Recombinant Heregulin-*Pseudomonas* Exotoxin Fusion Proteins: Interactions with the Heregulin Receptors and Antitumor Activity *in Vivo*[1]

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ABSTRACT

Growth factor receptors provide unique opportunities for development of targeted anticancer therapy. Members of the type I receptor tyrosine kinase family, including epidermal growth factor (EGF) receptor (EGFR) and ErbB-2/neu, are often overexpressed in various human cancer cells, including breast. Recently, it has been shown that both ErbB-3 and ErbB-4 are receptors for heregulin (HRG)/Neu differentiation factor. Eight chimeric toxins composed of the extracellular and EGF-like domains of four different HRG isoforms and truncated *Pseudomonas* exotoxin (PE38KDEL) were constructed. The fusion proteins exhibited activity similar to the native HRG in inducing ErbB receptors phosphorylation. The EGF-like domain of HRGβ1 and HRGβ2 fused to PE38KDEL showed the highest cytotoxic activity, with a IC_{50} of ≤0.001 ng/ml. The α isoforms that were fused to PE38KDEL were 100-fold less active than the β isoforms. The HRG-*Pseudomonas* exotoxin (PE) toxins show extremely high activity against cells expressing ErbB-4 receptor, alone or together with other members of the ErbB receptor family. Cells that do not express ErbB-4 but express ErbB-3 receptor, together with the ErbB-2 or EGFR, exhibited moderate sensitivity to HRG-PE toxins. HRG-PE toxins have little or no activity against cells expressing EGFR, ErbB-2, or ErbB-3 alone. More than an 80% tumor regression was achieved by intratumor injection of 1 μg of fusion proteins per day for 5 days. Continuous i.p. administration of EGF-like domain of HRGβ1-PE38KDEL for 7 days via a miniosmotic pump at a dose of 40 μg/kg/day inhibited the growth of ErbB-4 receptor positive but not ErbB-4 receptor negative cell lines in athymic nude mice. We conclude that there is therapeutic potential of HRG-PE toxins in the therapy of cancers overexpressing the ErbB-4 or ErbB-2 plus ErbB-3 receptors.

INTRODUCTION

EGFR[3] and ErbB-2/*neu* are prototypes for a family of structurally related transmembrane proteins (type I receptors) that play a role in the development and progression of cancer (1, 2). Members of this family, including the recently identified erbB-3 and erbB-4 genes, are often overexpressed in various human cancer cell lines, such as breast (3–9). The ligand-binding domains of ErbB-3 and ErbB-4 share significantly more homology to each other (~65%) than they do to the other two known members of the receptor family (41–46%). Recent immunostaining studies show that ErbB-3 is overexpressed in breast cancer (10–12), ovarian cancer (13) and prostate cancer (14). The expression of ErbB-4 is elevated in breast cancer cell lines (9), but clinical correlations are not yet available. The fact that many breast tumor cells preferentially co-overexpress ErbB-2 or EGFR with ErbB-3 and/or ErbB-4 receptors (3–9) supports the importance of all these receptors in the pathogenesis of breast cancer. The extracellular accessibility of these proteins makes them attractive potential targets for specific antitumor therapy.

Attempts to isolate a ligand for ErbB-2 have led to the discovery of a factor named alternatively by various groups, *e.g.*, gp30 (15), HRG (16), NDF (17), acetylcholine receptor inducing factor (18), and glial growth factor (19), all of which are products of the same gene and contain a single EGF-like domain. EGF-like domain is sufficient for the stimulation of phosphorylation of $p185^{erbB}$ receptor(s). The EGF-like domain of HRG contains six cysteine residues that are characteristic of the EGF family of growth factors; however, HRG does not bind to EGFR (16–17, 20–22). More recently, it has been discovered that both ErbB-3 and ErbB-4 are receptors for HRG and that the presence of one of these two receptors is required for the

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; HRG, heregulin; NDF, Neu differentiation factor; PE, *Pseudomonas* exotoxin; TGF, transforming growth factor; IMEM, improved MEM; FBS, fetal bovine serum; HEL, EGF-like domain of HRG; XTT, (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; Ab, antibody; mAb, monoclonal antibody; HSA, human serum albumin.

activation of ErbB-2 by HRG through heterodimerization (21–28). When ErbB-3 and/or ErbB-4 are expressed as the only receptor in cells, they bind to HRG/NDF, whereas ErbB-2 does not bind to HRG/NDF when it is expressed alone (22, 28). In addition, cells expressing both ErbB-2 and ErbB-3 bind HRG/NDF with increased affinity compared to those expressing ErbB-3 alone (22, 24, 27). ErbB-2 appears to indirectly participate in HRG-mediated signaling through receptor heterodimerization with ErbB-4 and/or ErbB-3 (22, 24, 29, 30). No ligand has been conclusively identified that can bind directly to the ErbB-2 receptor.

