3. Results

3.1. Selection of EGFR antibodies

For selections, phage were prepared from a 7.0×10^9 member human scFv phage antibody library (Sheets et al., 1998). To generate antibodies binding EGFR, phage was selected on the A431 cell line which overexpresses EGFR and on CHO cells transfected with the EGFR gene (CHO/EGFR). For selections on CHO/EGFR cells, the library was depleted of antibodies binding common cell surface receptors by adding phage to CHO/EGFR cells grown adherent to subconfluency with untransfected CHO cells in suspension. After 1 h at 4°C, CHO cells were removed from the culture flask and warm media at 37°C added to allow internalization into the target CHO/EGFR cells. For selections on A431 cells, which grow in suspension, the phage library was pre-depleted of antibodies binding common cell surface receptors by incubation with fibroblast cells. After 1 h at 4°C, the fibroblast cells were removed by centrifugation and the phage added to A431 cells in suspension at 4°C to allow binding followed by incubation at 37°C to allow phage internalization. After phage endocytosis, cells were extensively washed and then lysed with TEA. The cell lysate containing the internalized phage was used to infect *E. coli* to prepare phage for the next round of selection. Three rounds of selection were performed with the efficiency of selection monitored by titering the number of phage recovered from the cell lysate. For selections on both A431 and CHO/EGFR cells, the titer of phage recovered increased with each round of selection, consistent with enrichment for cell binding antibodies (Table 1).

3.2. Analysis of polyclonal phage for EGFR binding by ELISA and FACS

To evaluate the success of selections, polyclonal phage was prepared after each round of selection and

Table 1
Results of selection of α-EGFR scFv on whole cells in three rounds

Round	A431		CHO/EGFR	
	Phage titer (cfu)	Frequency of positives	Phage titer (cfu)	Frequency of positives
R1	1×10^{4}	ND	2×10^{3}	ND
R2	8×10^{5}	ND	8×10^{4}	ND
R3	8×10^{6}	10/94	5×10^{6}	4/282

analyzed for binding to recombinant EGFR-ECD by ELISA (Fig. 1). A signal significantly greater than background binding was observed after three rounds of selection on both A431 and CHO/EGFR cells (Fig. 1). No significant binding above background was observed after one or two rounds of selection on either cell type. Binding of polyclonal phage from the third round of selection to cell lines expressing different quantities of EGFR was studied further by flow cytometry. Phage selected on A431 cells showed a significantly greater fluorescent shift on CHO/EGFR cells than on CHO cells (Fig. 2 left panels) and on the high EGFR expressing tumor cell line MDA-MB-468 vs. the EGFR negative tumor cell line MDA-MB-453 (Fig. 2 left panels). For phage selected on CHO/EGFR cells, no significant difference in fluorescent shift was observed for binding to CHO/EGFR cells vs. CHO cells (Fig. 2 right panels). The strong shift on both cell lines indicates that the majority of the phage bind antigens common to CHO cells. Analysis of these phage for binding to high EGFR expressing MDA-MB-468 cells compared to EGFR negative MDA-MB-453 cells indicates, however, the presence of a relatively small number of phage binding EGFR (Fig. 2, right panels).

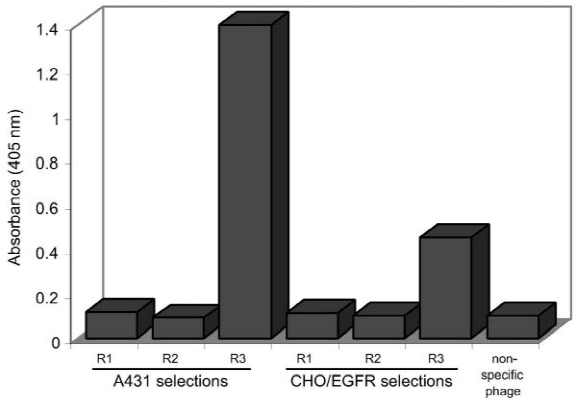

Fig. 1. Binding of polyclonal phage to recombinant EGFR as determined by ELISA. Phage was prepared from the first, second and third round of selections and analyzed for binding to recombinant EGFR by ELISA. After the third round of selection, binding was observed for selections performed on A431 cells and for selections performed on CHO/EGFR cells.

