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TARGETING TUMOR CELLS *VIA* EGF RECEPTORS: SELECTIVE TOXICITY OF AN HBEGF-TOXIN FUSION PROTEIN

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Over-expression of the epidermal growth factor receptor (EGFR) is a hallmark of numerous solid tumors, thus providing a means of selectively targeting therapeutic agents. Heparin-binding epidermal growth factor (HBEGF) binds to EGFRs with high affinity and to heparan sulfate proteoglycans, resulting in increased mitogenic potential compared to other EGF family members. We have investigated the feasibility of using HBEGF to selectively deliver a cytotoxic protein into EGFR-expressing tumor cells. Recombinant fusion proteins consisting of mature human HBEGF fused to the plant ribosome-inactivating protein saporin (SAP) were expressed in *Escherichia coli.* Purified HBEGF-SAP chimeras inhibited protein synthesis in a cell-free assay and competed with EGF for binding to receptors on intact cells. A construct with a 22-amino-acid flexible linker (L_{22}) between the HBEGF and SAP moieties exhibited an affinity for the EGFR that was comparable to that of HBEGF. The sensitivity to HBEGF-L_{22}-SAP was determined for a variety of human tumor cell lines, including the 60 cell lines comprising the National Cancer Institute Anticancer Drug Screen. HBEGF-L_{22}-SAP was cytotoxic *in vitro* to a variety of EGFR-bearing cell lines and inhibited growth of EGFR-over-expressing human breast carcinoma cells *in vivo*. In contrast, the fusion protein had no effect on small-cell lung carcinoma cells, which are EGFR-deficient. Our results demonstrate that fusion proteins composed of HBEGF and SAP exhibit targeting specificity and cytotoxicity that may be of therapeutic value in treating a variety of EGFR-bearing malignancies. *Int. J. Cancer* 78:106–111, 1998.

Numerous reports have demonstrated the ability of chimeric molecules containing growth factor and toxin moieties to selectively target and inhibit the growth of cells bearing their cognate growth factor receptors. These chimeras, termed mitotoxins or oncotoxins, bind to the surface of target cells *via* a growth factor receptor, are internalized by receptor-mediated endocytosis and trigger cell death. Several plant proteins that are toxic to eukaryotic cells as a result of catalytic inactivation of ribosomes have been identified (Stirpe *et al.*, 1992). The eukaryotic cytotoxicity of these plant ribosome-inactivating proteins (RIPs) has been exploited to create potent mitotoxins. For example, basic fibroblast growth factor (FGF2) has been linked to saporin (SAP) to target FGF receptor-expressing cells (McDonald *et al.*, 1996). This FGF2-SAP chimera exhibits targeting specificity to FGF receptors and high cytotoxicity *in vitro* and *in vivo* for transformed cells (Beitz *et al.*, 1992), lens epithelial cells (Behar-Cohen *et al.*, 1995) and smooth muscle cells (Farb *et al.*, 1997).

The epidermal growth factor (EGF) ligand family includes EGF, transforming growth factor alpha (TGF-α), amphiregulin, heparin-binding EGF (HBEGF) and related polypeptides that stimulate approx. 170-kDa transmembrane glycoprotein receptors with tyrosine kinase activity. Up-regulated expression of EGFRs and EGFR ligands has been implicated in numerous pathological conditions and is presumed to be one means by which cells acquire a selective growth advantage. For example, EGFR over-expression has been demonstrated in numerous tumor cell lines and solid human tumors, including glioblastoma, melanoma and lung, breast and bladder carcinomas, and is associated with reduced survival rates in human breast and bladder cancers (Davies and Chamberlin, 1996). Accordingly, EGFRs are an attractive target for the delivery of therapeutic agents.

HBEGF was first identified as a 22-kDa secreted product of cultured human macrophages and has since been identified in a variety of tissues (Raab and Klagsbrun, 1997). HBEGF is derived from a transmembrane-anchored precursor from which proteolysis generates a family of 8- to 10-kDa biologically active proteins. Like other members of the EGF family, HBEGF binds to EGFRs and triggers receptor tyrosine kinase activity and cell proliferation. Unlike EGF and TGF-α, HBEGF also interacts with cell-surface heparan sulfate proteoglycans, thus increasing its mitogenic potential (Raab and Klagsbrun, 1997).

Because of its increased potency compared to other EGF family members, we examined the feasibility of using HBEGF to target cytotoxic agents to EGFR-bearing cells. We report here that novel mitotoxins employing mature human HBEGF as the targeting agent and SAP as the cytotoxic agent can effectively compete with EGF for receptor binding and show selective cytotoxicity, *in vitro* and *in vivo*, to tumor cells bearing EGFRs.

