Int. J. Cancer: **60,** 137–144 (1995)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

EGF RECEPTOR AND p185^{erbB-2}-SPECIFIC SINGLE-CHAIN ANTIBODY TOXINS DIFFER IN THEIR CELL-KILLING ACTIVITY ON TUMOR CELLS EXPRESSING BOTH RECEPTOR PROTEINS

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Many human tumors over-express erbB-2 and EGF receptors. The membrane localization of these receptor tyrosine kinases make them appropriate targets for directed tumor therapy. We have used recombinant DNA technology to produce single-chain antibody exotoxin A (scFv-ETA) fusion proteins which specifically bind the erbB-2 and EGF receptors. The scFv portion is composed of the heavy- and light-chain variable domains of monoclonal antibodies which recognize the extracellular portion of each receptor. We have previously described the anti-tumor activity of the bacterially produced scFv(FRP5)-ETA directed to the erbB-2 receptor. In this paper we describe the characteristics of scFv(225)-ETA, a protein which binds the EGF receptor. The bacterially produced recombinant protein binds to the receptor with high affinity and inhibits the *in vitro* growth of the EGF receptor over-expressing tumor cell lines A431 and MDA-MB468. Combination treatment with scFv-(FRP5)-ETA and scFv(225)-ETA led to an additive inhibitory effect on the *in vitro* growth of A431 cells. SKBR3 cells expressing low levels of EGF receptor but high levels of p185^{erbB-2} were not affected by scFv(225)-ETA treatment but were sensitive to scFv(FRP5)-ETA. Stimulation of SKBR3 cells and HC11 R1#11 mouse mammary epithelial cells expressing the human erbB-2 with EGF led to an increase in scFv(FRP5)-ETA activity, showing that the EGF-induced activation of erbB-2 can potentiate the action of the erbB-2-directed toxin. Treatment of athymic nude mice with scFv(FRP5)-ETA and the combination of both scFv-ETA proteins led to the transient arrest of growth of established A431 tumors. scFv(225)-ETA treatment alone was the most effective, leading to tumor shrinkage during the course of treatment, whereas treatment with the parental monoclonal antibody 225 led to retarded tumor growth.

Members of the growth-factor-receptor tyrosine-kinase family play an important role in the development of human malignancies. Many tumors of epithelial origin, including glioblastoma and cancers of the lung, breast, head and neck, and bladder express increased EGF receptor levels on their cell-surface membranes (reviewed in Gullick, 1991). The tumors with increased receptor expression sometimes display increased production of TGF-α, allowing receptor activation by an autocrine pathway (Derynck *et al.,* 1987). The c-*erb*B-2/*neu* gene coding for p185^{erbB-2}/HER2, another member of the sub-class-I family of growth factor receptors is amplified and/or over-expressed in a high percentage of human adenocarcinomas arising at numerous sites, including breast, ovary, lung, stomach and salivary gland (reviewed in Hynes, 1993). Many clinical studies have shown that patients with tumors showing elevated expression of these receptors have a poorer prognosis. The elevated expression of the erbB-2- and EGF-receptor proteins on the membrane of tumor cells and their involvement in the transformation process make them appropriate targets for directed therapy.

We have described monoclonal antibodies (MAbs) directed to the extracellular portion of the erbB-2-receptor protein which inhibit tumor-cell growth *in vitro* and *in vivo* (Harwerth *et al.,* 1992, 1993). Antibody domains derived by recombinant DNA [illegible] order to enhance their tumoricidal potential. We have constructed an erbB-2-specific single-chain antibody toxin, scFv-(FRP5)-ETA, which displays potent *in vitro* and *in vivo* tumor-cell-killing activity (Wels *et al.,* 1992*a;* Harwerth *et al.,* 1993). The biological characteristics of the EGF-receptor-specific MAb 225 have been studied in great detail. MAb 225 competes with EGF for binding to the EGF receptor, thereby blocking ligand-dependent receptor activation (Fan *et al.,* 1993). Treatment with MAb 225 inhibits the growth of EGF-receptor-expressing tumor cells *in vitro* and *in vivo* (Masui *et al.,* 1984; Ennis *et al.,* 1989).

We have constructed a recombinant single-chain immunotoxin consisting of a scFv domain derived from the MAb 225 and truncated *Pseudomonas aeruginosa* exotoxin A. Comparison of the anti-tumor effects of this bacterially expressed scFv(225)-ETA with those of the similar erbB-2-specific scFv-(FRP5)-ETA on human tumor cells expressing various levels of the respective target receptors revealed differences in toxin sensitivity which cannot be simply attributed to the different expression levels of the receptor proteins.