3.3. Isolation and characterization of monoclonal EGFR antibodies

Based on the ELISA and flow cytometry data indicating the presence of EGFR phage antibodies, individual clones were picked into 96-well microtiter plates and expression of native soluble scFv induced. Bacterial culture supernatants containing scFv were analyzed by ELISA for their ability to bind recombinant EGFR-ECD. For the third round of selection on A431 cells, 10/94 clones (11%) bound EGFR-ECD, while for the third round of selection on CHO/EGFR cells, 4/282 (1%) clones bound EGFR-ECD (Table 1). The relative proportions of binders is consistent with the ELISA and flow cytometry analysis of the polyclonal phage. To determine the number of unique antibodies, the scFv gene of all EGFR-ECD binding clones was analyzed by BstN1 fingerprinting followed by DNA sequencing. Two unique EGFR antibodies (E12 and B11) were isolated from selections on A431 cells. For selections on CHO/EGFR cells, the B11 scFv was re-isolated along with another unique scFv (C10).

To determine whether the monoclonal antibodies bound native EGFR as expressed on cells, phage and native scFv were prepared from each of the three

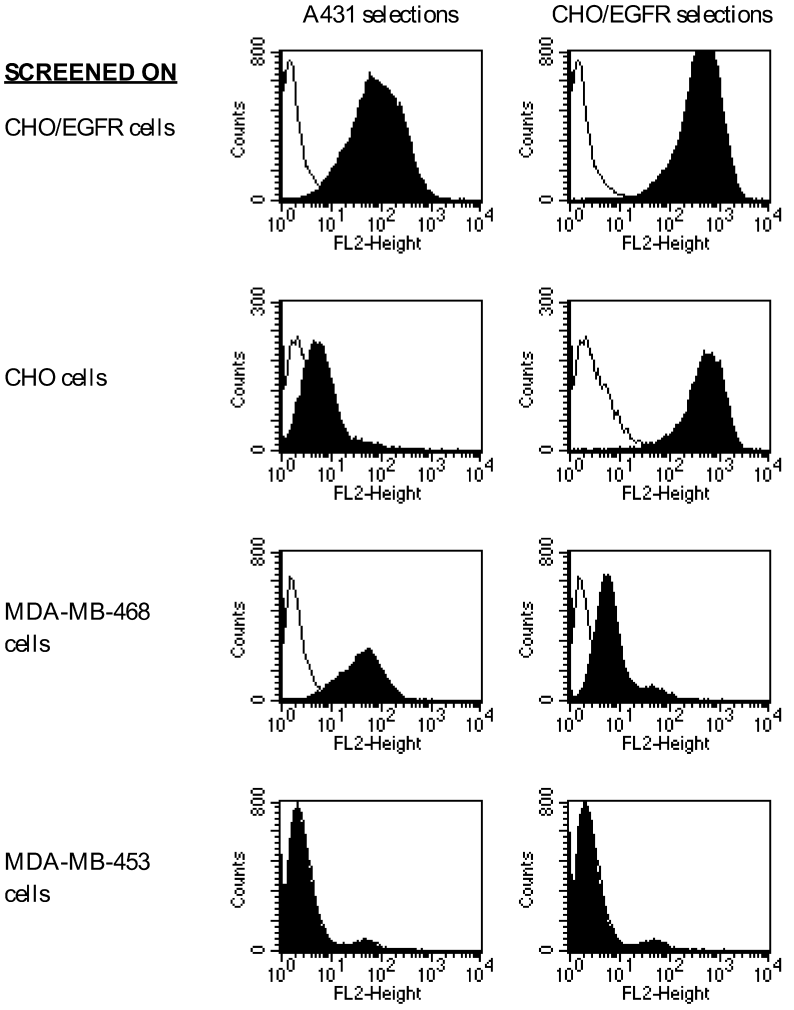