MATERIAL AND METHODS

Plasmid construction

All enzymes were obtained from Boehringer-Mannheim (Indianapolis, IN). DNA fragments were purified from agarose gels using the Geneclean II kit (Bio 101, Vista, CA). PCR and sequencing primers were synthesized on a Cyclone Plus DNA Synthesizer (Millipore, Bedford, MA). All PCR-generated fragments were sequenced using Sequenase Version 2.0 (Amersham, Arlington Heights, IL).

PCR was used to generate 2 overlapping *HBEGF* fragments from a plasmid containing cDNA encoding human HBEGF (pJMU2–1; generously provided by Dr. J. Abraham, Scios Nova, Mountain View, CA; GenBank accession number M60278). Internal *Nco*I sites were mutated without changing the amino acid composition of the corresponding protein. The 5' *HBEGF* fragment spans codons 13–129 in the HBEGF precursor and was generated with the "sense" primer 5′-CTG GCT GCA GTT CTC TCG GCA-3′, which contains a *Pst*I site, and the anti-sense primer 5′-AGC CCG GAG CTC CTT CAC ATA TTT GCA TTC TCC GTG GAT GCA GAA-G-3′. The 3′ *HBEGF* fragment spans from codon 124 to downstream of the HBEGF-coding region and was generated using the sense primer 5′-GTG AAG GAG CTC CGG GCT CCC TCC TGC ATC TGC CAC CCG GGT TAT CAT GGA GAG AGG-3′ and the anti-sense primer 5′-ATA TAG AAT TCT GTC TTC TCA GAG GTA-3′, which contains an *Eco*RI site. The 2 PCR-generated fragments overlapped at a *Sac*I site and were ligated into the *Pst*I and *Eco*RI sites of the vector pGEM-4 (Promega, Madison, WI). Using this plasmid as a template, the region encoding mature HBEGF (amino acids 73–149 in the precursor) was amplified by PCR using the sense primer 5′-CTG

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GAC CAT ATG AGA GTC ACT TTA-3′, which adds an *Nde*I site and a methionine codon to the 5’ end of HBEGF, and an anti-sense primer 5′- ATA TAC CAT GGC TGG GAG GCT CAG CCC ATG ACA-3′, which introduces an *Nco*I site immediately downstream of the coding region for HBEGF. The plasmid pZ1B contains the gene encoding the mitotoxin FGF2-SAP cloned into the prokaryotic expression vector pET11a (Novagen, Madison, WI) (McDonald *et al.*, 1996). *FGF2* sequences were specifically removed from pZ1B by digestion with *Nde*I and *Nco*I and replaced with the *HBEGF* sequence. The resulting plasmid encodes a fusion protein (designated HBEGF-SAP) that consists of 78 amino acids of HBEGF, an Ala-Met spacer encoded by the *Nco*I site and 253 amino acids of SAP, has a predicted m.w. of 37.6 kDa and an isoelectric point of 9.6.

Complementary single-stranded oligos were synthesized (Midland Certified Reagent Company, Midland, TX) and annealed to generate a linker with *Nco*I ends that encoded $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_{x4}$. The sequence of the sense oligo was 5′-CTA TAC CAT GGG CGG CGG CGG CTC TGG CGG CGG CGG CTC TGG CGG CGG CGG CTC TGG CGG CGG CGG CTC TGC CAT GGT ATA-3′. The plasmid encoding HBEGF-SAP was digested with *Nco*I, which cuts between the *HBEGF* and *SAP* moieties, and the *Nco*I-digested linker was ligated into the plasmid. Sequencing of the resulting plasmid revealed a 22-amino-acid linker encoding $Ala\text{-}Met\text{-}Gly_4\text{-}Ser\text{-}Gly_2\text{-}Ser\text{-}Gly_4\text{-}Ser\text{-}Gly_4\text{-}Ser\text{-}Ala\text{-}Met$ (L_{22}). The divergence from the intended linker sequence was likely caused by a secondary structure formed during primer annealing because of sequence redundancy and high GC content of the primers. HBEGF-L_{22}-SAP is 353 amino acids long, with a predicted m.w. of 38.9 kDa and an isoelectric point of 9.6.