PE is a bacterial toxin that irreversibly inactivates target cell protein synthesis by catalytically modifying key components of the translational machinery and is made of three structural domains (31). Domain Ia (amino acids 1–252) is responsible for cell binding; the middle domain, domain II (amino acids 253–364), mediates translocation; and domain III (amino acids 400–613) catalyzes ADP-ribosylation of elongation factor 2 (31). Domain Ib (amino acids 365–399) has no known function, and most of domain Ib can be deleted without loss of activity (31). PE38 consists of amino acids 252–613 of PE, with a deletion of amino acids 365–380. Modification of the COOH terminus by tetrapeptide Lys-Asp-Glu-Leu (KDEL) increases toxin activity (32). A number of recombinant toxins have been developed by fusing growth factors (ligand), single-chain antigen-binding proteins, and cytokines to PE. These chimeric immunotoxins or oncotoxins are very cytotoxic to target cells and have potent *in vivo* tumor-inhibiting activity. Several clinical trials are now underway (31). TGF-α-PE40, which targets EGFR, has been evaluated in a Phase I trial of bladder carcinomas (33).

Chimeric toxins, composed of HRG and PE38KDEL, may have a potential as a novel therapeutic agent for the treatment of breast cancer. We have previously described the construction of several recombinant HRG-PE fusion proteins, and they have been shown to have cytotoxic activities *in vitro* (34). We now report a series of studies of the cytotoxic efficacy of various HRG-PE38KDEL chimeric toxins for a panel of breast cancer cells *in vitro* and in nude mice and correlation between the specific expression of HRG receptors and cytotoxic activity.

MATERIALS AND METHODS

Cells and Cell Culture. Most cell lines were obtained from the American Type Culture Collection (Rockville, MD). The human gastric cancer cell line N87 was kindly provided by Dr. R. King (Lombardi Cancer Center, Georgetown University; Ref. 35). MDA-435/LCC6 cells were generously provided by Dr. R. Clarke (Lombardi Cancer Center, Georgetown University; Ref. 36). Cells were routinely maintained in IMEM (Biofluids, Rockville, MD) with 10% FBS. 32D cells and various ErbB receptor transfected 32D cells were cultured as in a previous report (49). Cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

Construction and Purification of the Chimeric Toxins. Fusion of HRG to the modified PE (PE38KDEL) was described previously (34). Briefly, the four isoforms of the extracellular domains (HRG) and the four isoforms of HEL (amino acids 175–242 for HRGα1, amino acids 175–247 for HRGβ1, amino acids 175–239 for HRGβ2, and amino acids 175–242 for HRGβ3) were fused to the PE38KDEL. Recombinant proteins were produced with the T7 promoter-based expression system in *Escherichia coli* (BL21), where each toxin accumulated within inclusion bodies. The recombinant proteins were dissolved in guanidine and renatured, purified through sequential use of Q-Sepharose, Mono Q HR 5/5, and TSK-250 columns. Extracellular domains of native HRG were purified by heparin-agarose instead of Q-Sepharose. SDS-PAGE was used to analyze each column fraction, and the final recombinant proteins were at least 95% pure.