Fig. 2. Binding of polyclonal phage to EGFR expressing cells. Phage was prepared from the third round of selections performed on A431 cells and CHO/EGFR cells, and binding to a panel of cells was analyzed by flow cytometry. For selections performed on A431 cells (left panels), phage stained EGFR expressing cells (CHO/EGFR and MDA-MB-468 cells) more strongly than EGFR negative cells (CHO and MDA-MB-453 cells). For selections performed on CHO/EGFR cells (right panels), no difference in staining was observed between CHO/EGFR cells and CHO cells, however EGFR expressing cells stained more intensely than EGFR negative cells. This result suggests that many antibodies were selected that bind antigens common to CHO cells with a minority of antibodies binding EGFR.

unique scFvs and used to stain cells which were analyzed by flow cytometry. Each of the monoclonal antibodies stained EGFR expressing cells (A431, MDA-MB-468 and CHO/EGFR) but not EGFR negative cells (MDA-MB-453 and CHO) both as phage antibodies (Fig. 3) and as native scFv (Fig. 4). The binding constant for EGFR of each of the native scFvs was determined on A431 cells and on MDA-MB-468 cells (for the E12 scFv). The K_D values ranged between 217 and 300 nM (Table 2).

3.4. Cell binding and internalization of phage antibodies and scFv

Since the phage antibodies were selected on the basis of internalization, we examined the ability of

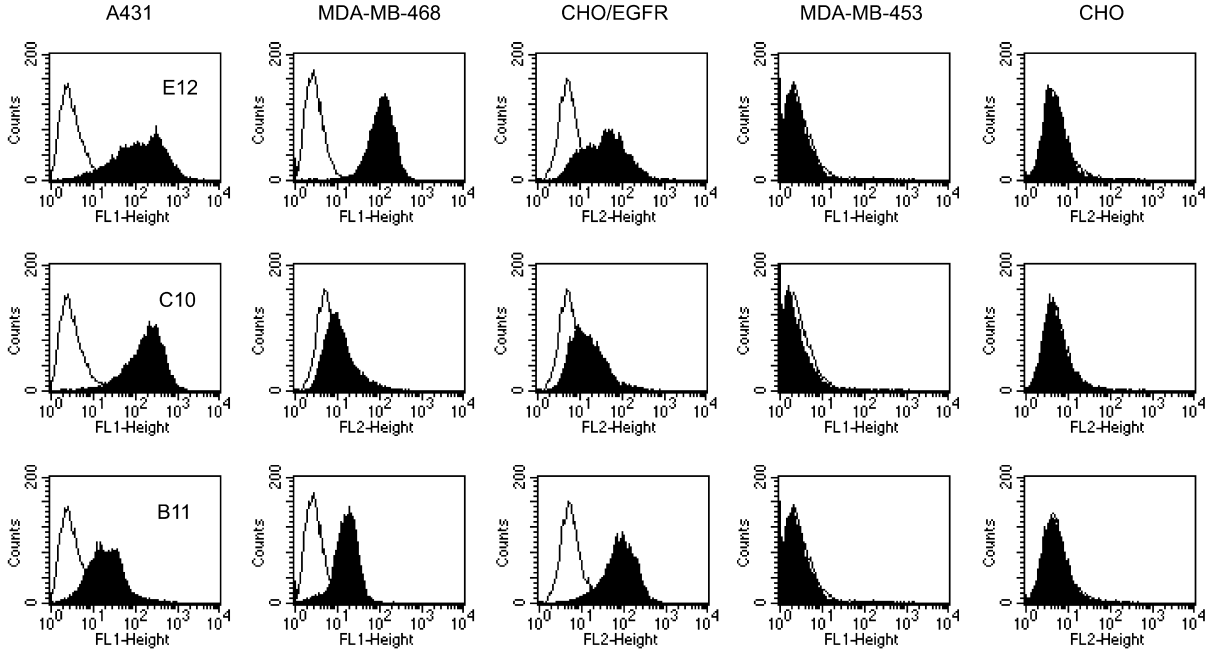

Fig. 3. Binding of monoclonal phage to EGFR positive and negative cell lines. Phage antibodies (E12, C10 and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