Expression and purification of HBEGF-SAP fusion proteins

For expression, plasmid DNAs were transformed into the *Escherichia coli* strain BL21(DE3) (Novagen), which contains chromosomal copies of the T7 RNA polymerase gene linked to an IPTG-inducible *lac*UV promoter. Transformed cells were grown in a 7L Applikon (Foster City, CA) fermenter in complex batch media as previously described for FGF2-SAP (McDonald *et al.*, 1996). The culture was induced with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) at an A_{600} of 85. Cells were harvested by centrifugation (8,000 *g*, 10 min) 4 hr after induction, and the culture paste was stored at –80°C until ready for processing.

The HBEGF-SAP fusion proteins were purified using steps similar to those employed for purification of recombinant FGF2-SAP (McDonald *et al.*, 1996). Briefly, bacterial pellets were resuspended in 3–4 volumes of 10 mM sodium phosphate, pH 6, containing 10 mM EDTA, 10 mM EGTA and 50 mM NaCl, then passed 3 times through a microfluidizer (Microfluidics, Newton, MA) at 18,000 lb/in^2. Lysates were subjected to expanded-bed adsorption chromatography using Streamline cation-exchange resin (Pharmacia, Piscataway, NJ). Partially purified fusion proteins were then purified to homogeneity by a combination of anion-exchange (Q-Sepharose, FF), cation-exchange (SP-Sepharose, HP) and hydrophobic interaction (Phenyl-Sepharose, HP) chromatographies before buffer exchanging the purified proteins into 10 mM sodium citrate, pH 6, containing 0.1 mM EDTA and 0.14 M NaCl by size-exclusion chromatography (Sephacryl S100, HR). The final materials were over 98% pure, as evaluated by SDS-PAGE, reverse-phase HPLC and size-exclusion HPLC (data not shown). All purified proteins were stored at –80°C.

Human tumor cell lines

The cell lines employed in this study were obtained from 3 sources. The 60 cell lines comprising the NCI Anticancer Screen have been described previously (Monks *et al.*, 1991). The colon lines N87 and SW948 and the ovarian lines OVCAR2 and OVCAR10 were generously provided by Dr. L.M. Weiner (Fox Chase Cancer Center, Philadelphia, PA). N87, OVCAR2 and OVCAR10 cells were maintained in RPMI 1640. SW948 cells were maintained in L-15 medium. All other cell lines were obtained from the ATCC (Rockville, MD) and maintained according to the conditions specified by the ATCC. MDA-MB-468 cells were maintained in medium containing 5% FBS, and all other cells were maintained in medium containing 10% FBS (Hyclone, Logan UT).

Protein synthesis inhibition assays

The protein synthesis-inhibitory activities of the fusion proteins and of native SAP were determined by measuring effects on *in vitro* translation of luciferase RNA. SAP was purified from the seeds of *Saponaria offıcinalis* as previously described (Stirpe *et al.*, 1983). Reagents for the assay were obtained from Promega unless specified otherwise. Briefly, samples were serially diluted in 20 mM Tricine, pH 7.8 (Sigma, St. Louis, MO), and 5 µl of diluted protein were combined with 5 µl of reaction mix (50 µg/ml luciferase RNA, 0.1 mM amino acid mixture minus leucine, 0.1 mM amino acid mixture minus methionine) and 15 µl of rabbit reticulocyte lysate. Samples were incubated for 1 hr at 30°C and diluted 1:11 in 20 mM Tricine, pH 7.8; 10 µl were transferred in duplicate to a Dynatech (Chantilly, VA) 96-well plate. Using a Dynatech ML3000 luminometer that was pre-warmed to 30°C, 50 µl of luciferase assay reagent were injected per well and relative light units were determined at room temperature with a 1-sec delay, a 5-sec integration and medium gain.

Receptor-binding studies

The relative affinities of the fusion proteins for the EGFR were determined by binding competition assays. A431 cells were plated onto 24-well plates at 16,000 cells per well. The next day, plates were put on ice for 10 min, then the media was aspirated and the cells were washed with 0.5 ml of ice-cold binding buffer (DMEM containing 1 mg/ml BSA and 50 mM *N,N*-bis[2-hydroxy-ethyl]-2-aminoethane sulfonic acid). Sample wells were treated in duplicate with 200 µl of binding buffer, 50 µl of test protein (or binding buffer for controls) and 50 µl (0.075 µCi) of [^{125}I]-EGF (Amersham). Recombinant human HBEGF was obtained from R&D Systems (Minneapolis, MN). Plates were incubated for 2 hr at 4°C with gentle agitation. Cells were then washed 3 times with cold binding buffer and lysed by the addition of 500 µl of lysis buffer (10 mM Tris, pH 8; 0.5% SDS; 1 mM EDTA). The radioactivity in each sample was determined using a Cobra II auto gamma counter (Packard, Meriden, CT).