MATERIAL AND METHODS

Cells and culture conditions

The SKBR3 and MDA-MB468 human breast-tumor cells and the A431 human vulvar squamous-carcinoma cells were maintained in DMEM containing 8% FCS. Hybridoma cells producing the MAb 225 (IgG1, kappa) (Kawamoto *et al.,* 1983) were grown in RPMI 1640 containing 20% heat-inactivated FCS, 4 mM glutamine, 1 mM sodium pyruvate and 14.2 mM β-mercaptoethanol. The HC11 R1#11 cells expressing the human erbB-2 receptor (Hynes *et al.,* 1990) were grown in RPMI 1640 supplemented with 8% FCS and 5 μg/ml insulin.

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Abbreviations: MAb, monoclonal antibody; ETA, exotoxin A; scFv, single-chain antigen-binding protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EF-2, elongation factor 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; ELISA, enzyme-linked immunosorbent assay; IC_{50}, 50% inhibitory concentration; BSA, bovine serum albumin.

cDNA synthesis and construction of scFv

Total RNA was extracted from the 225 hybridoma cells by the acid-guanidium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). First-strand cDNA synthesis, carried out using a cDNA synthesis kit (Pharmacia Biotech, Brussels, Belgium), was in a standard 33-μl reaction containing 5 μg total RNA and 0.2 μg Not1-d$(T)_{18}$ primer. For amplification of V_H and V_L domains using PCR, 5 μl of the first-strand cDNA reaction was used as a template in a PCR as described (Wels *et al.,* 1992*b*). For amplification of the V_H domain, 50 pmol each of the oligonucleotides VH1FOR 5′-TGAGGAGACGGTGACCGTGGTCCCTTG-GCCCCAG-3′ and VH1BACK 5′-AGGTSMARCTGCAG-SAGTCWGG-3′ were used, for amplification of the V_L kappa domain, 50 pmol each of the oligonucleotides VKW1FOR 5′-GTTAGATCTCCARYTTKGTSCS-3′ and VK1BACK 5′-GACATTCAGCTGACCCAGTCTCCA-3′ were used (M = A + C, R = A + G, S = C + G, W = A + T, Y = C + T, K = G + T). PCR products were digested with PstI and BstEII (V_H) or PvuII and BglII (V_L). MAb 225 V_H cDNA was inserted into PstI/BstEII-digested plasmid pWW152, a derivative of the modified bluescript plasmid pWW15 (Wels *et al.,* 1992*b*) containing a sequence encoding the 15-amino-acid linker $(GGGGS)_3$. Subsequently the 225 V_L fragment was inserted 3′ of the V_H and linker sequences resulting in the scFv(225)-encoding plasmid pWW152-225. The scFv(FRP5) gene encoding an erbB-2-specific antibody domain was sub-cloned into pWW152 as a PstI/XbaI fragment derived from pWW15-5 (Wels *et al.,* 1992*b*). The plasmid pFLAG-1 (IBI Biochemicals, New Haven, CT, USA) was digested with HindIII and XbaI and a double-stranded DNA linker encoding 6 His residues at its 5′ end and the original HindIII-, EcoRI- and XbaI-restriction sites of pFLAG-1 at its 3′ end was inserted 3′ of the FLAG epitope. The resulting plasmid, pSW50, was digested with HindIII and XbaI and the scFv genes which were isolated from pWW152-225 and pWW152-5 as HindIII/XbaI fragments were inserted yielding the scFv expression plasmids pSW50-5 and pSW50-225.