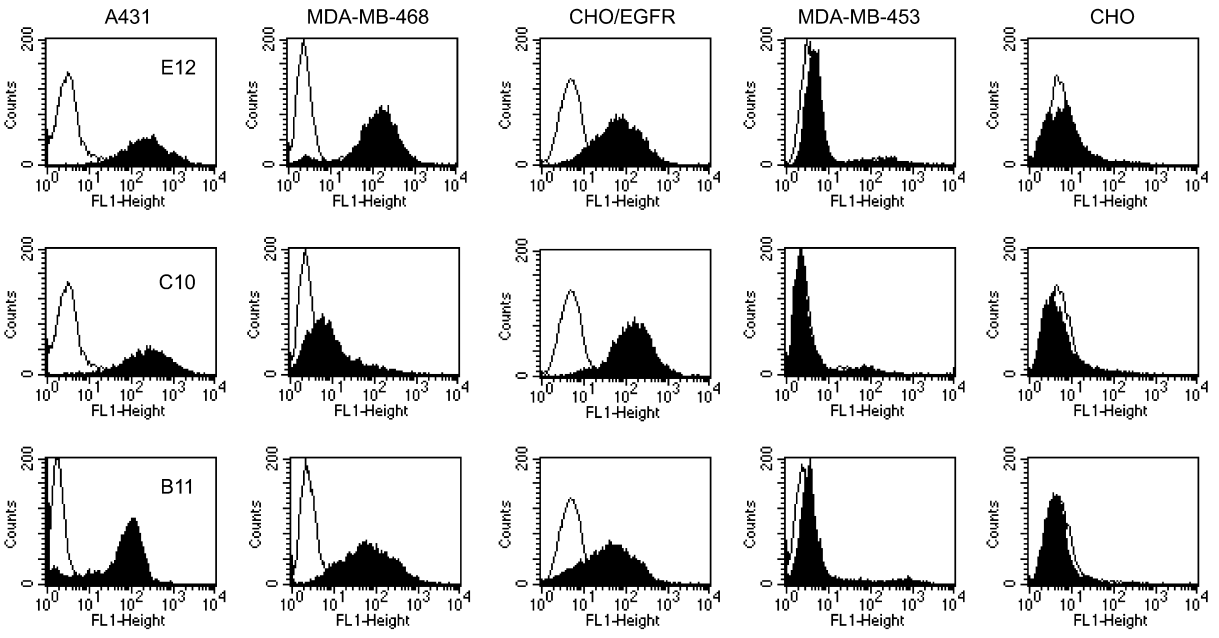

Fig. 4. Binding of monoclonal scFv to EGFR positive and negative cell lines. Purified scFvs (E12, C10, and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