In vitro *cytotoxicity assays*

HBEGF-L_{22}-SAP was submitted to the National Cancer Institute Anticancer Screen (NSC D672640) to measure growth inhibition in a panel of 60 human tumor cell lines representing 9 tumor types (Monks *et al.*, 1991). Cells were treated in duplicate over a range of concentrations from 0.012 to 120 nM, and after 48 hr a sulforhodamine B (SRB) assay was performed (Monks *et al.*, 1991). All other cell lines were evaluated for sensitivity to HBEGF-L_{22}-SAP, using the MTT colorimetric assay, as previously described (McDonald *et al.*, 1996). Briefly, culture media were removed 24 hr after plating and duplicate wells were treated with fresh media containing serially diluted test samples. After an additional 48 hr, cell survival was estimated by the ability of live cells to reduce MTT. Absorbance was read at 550 nm on a Molecular Devices (Sunnyvale, CA) plate reader using Softmax Pro software.

Inhibition of tumor growth in vivo

MDA-MB-468 human breast cancer cells (2 x 10^6) in 0.1 ml of PBS were injected into the thoracic mammary fatpad of female nude mice. Tumor growth was monitored weekly, using calipers to measure 2 orthogonal diameters. When mean tumor diameters reached 5 mm (day 52), HBEGF-L_{22}-SAP (10 ng/g in 0.05 ml PBS) was injected s.c. adjacent to the tumors twice weekly for 4 weeks. The treated animals showed no signs of drug-induced toxicity. Tumor growth was monitored for up to 35 days after the last treatment. At the end of the experiment, mice were killed and residual tumors weighed. Student’s *t*-test was used to evaluate the statistical significance of differences in tumor weight. Mice were maintained in specific pathogen-free conditions in a facility

approved by the AALAC (Rockville, MD). Animal care and experimental procedures were in accordance with the regulations and standards of the USDA, DHSS and NIH.

RESULTS

Expression and purification of recombinant HBEGF-SAP mitotoxins

A bacterial expression system was employed for the production of HBEGF-SAP fusion proteins. The initial HBEGF-SAP fusion protein consisted of a 78-amino-acid isoform of HBEGF, an Ala-Met linker and the coding sequence of SAP. Because the receptor-binding domain is in the C-terminal half of HBEGF, we reasoned that receptor binding, and thus activity of the mitotoxin, may be improved by introducing a long, flexible linker between the HBEGF and SAP moieties. Therefore, DNA encoding a flexible linker consisting of $AlaMetGly_4SerGly_2SerGly_4SerGly_4SerAlaMet$ (L_{22}) was inserted between the sequences encoding HBEGF and SAP. *E. coli* fermentation resulted in accumulation of appropriately sized proteins that were readily detectable in crude lysates by Western blotting with anti-SAP anti-sera (data not shown). Fusion proteins were purified from the soluble fraction of bacterial lysates using a straightforward strategy that yielded highly pure material (see ''Material and Methods'').

In vitro *activities of HBEGF-SAP mitotoxins*

HBEGF-SAP and HBEGF-L_{22}-SAP were tested for the ability to inhibit protein synthesis in a cell-free system (Fig. 1). Native SAP exhibited an IC_{50} of 7 pM in this assay, and although significantly less active than native SAP, HBEGF-SAP showed a dose-dependent inhibition of protein synthesis with an IC_{50} of 0.16 nM. HBEGF-L_{22}-SAP exhibited activity comparable to that of HBEGF-SAP (IC_{50} = 0.15 nM), suggesting that the flexible linker had no effect on the RIP activity of the fusion protein.

A431 is a human epidermoid carcinoma cell line that expresses high numbers of cell-surface EGFRs (approx. 3 x 10^6/cell). HBEGF-SAP and HBEGF-L_{22}-SAP were tested for the ability to compete with [^{125}I]-labeled EGF for binding to EGFRs on human A431 cells (Fig. 2). While HBEGF-SAP competed for receptor binding, though not as effectively as HBEGF, HBEGF-L_{22}-SAP was nearly as effective as recombinant HBEGF. Therefore, insertion of a long, flexible linker between HBEGF and SAP is favorable for EGFR binding. Taken together, the RIP and receptor-binding assays demonstrate that while the HBEGF and SAP