Construction, expression and purification of scFv-ETA proteins

pFLAG-1 was digested with SalI and treated with the Klenow enzyme to create blunt ends; the linearized fragment was digested with XbaI. A truncated *Pseudomonas* ETA gene lacking the cell-binding domain Ia, was isolated from pWW20 (Wels *et al.,* 1992*a*) by EcoRI cleavage, Klenow fill-in and subsequent XbaI digestion. This blunt-ended XbaI fragment was inserted into the blunt-ended XbaI pFLAG-1 vector. The resulting plasmid, pSG100, was digested with HindIII and XbaI, and a double-stranded DNA linker encoding 6 His residues was inserted in frame 5′ of the ETA sequences yielding pSW200. DNA fragments containing the erbB-2- and EGF-receptor-specific scFv genes, scFv(FRP5) and scFv(225), including the ompA signal peptide, the FLAG epitope and the N-terminal His-encoding sequences from pSW50 were isolated from pSW50-5 and pSW50-225 by NdeI and XbaI digestion and inserted into NdeI/XbaI-digested pSW200. For expression of the scFv-ETA fusion proteins, the resulting plasmids pSW202-5 and pSW202-225 were transformed into *E. coli* strain CC118 (Manoil and Beckwith, 1985). A single colony was grown overnight at 37°C in LB medium containing 0.6% glucose and 100 μg/ml ampicillin. The culture was diluted 30-fold in the same medium, grown at 37°C to an OD_{550} of 0.5 and induced 45 min at 37°C with 0.5 mM IPTG. Cells were harvested by centrifugation at 4000 *g* for 15 min at 4°C and the cell pellet from 1 l of culture was lysed by freeze/thaw in 0.5 ml of 100 mM Tris-HCl, pH 8.0, 0.1 mg/ml lysozyme, 0.3 mM PMSF and 10 μg/ml DNAse I. PBS (15 ml) containing 8 M urea were added and incubated for 30 min at RT. The lysate was clarified by ultracentrifugation at 45,000 *g* for 30 min at [illegible] by centrifugation. scFv-ETA proteins were purified via binding of the 2 His clusters to Ni^{2+} loaded chelating sepharose (Pharmacia Biotech) followed by elution with a step gradient of 50 to 400 mM imidazole in PBS. Fractions containing the scFv-ETA proteins were pooled, imidazole was removed by dialysis against PBS, and the proteins were concentrated by ultrafiltration through a YMT10 membrane (Amicon, Beverly, MA). Purified proteins were analyzed by SDS-PAGE and quantitated by densitometry after Coomassie staining in comparison with BSA standards.

scFv(225)-ETA binding assay

The binding of scFv(225)-ETA to the EGF receptor on MDA-MB468 and SKBR3 cells was measured by ELISA (Wels *et al.,* 1992*a*). Cells were grown on 96-well microtiter plates, fixed with 2% formaldehyde in PBS and blocked with 3% BSA in PBS. scFv(225)-ETA (100 μl) at concentrations ranging from 1.2 nM to 150 nM was added to each well and the plates were incubated for 1 hr at 37°C. Unbound scFv(225)-ETA was removed and the cells were washed and incubated for 1 hr at 37°C with 100 μl of rabbit anti-ETA serum, then incubated with 100 μl of goat anti-rabbit IgG coupled to alkaline-phosphatase (Sigma, St. Louis, MO). The specifically bound scFv(225)-ETA was detected by 30-min incubation at 37°C with a solution of 1 M Tris-HCl (pH 8.0) and 0.4 mg/ml p-nitrophenylphosphate disodium (Sigma). The absorbance at 405 nm was measured.

EGF-receptor-activation assay

NE1 mouse fibroblasts expressing the human EGF receptor cDNA were grown for 16 hr in DMEM containing 0.5% FCS, then treated for 5 min at 37°C with 10 ng/ml EGF in the presence or absence of a 500-fold molar excess of competitor. Cell lysates were prepared using lysis buffer containing 200 μM sodium-orthovanadate and total proteins were separated by 7.5% SDS-PAGE and electroblotted onto polyvinylidenedifluoride membranes as described (Harwerth *et al.,* 1992). Phosphotyrosine-containing proteins were detected with a specific MAb; the filter was then treated with peroxidase-coupled anti-mouse IgG and bound antibody was visualized using the ECL system (Amersham, Aylesbury, UK).

Cell-killing assay

The cell-killing activity of scFv-ETA proteins was measured with the Cell Titer 96 Kit (Promega, Madison, WI) exactly as described (Wels *et al.,* 1992*a*).

Sub-cellular distribution of EGF receptor in EGF-treated SKBR3 cells

The kinetics of EGF-receptor internalization and its sub-cellular distribution were determined as described (Kornilova *et al.,* 1992). Briefly, iodinated EGF [20 ng/ml (^{125}I)EGF, Amersham; specific activity 100 mCi/mg] was bound to surface EGF receptors on SKBR3 cells for 60 min at 4°C in a working medium (WM) consisting of DMEM, 0.1% BSA and 20 mM HEPES pH 7.3. The cells were washed with cold WM to remove unbound (^{125}I)EGF and one plate was set aside at 4°C to determine the position of plasma membrane-bound EGF. EGF-receptor internalization was stimulated by placing cells in WM at 37°C. After 5, 15 or 30 min one plate was placed on ice and the surface (^{125}I)EGF was removed by washing for 3 min with 0.2 M acetic acid, pH 4.5, containing 0.5 M NaCl. The sub-cellular fractionation procedure was carried out on 17% Percoll gradients as described (Kornilova *et al.,* 1992).

In vivo *anti-tumor activity*

In vivo anti-tumor activity of MAb 225 and the scFv-ETA proteins was tested using A431 epidermoid-tumor xenografts in athymic nude mice. Approximately 25 mg of tumor tissue was implanted s.c. in each mouse (5 mice/group). Six days later [illegible]

or the scFv-ETA proteins. The control group received PBS. Tumor growth was followed as described (Wels *et al.,* 1992*a*).