Table 2
Binding affinity of α-EGFR scFv

scFv	K_D (nM)	
	A431	MDA-MB-468
E12	300	265
C10	217	–
B11	280	–

the phage antibodies to be endocytosed by EGFR expressing cells. After incubation of EGFR expressing and EGFR negative cells with phage antibodies, cells were fixed, surface phage removed with low-pH glycine and intracellular phage detected with anti-M13 antibody and confocal microscopy. Intracellular phage were detected in EGFR expressing cells (e.g., A431, MDA-MB-468 and CHO/EGFR) but not in EGFR negative cells (MDA-MB-453 and CHO). Representative results are shown for the E12 scFv on CHO/EGFR and CHO cells (Fig. 5) and on the tumor cell lines MDA-MB-468 and MDA-MB-453 (Fig. 6). To determine if native scFv were endocytosed by EGFR expressing cells, scFv were directly FITC labeled and incubated with live cells. After incubation, cells were analyzed directly by confocal microscopy allowing observation of surface bound and intracellular scFv. Staining of EGFR expressing cells was observed (e.g., A431, MDA-MB-468 and CHO/EGFR) but no staining was seen for EGFR negative cells (MDA-MB-453 and CHO). Much of the scFv remained surface bound, with some intracellular staining observed (Figs. 5 and 6 for representative results with E12 scFv). To determine the ability of the scFv to deliver a drug to EGFR expressing cells, immunoliposomes were constructed by fusing E12 scFv to the surface of HPTS containing liposomes. Strong intracellular fluorescence was observed for EGFR expressing cells, with no fluorescence observed for EGFR negative cells.

4. Discussion

The first step in developing a targeted cancer therapeutic is generating a ligand that specifically binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. Antibodies have proved to be important targeting ligands for cell surface receptors, especially with recent engineering techniques to generate antibodies which are entirely human in sequence. Libraries of antibodies displayed on phage can rapidly generate panels of human antibodies to a target antigen without the need for immunization (Marks et al., 1991; Sheets et al., 1998). To generate antibodies which bind native cell surface receptors, we recently demonstrated that phage could be directly selected on tumor cell lines by recovering endocytosed phage from within the target cell (Poul et al., 2000). Compared to simply recovering phage from the cell surface, intracellular phage recovery increases specific enrichment of antigen binding antibodies more than 10- to 30-fold (Becerril et al., 1999). High enrichment ratios are essential for successful selection of antibodies on heterogeneous antigens such as the surface of cells. In our previous publication, more than 10 unique tumor specific antibodies were generated, two of which were determined to bind ErbB2 and the transferrin receptor (Poul et al., 2000).

For this work, we demonstrate that this approach can be used to generate human scFv antibodies to a known tumor antigen (EGFR). EGFR is a 170-kDa transmembrane glycoprotein overexpressed in a number of human cancers. Ligand binding induces receptor dimerization which results in autophosphorylation of the kinase domain (Odaka et al., 1997; Tzahar et al., 1997). Receptor internalization occurs following dimerization and is believed to be a mechanism of receptor signal downregulation. EGFR antibodies were generated both by selecting on an overexpressing cell line or by using a cell line transfected with the target gene. The transfected human EGFR has been shown to function normally in its foreign environment: stimulation with EGF leads to receptor phosphorylation and receptor internalization follows activation. Selection on the EGFR transfected cell line permits use of the untransfected parental cell line to deplete the library of phage binding irrelevant receptors, enhancing enrichment ratios. The two cell lines should differ only in the presence of the target receptor. The availability of the untransfected cell line also provides an ideal reagent for the screening and characterization of antigen specific clones following selection. The ability to select on a transfected cell also eliminates