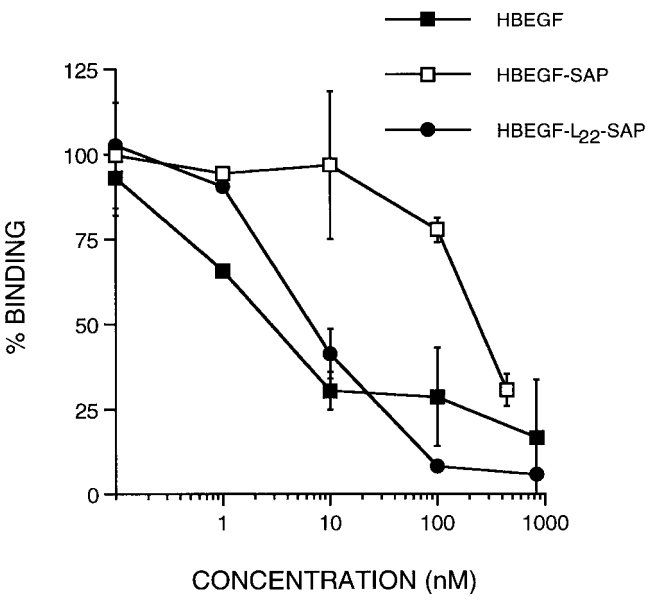

FIGURE 2 – Receptor-binding analysis of HBEGF-SAP mitotoxins. The ability of rHBEGF and the HBEGF-SAP mitotoxins to compete with [^{125}I]-EGF for receptor binding on A431 cells was determined as described in ''Material and Methods''. The plotted values are the means ± SD (n =2), and the data are representative of at least 2 independent experiments.

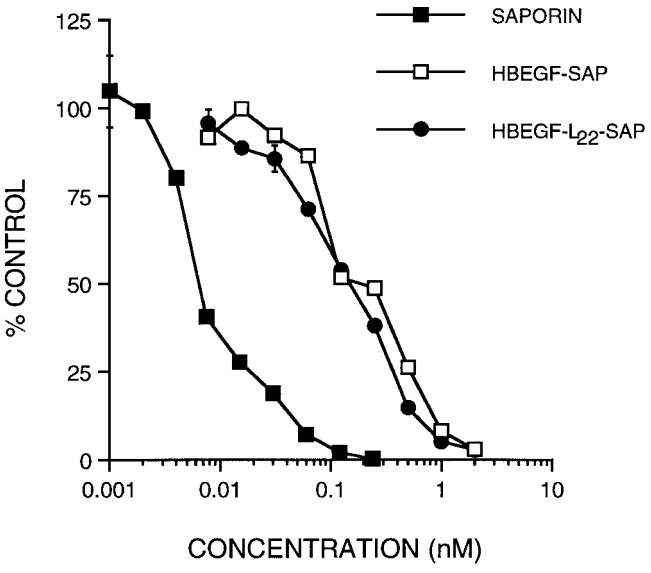

FIGURE 1 – Inhibition of protein synthesis *in vitro* by HBEGF-SAP mitotoxins. The inhibition of luciferase RNA translation in a cell-free system was measured as described in ''Material and Methods''. The plotted values are the means ± SD (n =2) and the data are representative of 2 independent experiments. The calculated IC_{50} values are saporin, 0.007 nM; HBEGF-SAP 0.16 nM; HBEGF-L$_{22}$-SAP 0.15 nM.

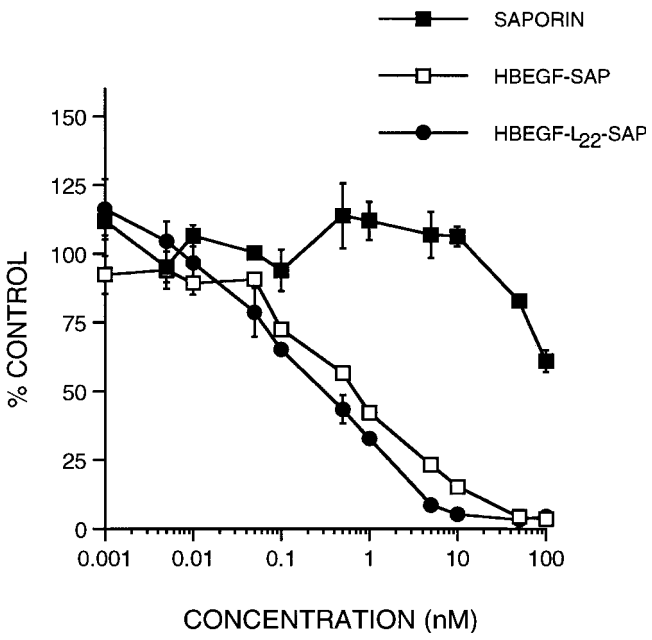

FIGURE 3 – *In vitro* cytotoxicity of HBEGF-SAP mitotoxins to A431 cells. The cytotoxic activities of HBEGF-SAP and HBEGF-L_{22}-SAP to human A431 epidermoid carcinoma cells were compared to that of SAP alone. The plotted values are the means ± SD (n =2), and the data are representative of at least 2 independent experiments. The calculated IC_{50} values are HBEGF-SAP 0.7 nM; HBEGF-L$_{22}$-SAP 0.3 nM.