RESULTS

Construction and expression of a gene encoding the chimeric scFv(225)-ETA protein

A gene encoding a protein consisting of the scFv of the EGF-receptor-specific MAb 225 (Sunada *et al.,* 1986) fused to domains II, Ib and III of the *Pseudomonas aeruginosa* ETA was constructed in the pFLAG-1 expression vector. Domain Ia of ETA is responsible for cell recognition, but is not necessary for its enzymatic activity, which is the ADP-ribosylation of EF-2 (Siegall *et al.,* 1989). The bacterial expression vector pSW202-225, shown schematically in Figure 1*a,* has an IPTG-inducible tac promoter followed by sequences encoding the ompA signal peptide, the FLAG epitope, 6 His residues, the V_H, linker, the V_L, 6 His residues and the ETA domains II, Ib and III. Figure 1*b* presents the sequence of the scFv(225) portion of the chimeric gene. The sequence of the toxin has been published (Gray *et al.,* 1984). Likewise, 2 clusters coding for 6 His residues each were introduced 5′ and 3′ of the scFv domain in the coding region of the erbB-2-specific scFv(FRP5)-ETA (Wels *et al.,* 1992*a*), resulting in the expression plasmid pSW202-5.

The scFv(225)-ETA and scFv(FRP5)-ETA fusion proteins were expressed in *E. coli* strain CC118. Total bacterial lysates were prepared in 8 M urea, the lysates were dialyzed against PBS, and the soluble scFv-ETA proteins were purified by binding to Ni^{2+} columns and elution with imidazole step gradients. Fractions containing the recombinant scFv-ETA proteins were pooled, imidazole was removed by dialysis and the proteins were concentrated by ultrafiltration. SDS-PAGE analysis of the purified material revealed a purity of more than 70% after a single round of Ni^{2+}-affinity purification (data not shown). The yield of purified scFv(225)-ETA and scFv(FRP5)-ETA from 1 l of bacterial culture was between 1 and 1.5 mg.

Binding properties of scFv(225)-ETA

The affinity of the purified recombinant scFv(225)-ETA for the EGF receptor was measured in an ELISA using MDA-MB468 and SKBR3 breast-tumor cells. MDA-MB468 cells have an amplified EGF-receptor gene and express approximately 1.5×10^6 receptors per cell (Filmus *et al.,* 1987). SKBR3 cells which have an amplified c-*erb*B-2 gene (Hynes *et al.,* 1989), but a single copy of the EGF-receptor gene, express approximately 9×10^4 EGF receptors per cell (data not shown). The cells were grown in 96-well dishes, scFv(225)-ETA at concentrations ranging from 1.2 to 150 nM was added to the wells, and the plates were incubated at 37°C for 1 hr. Specifically bound protein was determined after incubation with a rabbit anti-ETA serum followed by goat anti-rabbit IgG coupled to alkaline phosphatase. The phosphatase reaction product was measured as absorbance at 405 nm and the results are shown in Figure 2*a*. The apparent binding affinity of scFv(225)-ETA to the EGF receptor, measured as the half-maximal saturation value, is 12 nM. The apparent binding affinity for the parental MAb 225 was determined as 1 nM in a similar experiment (data not shown). The reported affinity of the 225 Fab′ fragment to the EGF receptor is 5 nM (Fan *et al.,* 1993), therefore the binding affinity of the monovalent recombinant protein is approximately 2-fold lower than that of the monovalent Fab′ fragment and 10-fold lower than that of the bivalent MAb.

In a similar experiment, the binding of purified scFv(FRP5)-ETA to the erbB-2 receptor on SKBR3 cells was measured. The apparent binding affinity of this version of the scFv(FRP5)-ETA containing 2 His clusters N- and C-terminal of the scFv domain specific for the erbB-2 receptor is 4.2 nM (data not