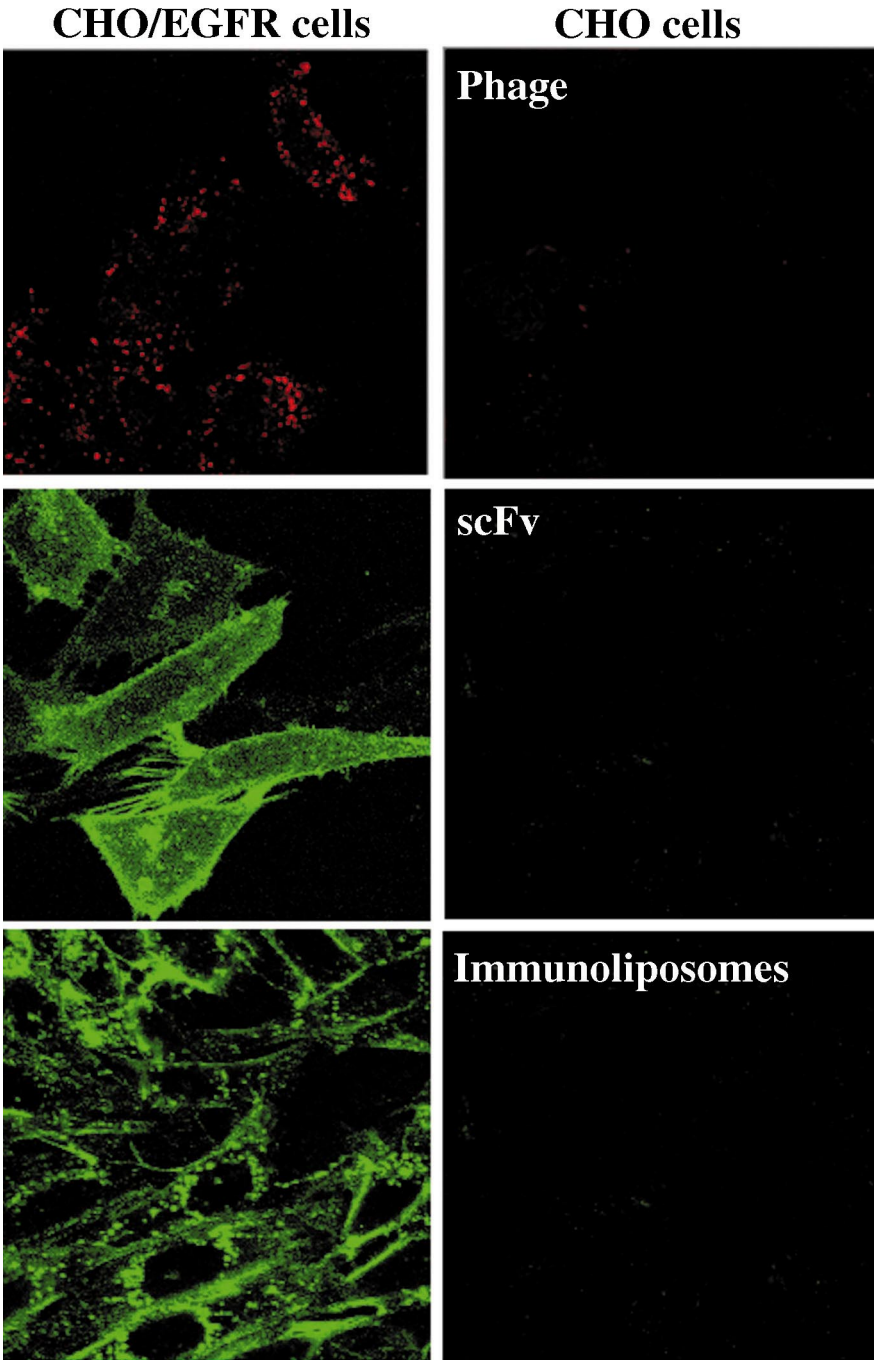

Fig. 5. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into CHO/EGFR and CHO cells. The E12 phage antibody was detected with α-M13-biotin followed by streptavidin–phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into CHO/EGFR and CHO cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv, and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