moieties retain their individual functions, steric hindrance can compromise receptor binding.

The linker in HBEGF-L_{22}-SAP most likely improves receptor binding by reducing the steric constraints on the C-terminal receptor-binding domain of HBEGF. We therefore reasoned that the effectiveness of the HBEGF-SAP mitotoxins might be improved by reversing the orientation of the protein moieties, thereby making the C-terminus of HBEGF more readily available for receptor binding. Plasmids that encode SAP-HBEGF fusion proteins with and without the long, flexible linker were constructed. These fusion proteins were highly unstable, as shown by Western blots developed with anti-SAP anti-sera (data not shown), and one of the reactive bands co-migrated with free SAP. Consistent with breakdown to free SAP, these materials were nearly as active as native SAP in the cell-free protein synthesis inhibition assay (data not shown). Due to their marked instability, the SAP-HBEGF molecules were not further characterized.

HBEGF-SAP and HBEGF-L_{22}-SAP were also evaluated for cytotoxicity to A431 cells (Fig. 3). HBEGF-SAP had a significant, dose-dependent cytotoxic effect on A431 cells (IC_{50} = 0.7 nM), and complete inhibition of cell growth was achieved at high concentrations. HBEGF-L_{22}-SAP exhibited a small increase in toxicity to A431 cells (IC_{50} = 0.3 nM) compared to HBEGF-SAP. Therefore, even though HBEGF-SAP and HBEGF-L_{22}-SAP had significantly different activities in the A431 receptor-binding assay (Fig. 2), the toxicity of the 2 mitotoxins is comparable. These results illustrate the potency of RIPs such as SAP in that internalization of very few molecules is sufficient to kill a cell (Yamaizumi *et al.*, 1978). A431 cells were resistant to SAP alone (IC_{50} >100 nM), demonstrating the dependence on HBEGF for internalization of the toxin and effectiveness of the mitotoxin.

TABLE I – CYTOTOXICITY OF HBEGF-L_{22}-SAP TO HUMAN TUMOR CELL LINES[1]

Cell line	IC_{50} (nM)	Cell line	IC_{50} (nM)
Epidermoid		Breast	
*A431	0.3	T47D	36
*KB	3.9	BT-549	3.2
Bladder		MDA-N	2.4
*EJ6	10	MDA-MB435	1.7
*HT1197	1	MDA-MB231	>73
*J82	1.6	HS578T	0.6
*RT4	1.5	MCF-7/ADR	5.8
*T24	17	MCF-7	27
*TCCSUP	5	Colon	
Leukemia		KM-12	32
SR	3.1	HCT-15	36
RPMI 8226	10.3	COLO-205	2.3
HL-60 TB	15.1	SW-620	42
MOLT-4	>73	HCT-116	0.6
K-562	4.6	HCC-2998	1.1
CCRF-CEM	>73	HT-29	1.9
Non-small cell lung		*N87	6.6
A549	7.8	*SW948	>100
NCI-H226	0.1	Ovarian	
NCI-H23	12.7	SKOV-3	>73
NCI-H522	7.4	OVCAR-8	1.2
NCI-H460	13.9	IGROV-1	2
NCI-H322	4.7	OVCAR-5	1.3
HOP-92	4.7	OVCAR-4	16.2
HOP-62	13	OVCAR-3	0.4
EKVX	>73	*OVCAR2	>100
Prostate		*OVCAR10	12.1
DU-145	10.1	Brain	
PC-3	0.04	SNB-19	4
Melanoma		SF-539	0.2
UACC-257	5.8	SF-295	1.6
SK-MEL-5	0.3	SF-268	0.2
UACC-62	1.8	U-251	0.1
SK-MEL-28	0.8	SNB-75	4.4
M14	0.2	*A172	12.4
MALME-3M	0.2	*DAOY	2
LOX-IMVI	1.5	*H4	0.3
Renal		*Hs683	>100
A498	0.05	*T98G	8.4
SN-12C	5.6	*U87MG	1
UO-31	4.2	Small cell lung	
TK-10	59	NCI-H345	>100
RXF-393	1.2	NCI-H510	>100
CAKI-1	6.3	NCI-H526	>100
ACHN	2.6	NCI-H69	>100
786-0	1.1		

[1]IC_{50} values represent the concentration of mitotoxin required to achieve 50% inhibition of cell growth as determined by the MTT assay for cells with an asterisk and the SRB assay for cells without an asterisk (see "Material and Methods").