Assay of Biological Activity of Chimeric Toxins. The effect of chimeric toxins on protein synthesis was determined as described previously (34). Assays of inhibition of cell proliferation were carried out on plastics to directly measure the cell-killing activity. A soluble tetrazolium/formazan (XTT) assay for cell growth in a 96-well plate was performed (37). Cells (2000–4000 cells/well) were grown in IMEM with 10% FBS and were treated with increasing concentrations of recombinant toxins (0.001–10 μg/ml). After 4 days of culture, XTT (1.0 mg/ml plus phenazine methosulphate at 1.53 mg/ml) was added to each well and incubated for 4 h at 37°C. $A_{450\,nm}$ was measured with the Dynatech Model MR700. To examine the ability of a fusion protein to penetrate through soft-agar colonies, cells in suspension (10,000 cells/ml) were mixed with 0.33% of agarose and plated on top of a bottom layer with 1% agarose. The next day, different concentrations of HELβ1-PE38KDEL mixed with 1 ml of culture medium were added to the top layer and incubated for 2 weeks. The number of colonies that were >60 μm in diameter and formed after the incubation period was counted using a Bausch and Lomb Image Analysis system.

Western Blot of ErbB Receptors. Cells ($1–2 \times 10^6$) were plated into 100-mm dishes with 10% FBS. Cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 2 mM sodium orthovanadate]. A protein concentration was determined by bicinchoninic acid method (Pierce, Rockford, IL). Thirty μg of protein were subjected to 8–20% SDS-PAGE (Novex, San Diego, CA) and transferred to nitrocellulose membrane. Each receptor was detected with a specific Ab, *e.g.*, EGFR mAb (Upstate Biotechnology, Inc., Lake Placid, NY), ErbB-2 mAb (Oncogene Sciences Inc., Cambridge, MA), ErbB-3 (NeoMarkers Inc., Fremont, CA), or antiphosphotyrosine mAb (Upstate Biotechnology, Inc.), and visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Individual Receptor Tyrosine Phosphorylation. To investigate receptor activation by the fusion proteins, cells treated with or without HELβ1-PE38KDEL were immunoprecipitated with specific EGFR, ErbB-2, ErbB-3, and ErbB-4 antibodies separately and then blotted with an antiphosphotyrosine Ab. Briefly, $1–2 \times 10^6$ cells were plated into 100-mm dishes with 10% FBS. The next day, cells were washed twice with serum-free IMEM and incubated in that medium for 5 h. Cells were then stimulated with 10 nM of HELβ1-PE38KDEL for 10 min at 37°C. The cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (same as above). Five hundred μg of protein were incubated with EGFR mAb (Upstate Biotechnol-

ogy, Inc.), ErbB-2 mAb (Oncogene Science Inc.), or ErbB-3 or ErbB-4 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight with gentle rotation. Immunocomplexes were then incubated with protein A-Sepharose (CL-4B; Pharmacia, Piscataway, NJ) at 4°C for 1 h and collected by ultracentrifuge. Proteins were electroblotted onto nitrocellulose and probed with antiphosphotyrosine mAb. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham). In addition, the same immunoprecipitated proteins were blotted against the EGFR, ErbB-2, ErbB-3, and ErbB-4 antibodies, respectively.

Animal Toxicity Studies. Groups of four or five female nude mice (NCr, *nu/nu*) were given single injections i.v. through the tail vein with 100 μl of different amounts of HELβ1-PE38KDEL diluted in PBS-HSA (0.2%). Animals were monitored daily for weight loss and mortality for 2 weeks after injection. The LD_{50} was obtained by noting the doses that caused 50% death of animals. Some of the animals were sacrificed, and major organs were removed. Tissues were examined by H&E-stained sections.

Pharmacokinetics. To evaluate the pharmacokinetics of the chimeric toxin in blood, female nude mice (weight, 22–25 g) were given a single dose of 1 μg of HELβ1-PE38KDEL in 200 μl of PBS-HSA by injection into the tail vein. Blood samples were drawn from the orbital sinus or chest cavity at 2, 5, 10, 20, 30, 45, 60, 120, 180, 240, and 300 min after injection. Each mouse was bled three times, and each time point represents the mean of results obtained from three mice. The concentration of the recombinant toxin was measured by bioassay, in which serum samples were diluted in PBS and incubated with N87 cells. A standard curve was made using purified HELβ1-PE38KDEL. Pharmacokinetic parameters were calculated using an exponential curve fitting program, RSTRIP (MicroMath Scientific Software, Salt Lake City, UT).