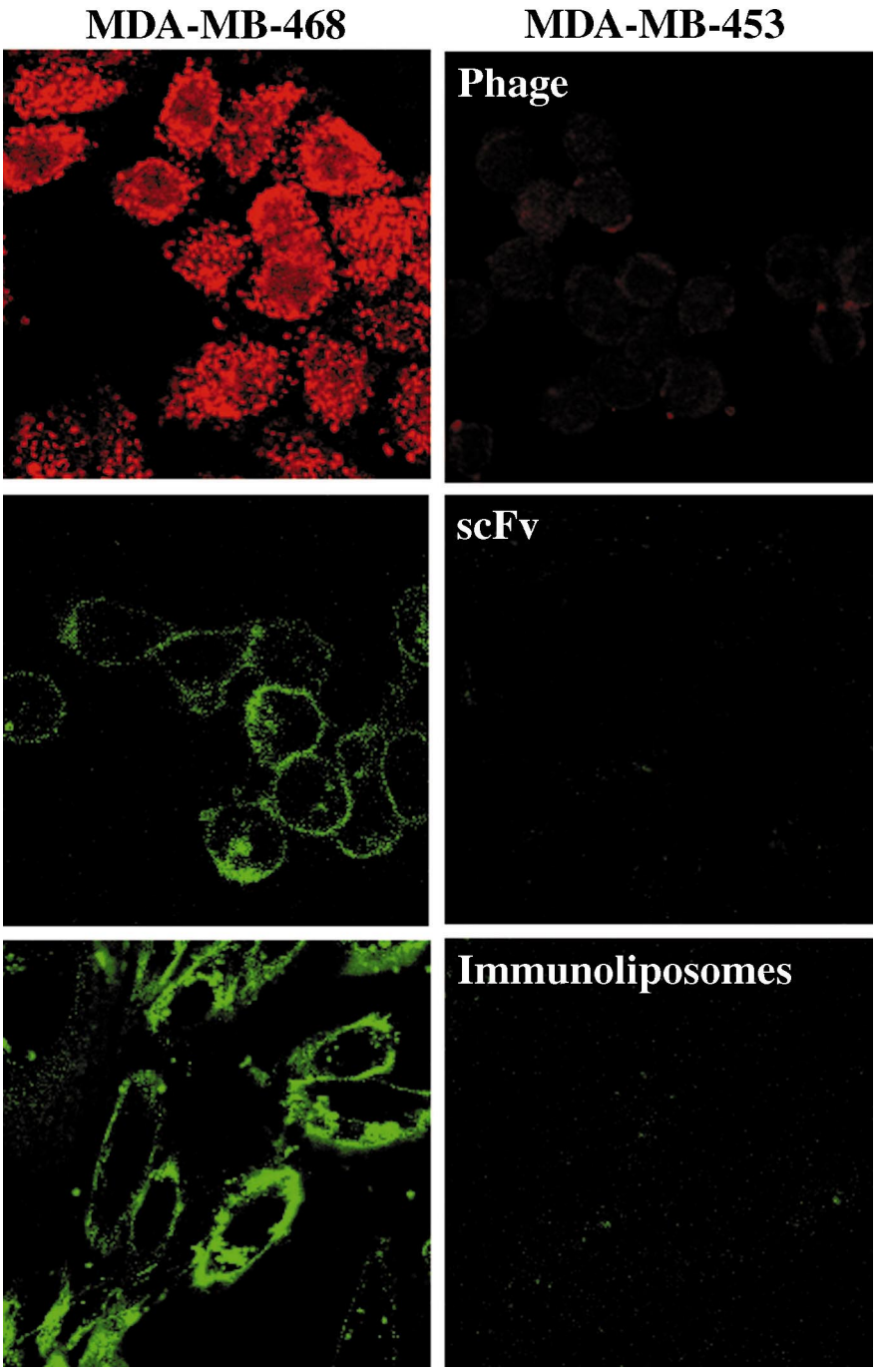

Fig. 6. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells. The E12 phage antibody was detected with α-M13-biotin followed by streptavidin–phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

the need to express and purify the target antigen in order to select antibodies. This could significantly speed development of antibodies to genes discovered as part of genomic sequences.

Selection on EGFR overexpressing A431 cells resulted in more efficient selection of EGFR antibodies than selection on CHO/EGFR cells (a higher percentage of antigen binding clones, although both selections yielded two unique antibodies). This occurred despite depletion of non-EGFR binding phage using the parental CHO cell line. In fact, the depletion was found to have been insufficient as FACS analysis showed that polyclonal phage bound both CHO/EGFR cells and CHO cells. The difference in efficiency between the two selections could potentially be attributed to a greater cell surface receptor density on A431 cells than on CHO/EGFR cells. Although not quantified, a Western blot of the cell lysates demonstrated a greater signal for A431 cells as compared to CHO/EGFR cells. Interestingly, one antibody was common to both selections (A431 or CHO/EGFR), whereas each of the remaining two antibodies were only selected on one of the cell types (A431 or CHO/EGFR). This result suggests that selection on multiple cell types may yield a greater number of antibodies.