Cytotoxicity of HBEGF-L_{22}-SAP to tumor cells in vitro

The cytotoxicity of HBEGF-L_{22}-SAP was tested on a panel of human cell lines representing tumors of 12 different cell types, including the 60 cell lines comprising the NCI Anticancer Screen (Monks *et al.*, 1991). The results of this analysis are summarized in

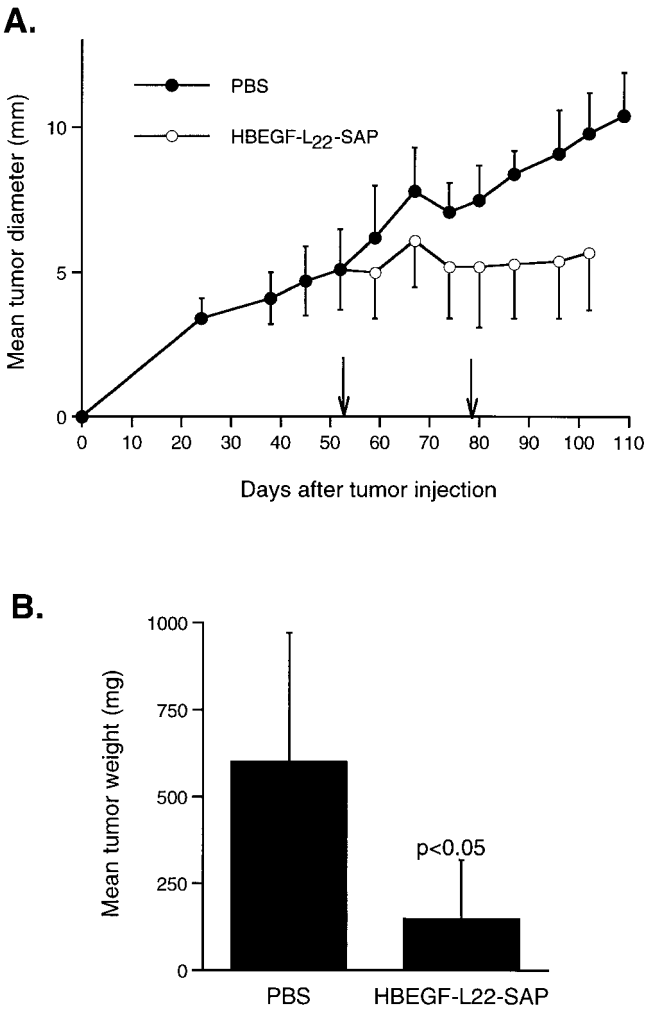

FIGURE 4 – Inhibition of MDA-MB-468 tumor growth by HBEGF-L_{22}-SAP. MDA-MB-468 tumor cells were injected into the mammary fatpad of a nude mouse, and effects of s.c. injection of HBEGF-L_{22}-SAP adjacent to the tumor were assessed. *a:* Growth curves of tumors in mice treated with s.c. injection of HBEGF-L_{22}-SAP (10 ng/g, twice weekly for 4 weeks). Plotted values are mean tumor diameters in millimeters (bar = SD). Arrows indicate period of treatment. Closed circles, PBS alone (n = 6). Open circles, HBEGF-L_{22}-SAP (n = 7). *b:* Mean tumor weights at end of experiment presented in *(a)*. $p < 0.05$ (Students t test).

Table I. Each of the cell lines was also treated with native SAP and found to be largely unaffected (data not shown). Cell lines derived from small-cell lung carcinoma (SCLC) were uniformly resistant to HBEGF-L_{22}-SAP, with IC_{50} values greater than 100 nM. In contrast, many of the cell lines representing the remaining 11 tumor types (i.e. melanoma, breast, bladder, colon, ovarian) exhibited IC_{50} values in the low nanomolar range.