***In Vivo* Antitumor Activity.** We have developed a subline of the MDA-361 cells termed MDA-361/DYT2. This derivative cell line forms large tumors that grow progressively and are highly vascularized, but there are no changes in the expression of the ErbB receptors or sensitivity to the HRG-PE *in vitro* (data not shown). Details of this cell line will be described elsewhere. Tail vein injection of fusion proteins up to 50 μg/kg (LD_{10}) of HELβ1-PE38KDEL was injected every other day for 8 days into mice bearing tumor xenograft. Local (intratumor) administration was carried out by direct injection of the chimeric toxin into the right side of tumor at 1 μg/tumor daily for 5 days, whereas the left side of tumor of the same mouse was treated with PBS. In other animals, miniosmotic pumps (model 107, Alza Corp., Palo Alto, CA; 0.5 μl/h for 7 days) were used to deliver fusion proteins through i.p. implantation. Osmotic pumps containing escalating doses of HRG chimeric toxins were placed through i.p. implantation, and the LD_{50} for chimeric toxins using osmotic pumps was determined in pilot experiments. Minipumps were filled with 100 μl of HELβ1-PE38KDEL at doses of 40 or 80 μg/kg/day for 7 days in sterile PBS containing 0.2% HSA or with PBS alone. Cells ($1–5 \times 10^6$) were injected on day 0 into the mammary fat pad of the female athymic nude mice, and filled osmotic pumps were placed i.p. 5 days after tumor implantation. Each treatment group consisted of five animals. Tumor volume was calculated by using formula volume = length × width^2 × 0.4.

RESULTS

Activity of Different Isoforms of HRG-PE Toxins. Initially, the activity of each purified toxin was examined by the protein synthesis inhibition assay using [^{3}H]leucine labeling. As shown in Fig. 1, chimeric toxins containing the HELβ1 and HELβ2 are the most active compounds, with IC_{50}s of 0.2–5 ng/ml, which are similar to results obtained in previous studies (34). The whole extracellular domain fusion proteins (HRG-PE38KDEL) are less cytotoxic and less specific (comparing with HUT102 cells, which express no detectable receptors) than the chimeric toxins made with the HEL (HEL-PE38KDEL). One possible explanation is that the molecule size and presence of other structure domains, such as the immunoglobulin-like domain in the intact HRG may contribute the reduction in the killing and specificity. The cytotoxic activity of the HELα1 fused protein is more than 100-fold less than that of the β isoforms. This latter result may be due to a 10-fold reduction in binding affinity for the α1 isoform as compared to the β isoforms and is similar to previous findings (34, 38). We carried out most of the following experiments using HELβ1s fused to PE (HELβ1-PE38KDEL). The specificity of HEL-PE38KDEL was verified in a competition assay and the cytotoxicity of HELβ1-PE38KDEL was completely blocked at low concentration and partially blocked at high concentration by HRGβ1 in N87 cells (Ref. 34 and data not shown).

Cytotoxicity and Correlation with the Status of ErbB-1 (EGFR), ErbB-2, ErbB-3, and ErbB-4 Expression. We next evaluated the cytotoxicity of HELβ1-PE38KDEL in a panel of cancer cell lines from breast, ovary, gastric, and epidermoid carcinomas using a soluble tetrazolium/formazan (XTT) assay to directly measure the cell killing effects. Table 1 summarizes IC_{50} data. The responsiveness of these cells to the HRG-PE toxin-mediated cell killing can be divided into three categories. Cells from breast cancer cell lines MDA-453, BT-474, and MDA-361 and the gastric carcinoma cell line N87 are extremely sensitive to the cytotoxic effects of the fusion protein, with IC_{50}s of <0.01 ng/ml. Cells from MCF-7, SKBr-3, MDA-468, and A431 showed intermediate response to the HRG-PE toxin killing, with IC_{50}s of 0.1–0.5 ng/ml. MDA-231, MDA-435/LCC6, and SKOV-3 cells are resistant to HRG-PE toxin, with IC_{50}s of >1000 ng/ml.

In general, colony-formation in soft-agarose correlates well with *in vivo* tumorigenicity, and precise quantitation of number of colonies can be done with an image analysis system. Post *et al.* (39) recently suggested that the best method to evaluate immunotoxin efficacy is the clonogenic assay. As shown in Fig. 2, MDA-231 and SKOV3 cells are highly resistant, whereas MDA-453 and BT-474 are extremely sensitive. A431 and MDA-468 are intermediate. Therefore, the chimeric toxin is able to penetrate through the semisolid agarose and resulted in specific cell killing that is comparable to that seen in the XTT assay.