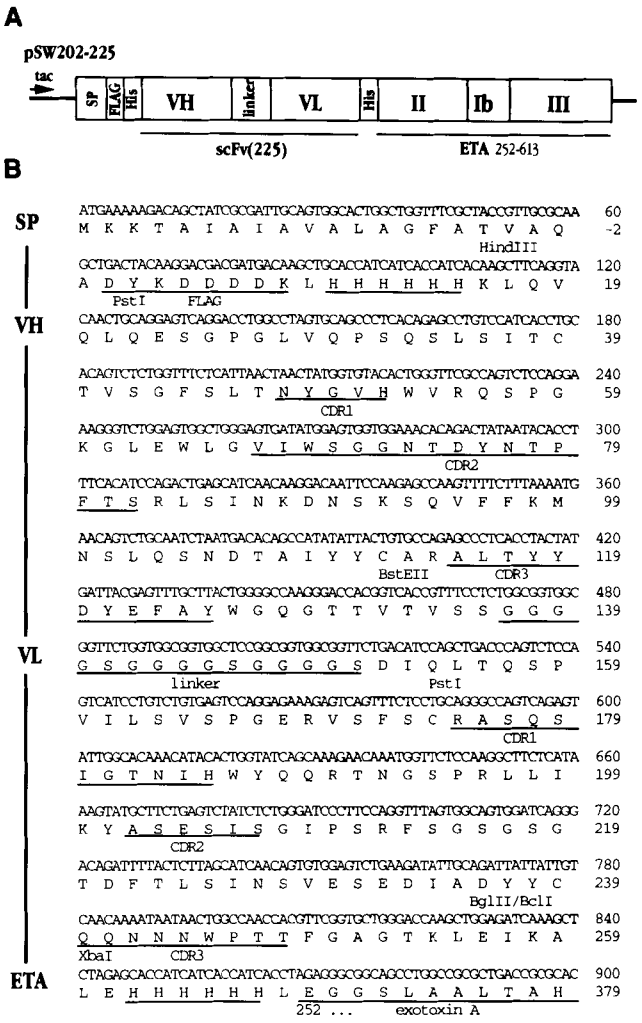

FIGURE 1 – *(a)* The scFv(225)-ETA expression plasmid pSW202-225. The plasmid contains the IPTG inducible tac promoter (tac), the ompA signal peptide (SP), the synthetic FLAG epitope (FLAG), a 6-residue His cluster (His), the PCR-amplified V_H cDNA of MAb 225, a sequence coding for a 15-amino-acid linker, the PCR-amplified V_L cDNA of MAb 225, a second His cluster, and the truncated *Pseudomonas* exotoxin A gene encoding domains II, Ib and III (amino acids 252-631 of ETA). *(b)* Partial nucleotide and deduced amino-acid sequence of the scFv(225)-ETA gene. The sequence shows: the ompA signal peptide (bp 1-63); the FLAG epitope (bp 64-87); the first His cluster (bp 91-108); the PstI/BstEII fragment encoding the V_H domain (bp 124-462); the 15-amino-acid linker (bp 472-516); the PvuII/BglII fragment encoding the V_L domain (bp 523-835); the second His cluster (bp 847-864); a partial sequence of the fragment encoding amino acids 252-613 of the *Pseudomonas* ETA protein (bp 868-900). The complementarity-determining regions (CDR) in the deduced amino-acid sequence of the 225 V_H and V_L and the linker sequences are underlined. The additional nucleotides represent vector sequences used for cloning.

previously described scFv(FRP5)-ETA protein purified via FLAG-affinity chromatography have very similar binding characteristics and cell-killing activity (Wels *et al.,* 1992*a*), indicating that the His clusters included in the molecule do not alter its biological activity.