Inhibition of tumor growth in vivo *by HBEGF-L_{22}-SAP*

Since many breast tumor cell lines were sensitive *in vitro*, the effect of HBEGF-L_{22}-SAP on MDA-MB-468 human breast cancer cells was evaluated *in vivo*. MDA-MB-468 cells are estrogen receptor-negative, grow in the mammary fatpad of female nude mice without estrogen supplementation and have an amplified *EGFR* gene, resulting in unusually high numbers (approx. 3.6 x 10^6) of cell-surface EGFRs (Filmus *et al.*, 1985). With peri-tumoral administration of HBEGF-L_{22}-SAP, the mean tumor diameter and mean tumor weight were significantly less than in the control groups (Fig. 4). In fact, treated tumors showed arrest of tumor growth lasting longer than 3 weeks after cessation of treatment.

DISCUSSION

In recent years, the over-expression of cell-surface growth factor receptors in human disease has been exploited to develop novel therapeutic agents that target the diseased cell. For example, chimeric molecules containing growth factors fused to toxins (termed mitotoxins) are emerging as potentially powerful and versatile therapeutic agents. Because over-expression of EGFRs has been demonstrated in numerous solid human tumors and is associated with increased metastatic potential and poor prognosis, EGFRs are particularly attractive therapeutic targets (Davies and Chamberlin, 1996). Although normal cells do express EGFRs, the elevated number of receptors on tumor cells confers a degree of targeting specificity in that the tumor cells can bind proportionately more mitotoxin.

The biological activity of mitotoxins relies on the ability of each component to perform its individual biological function. The recombinant HBEGF-SAP mitotoxins described in this study retain the ability of HBEGF to bind EGFRs and the ability of native SAP to inhibit protein synthesis. Together, the complex can kill eukaryotic cells upon internalization, and by separating the HBEGF domain from the SAP domain with a 22-amino-acid spacer, some improvement of function was realized. The intracellular mechanism by which the fusion proteins are metabolized is unknown. However, since SAP is highly resistant to proteolysis (Stirpe *et al.*, 1983), it is tempting to speculate that upon entry into cells, HBEGF is proteolytically degraded to release a fully functional SAP moiety.

In this study, cell lines derived from SCLC were unique in their uniform resistance to HBEGF-L_{22}-SAP. This result is consistent with binding studies which have demonstrated few and even undetectable levels of EGFRs on human SCLC cell lines (Haeder *et al.*, 1988). In contrast, HBEGF-L_{22}-SAP was cytotoxic to cell lines representing all of the remaining 11 tumor types, with many of the cell lines having IC_{50} values in the low nanomolar range. The tumor cell lines that are sensitive to HBEGF-L_{22}-SAP represent cancers generally considered to express EGFRs. A431 and KB epidermoid carcinoma cells, for example, have been shown to have 2 to 3 x 10^6 and 1 to 2 x 10^5 EGFRs/cell, respectively, and to be sensitive to TGF-α-PE (Siegall *et al.*, 1989). Expression of EGFRs has also been demonstrated in human bladder cancer (Wood *et al.*, 1992), primary human NSCLC (Rusch *et al.*, 1993), primary human renal carcinomas (Petrides *et al.*, 1990), human colon carcinoma cell lines (Radinsky *et al.*, 1995), human ovarian tumors (Henzen-Logmans *et al.*, 1992) and human gliomas (Ekstrand *et al.*, 1991). In addition, the ability of HBEGF-L_{22}-SAP to kill EGFR-expressing breast tumor cells *in vivo* (Fig. 4) further validates the use of HBEGF-containing mitotoxins for selective targeting to EGFR-bearing tumor cells.

HBEGF is an attractive ligand for targeted drug delivery because of its high affinity for EGFRs, which are over-expressed in a variety of human malignancies and other proliferative disorders. The heparin-binding capacity of HBEGF facilitates binding to EGFRs, thus making HBEGF preferred over other EGF family members as a targeting ligand. Consistent with this observation, it has been demonstrated that fusing the heparin-binding domain of HBEGF to the amino terminus of TGF-α-PE generates mitotoxins with improved cytotoxicity to proliferating smooth muscle cells (Mesri *et al.*, 1993). Biologically active mitotoxins consisting of mature HBEGF fused to PE have also been generated (Mesri *et al.*, 1994). SAP is an attractive alternative to the bacterial toxins, such as *Pseudomonas* exotoxin, because it has no high-affinity cell-binding domain and is highly resistant to proteolysis (Stirpe *et al.*, 1983). The HBEGF-SAP mitotoxins described in this report represent a novel, potent and versatile class of mitotoxins with potentially widespread therapeutic applications.

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