The phage antibodies generated in this and previous work (Poul et al., 2000) were internalized by cells as determined by immunofluorescence and confocal microscopy. In both reports, the phage antibodies were selected from libraries where monomeric scFv were displayed as single copies in a phagemid system. In fact, all large non-immune libraries display monovalent antibody fragments (either scFv or Fab) as single copies using a phagemid vector. Since antibodies typically need to be bivalent to crosslink receptors and trigger endocytosis (Heldin, 1995; Yarden, 1990), successful selection of internalizing antibodies from phagemid libraries would require that: (1) the scFv formed spontaneous scFv dimers (diabodies) on the phage surface, as has been reported for some scFvs; (2) the monovalent scFv mimicked the natural receptor ligand leading to receptor aggregation and endocytosis or (3) increased phage display levels led to greater than one scFv per phage. In our previous work, the two scFvs studied extensively (anti-ErbB2 and anti-transferrin receptor) were stable scFv monomers in solution and were significantly endocytosed into cells as monomeric scFv. In the case of the transferrin receptor antibody, the scFv was a ligand mimetic and could compete with the natural ligand transferrin for binding to the receptor. In the case of the ErbB2 scFv, the mechanism by which it was endocytosed as a monomer is unknown. In the present work, the E12 scFv shows evidence of spontaneous dimerization (diabody formation) by gel filtration which could explain how it could crosslink receptors and trigger endocytosis. Interestingly, the purified scFv monomer (separated from dimer) shows significantly more surface membrane staining than intracellular staining (Figs. 5 and 6), especially compared to the multimeric immunoliposomes or to phage (which could be displaying dimeric scFv). In the case of the other two EGFR scFvs (which form stable monomers) the mechanism of endocytosis is unclear. We did not study whether the EGFR antibodies were ligand mimetics.

The approach described would be limited to those receptors capable of undergoing endocytosis. While this eliminates some useful cell surface targets, ligand binding and receptor internalization is a common mechanism for receptor and signaling regulation. Since most antibodies need to be bivalent to crosslink receptors and be efficiently endocytosed, one mechanism to increase the applicability of this selection methodology would be to construct bivalent diabody libraries in a phagemid vector or scFv libraries in a multivalent phage vector. This should open up the selection approach to more epitopes on more target antigens. Our model system results indicate that the most efficient selection format would be display on phage (Becerril et al., 1999), an approach which is presently under investigation.

The therapeutic utility of scFvs generated by this approach depends on the specific molecules to be targeted by the antibodies and the properties of the antibody. For many therapeutic approaches (immunotoxins, immunoliposomes, gene therapy) intracellular delivery of the toxic molecule is essential. Other approaches, for example bispecific antibodies or enzyme activated prodrugs, require that the antibody and effector molecule remain on the cell surface. Based on the present results (and our prior publication), the selection strategy described generates two types of scFv: those that are endocytosed in

their monomeric form (probably the majority of scFvs) and those that remain on the cell surface as monomers but are endocytosed when dimeric or multimeric. scFvs which are endocytosed as monomers could only be used for targeting effector molecules that are active intracellularly. scFvs which are primarily endocytosed as dimers could be used to leave effector molecules on the cell surface (when used as monomeric antibody fragments) or to deliver drugs intracellularly (when used as bivalent diabodies or IgG or when targeting multivalent nanoparticles).

In conclusion, we report the successful selection of EGFR antibodies from a phage library by selection for internalization into overexpressing cells or transfected cells. The scFvs are specific for EGFR expressing cells and can be used to target nanoparticles for intracellular drug delivery. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences.

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