scFv(225)-ETA competes with EGF for receptor binding

MAb 225 competes with EGF for receptor binding (Sunada

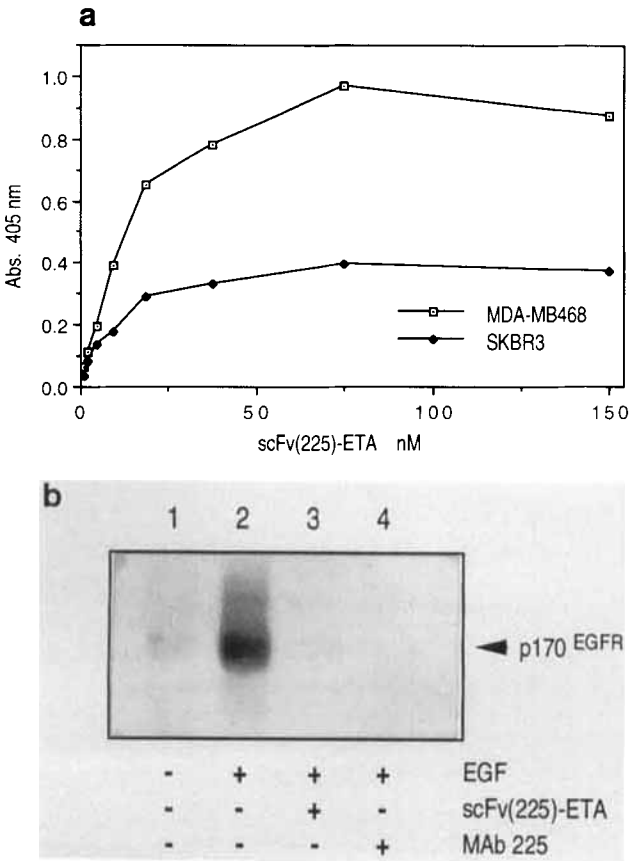

FIGURE 2 – *(a)* Binding of scFv(225)-ETA to the EGF receptor of MDA-MB468 and SKBR3 breast-tumor cells. Cells were fixed with 2% formaldehyde and incubated with various concentrations of scFv(225)-ETA. The amount of specifically bound scFv(225)-ETA was measured, after incubation with rabbit anti-ETA followed by AP-coupled goat anti-rabbit IgG, as the absorbance at 405 nm. Each point was determined in triplicate. The apparent binding affinity of scFv(225)-ETA to the EGF receptor on both cell lines is 12 nM. *(b)* Competition of EGF binding to the EGF receptor and inhibition of receptor activation by scFv(225)-ETA. NE1 mouse fibroblasts expressing human EGF-receptor cDNA were treated for 5 min at 37°C with 10 ng/ml EGF (lane 2), in the presence of a 500-fold molar excess of scFv(225)-ETA (lane 3) or a 500-fold excess of MAb 225 (lane 4) or were mock treated (lane 1). Equal amounts of cell lysates were analyzed by SDS-PAGE and phosphotyrosine was detected by immunoblotting with a specific anti-phosphotyrosine MAb. The position of the $p170^{EGFR}$ is indicated.

the recombinant scFv(225)-ETA protein was carried out by measuring inhibition of EGF-induced tyrosine phosphorylation with an excess of scFv(225)-ETA. Figure 2*b* shows that the increase in phosphotyrosine caused by 5-min incubation of NE1 mouse fibroblasts expressing a human EGF receptor cDNA with 10 ng/ml EGF (lane 2) was inhibited by a 500-fold molar excess of MAb 225 (lane 4) and scFv(225)-ETA (lane 3).

In vitro *toxicity and specificity of scFv(225)-ETA*

The cell-killing activity of scFv(225)-ETA was tested on MDA-MB468 cells using an enzymatic assay (Wels *et al.*, 1992*a*). The cells were incubated for 40 hr with 100 ng/ml (1.5 nM) of scFv(225)-ETA in the absence or presence of a 100-fold molar excess of MAb 225, and cell viability was measured in comparison with PBS-treated cells. The results are shown in Figure 3. Approximately 44% of the cells were

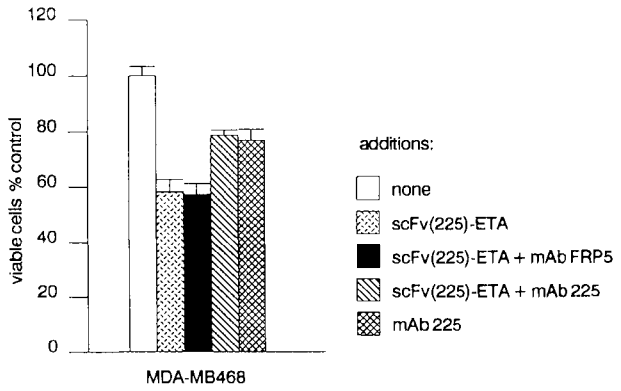

FIGURE 3 – Inhibition of scFv(225)-ETA cell-killing activity by competition with MAb 225. MDA-MB468 cells were incubated for 40 hr with 100 ng/ml scFv(225)-ETA without the addition of competitor or in the presence of 10 μg/ml MAb 225 or control MAb FRP5. The effect of MAb 225 was also tested. The relative number of viable cells was determined with an enzymatic assay as the absorbance at 570 nm as described in "Material and Methods". Each point was determined in triplicate.

the specific competitor, MAb 225, the cell-growth-inhibiting activity was reduced; whereas an excess of a non-specific MAb, FRP5, had no effect on the inhibiting activity of scFv(225)-ETA. The non-specific MAb FRP5 alone had no effect on the growth of MDA-MB468 cells (data not shown). MAb 225 itself inhibits the growth of MDA-MB468 cells via blocking autocrine stimulation by TGF-α (Ennis *et al.*, 1989). Similarly, MAb 225 alone at a concentration of 150 nM inhibits the growth of MDA-MB468 cells by 20% in this assay, indicating that the remaining growth-inhibiting activity observed with the scFv(225)-ETA/MAb 225 combination is probably caused by the excess of the parental MAb. The results also show that the cytotoxic fusion protein scFv(225)-ETA is more potent in inhibiting the *in vitro* growth of MDA-MB468 than a 100-fold molar excess of the original MAb 225.

In vitro *toxicity of scFv(225)-ETA and scFv(FRP5)-ETA*

We have described the properties of scFv(FRP5)-ETA a protein which specifically kills cells expressing the erbB-2 receptor (Wels *et al.*, 1992*a*). Since many tumor cells express erbB-2 and EGF receptors, we tested the killing activity of a combination of scFv(225)-ETA and scFv(FRP5)-ETA on 3 human tumor cell lines. A quantitative Western analysis for the relative level of erbB-2 and EGF receptor in SKBR3, A431 and MDA-MB468 cells is shown in Figure 4. A431 cells express approximately 50-fold less erbB-2 receptor than SKBR3 cells, and MDA-MB468 cells express no detectable erbB-2 (Fig. 4*a*); A431 and MDA-MB468 cells express high amounts of EGF receptors and SKBR3 cells express approximately 50-fold less (Fig. 4*b*).

The *in vitro* toxicity of the scFv(225)-ETA and the scFv(FRP5)-ETA proteins and a 1:1 combination of both were tested on the 3 human tumor cell lines. The cells were incubated for 40 hr with various concentrations of the scFv-ETA proteins, and the relative number of viable cells was determined using an enzymatic assay (Wels *et al.*, 1992*a*). The results are shown in Figure 5, and the IC_{50} values are summarized in Table I. As anticipated, the A431 cells were very sensitive to scFv(225)-ETA with an IC_{50} of 5.8 ng/ml. The A431 cells were also as sensitive to the scFv(FRP5)-ETA as were the SKBR3 cells (IC_{50} 33 *vs.* 34 ng/ml). For A431 cells, the combination of the 2 toxins was as active as the more potent (as a single modality) scFv(225)-ETA, suggesting an additive effect on these cells. MDA-MB468 cells were killed by

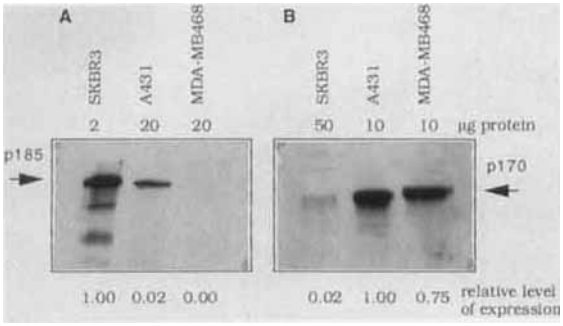

FIGURE 4 – Immunoblot of human tumor-cell extracts. The indicated amounts of protein from cellular extracts of SKBR3, A431 and MDA-MB468 human tumor cells were separated by 7.5% SDS-PAGE and blotted onto PVDF membranes. *(a)* The erbB-2 protein was detected with the 21N anti-serum. *(b)* The EGF receptor was detected with the 15E anti-serum (Ennis *et al.*, 1989). The position of the p185^{erbB-2} and the p170^{EGF} receptor and the relative level of expression are indicated.

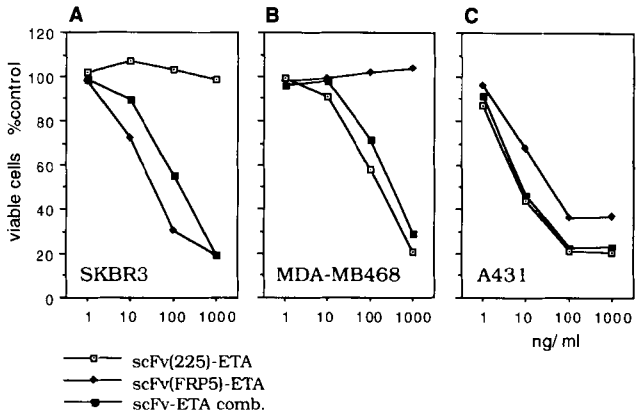

FIGURE 5 – Inhibition of the growth of human tumor cell lines by recombinant scFv-ETA proteins. SKBR3 *(a)*, MDA-MB468 *(b)* and A431 *(c)* tumor cells were incubated for 40 hr with the indicated concentrations of scFv(225)-ETA, scFv(FRP5)-ETA or a 1:1 scFv(225)-ETA/scFv(FRP5)-ETA combination. The relative number of viable cells was determined with an enzymatic assay as the absorption at 570 nm as described in "Material and Methods". Each point was determined in triplicate in 2 independent experiments.

TABLE I – *IN VITRO* TOXICITY OF scFv-ETA IMMUNOTOXINS

Cell line	IC_{50} ng/ml scFv-ETA immunotoxin[1]		
	scFv(225)-ETA	scFv(FRP5)-ETA	scFv-ETA combination
SKBR3	> 1000	34	108
MDA-MB468	112	> 1000	320
A431	5.8	33	6.8

[1]IC_{50} was determined in a cell-viability assay. The data are from Figure 5.

were not sensitive to the erbB-2-directed recombinant toxin. As shown (Wels *et al.*, 1992*a*), SKBR3 cells were killed by a low dose of scFv(FRP5)-ETA. Despite the fact that these cells have approximately 9 × 10^4 EGF receptors and bind the scFv(225)-ETA with the same kinetics as the MDA-MB468 cells which are killed by the toxin, the SKBR3 cells were not killed by up to 1000 ng/ml of scFv(225)-ETA. The parental MAb 225 at a concentration of 10 µg/ml also had no effect on

erbB-2-directed toxin efficiently kills the A431 cells, in which the numbers of erbB-2 receptors are similar to those of EGF receptors in SKBR3 cells, the EGF receptor in SKBR3 cells was examined further.

For their activation, ETA and scFv-ETA fusion proteins require internalization and cleavage of the ETA portion by a cellular protease (Zdanovsky *et al.*, 1993). We have reported that short-term treatment of SKBR3 cells with EGF leads to an increase in phosphotyrosine on erbB-2 and on other cellular proteins (Harwerth *et al.*, 1992), showing that the receptors are active kinases. The cleavage and activation of ETA occurs in the low-pH compartment of the endosomes (Zdanovsky *et al.*, 1993). Activated EGF receptors normally pass through this compartment on the way to degradation in the lysosomes (Sorkin *et al.*, 1988). The dynamics of EGF-receptor internalization in SKBR3 cells was examined by cellular-fractionation experiments (Kornilova *et al.*, 1992). SKBR3 cells were incubated at 4°C with (^{125}I)-EGF. Receptor internalization was promoted by raising the temperature of the cells to 37°C. At various times, cells were placed on ice, the surface-bound (^{125}I)-EGF was removed by an acid-salt wash, and the subcellular fractions were analyzed on Percoll gradients for the content of receptor-bound (^{125}I)-EGF. The results are shown in Figure 6. Fifteen minutes after the cell temperature is raised to 37°C most of the internalized EGF receptors can be found in the heavy endosomes and lysosomes. After 30 min, a substantial portion of the receptors have been degraded. The results suggest that the inability of scFv(225)-ETA to kill SKBR3 cells is not due to the EGF receptor *per se,* since it is activated and internalized rapidly following ligand treatment.

Effect of EGF on the activity of scFv(FRP5)-ETA

We have reported that EGF treatment accelerates erbB-2-receptor turnover in HC11 cells (Kornilova *et al.*, 1992). A431 cells express TGF-α, which is transported to the cell surface, leading to autocrine stimulation of the EGF receptor (Van de Vijver *et al.*, 1991) which might in turn accelerate erbB-2 turnover. To test whether activation of the EGF receptor can influence the activity of the erbB-2-directed scFv(FRP5)-ETA on cells which lack a TGF-α autocrine-stimulatory loop, SKBR3 and HC11 R1#11 cells were treated with increasing concentrations of scFv(FRP5)-ETA, with or without the addition of 20 ng/ml EGF (SKBR3) or 10 ng/ml EGF (HC11 R1#11) respectively. HC11 R1#11 cells are mouse mammary epithelial cells expressing human *erb*B-2 cDNA (Hynes *et al.*, 1990). Cells were treated for 40 hr and cell viability was determined using an enzymatic assay as described. Figure 7 shows that SKBR3 (upper panel) and HC11 R1#11 cells (lower panel) were, respectively, 1.8 and 3.1 times more sensitive to scFv(FRP5)-ETA in the presence of EGF, with IC_{50} values in this particular set of experiments of 38 *vs.* 70 ng/ml for SKBR3 cells and 17 *vs.* 58 ng/ml for HC11 R1#11 cells. This suggests that the EGF transactivation of erbB-2 stimulates toxin internalization or activation in these cells.

scFv(225)-ETA and scFv(FRP5)-ETA suppress tumor growth

The *in vivo* anti-tumor activity of scFv(225)-ETA was tested on A431 xenografts in nude mice. A431 tumor tissue (25 mg) was implanted s.c. into 5 groups of 5 mice each on day 0. Six days later, when the tumors had reached a size of approximately 100 mm^3, treatment was begun. The mice received twice-daily i.p. injections of 5 µg of MAb 225, scFv(225)-ETA, scFv(FRP5)-ETA, or a combination of 2.5 µg of each scFv-ETA protein for a total of 10 days. Control mice received PBS. The results are shown in Figure 8. None of the treatments led to complete inhibition of tumor-cell growth. The scFv(225)-ETA-treated mice showed greater inhibition of tumor growth than MAb 225-treated mice (Fig. 8*a*). The onset of tumor-cell growth was delayed by 9 *vs.* 6.5 days. By day 25, when the

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