It is known that many human cancer cell lines overexpress one or more members of the EGFR family and can be killed by recombinant toxins, including TGF-α (40–42), anti-ErbB-2 Ab

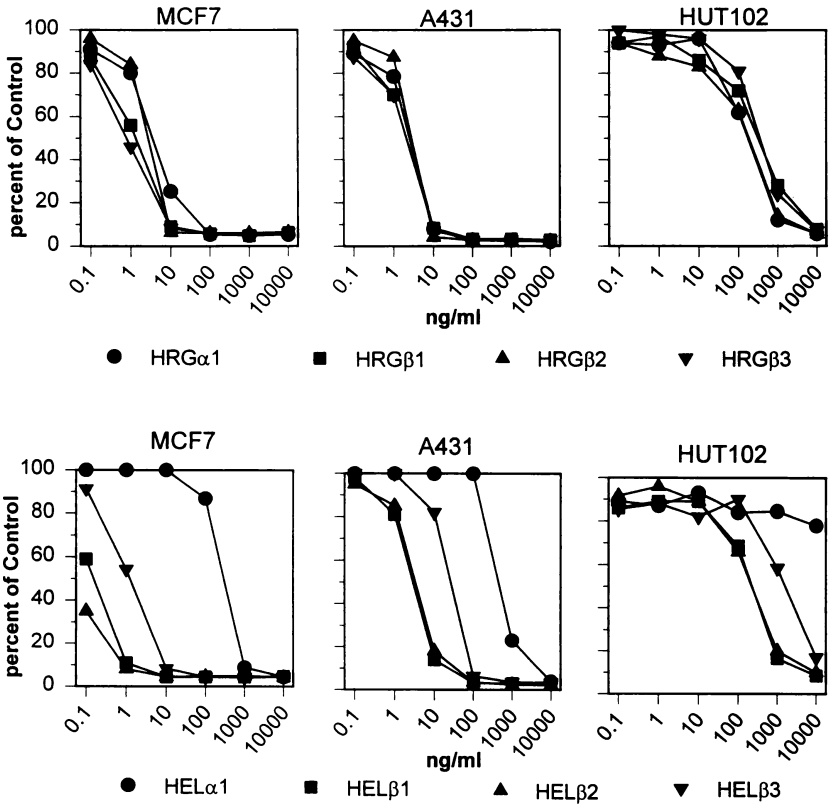

Fig. 1 Inhibition of protein synthesis in MCF7, A431, and HUT102 (adult T-cell leukemia, receptor negative) cells by various isoforms of HRG-fused PE38KDEL (*top*) or HEL-fused PE38KDEL (*bottom*). *Data points*, percentages of untreated control cells exhibiting [^{3}H]leucine incorporation.

Table 1 Expression of EGFR, ErbB-2, ErbB-3, ErbB-4, and HRG/NDF and cytotoxic activity of chimeric toxin HELβ1-PE38KDEL[a]

Cell line	Expression of					Cytotoxic activity, IC_{50} (ng/ml)	Tumor[b]
	EGFR	ErbB-2	ErbB-3	ErbB-4	HRG		
MDA-453	−[c]	+++	++	++	−	0.001	−
BT-474	+/−	++++	+++	++	−	0.001	+ (E2)
MDA-361	−	+++	++	+	−	0.001	+ (E2)
N87	−	+++++	+++	+++	ND	0.001	+++
MCF-7	+/−	+	++	+++	−	0.1	+ (E2)
SKBr3	+	++++	++	+/−	−	0.5	−
MDA-468	++++	−	++	−	−	0.5	+
A431	+++++	+	++	−	ND	0.5	++++
MDA-231	++	+/−	−	−	+++	>1000	++++
MDA-435/LCC6	−	−	++	−	ND	>1000	++++
SKOV3	−	+++++	+/−	−	−	>1000	+++
MCF10A	+	−	++	−	ND	>1000	−

[a] EGFR, ErbB-2, and ErbB-3 expression was determined by Western blotting and immunoblotting analysis; ErbB-4 expression was determined by immunoblotting, FACS analysis (with NeoMarker's Ab-1), and RT-PCR analysis; HRG expression was determined by RT-PCR and Refs. 35 and 36.

[b] Capacity and rate of tumor growth on nude mice.

[c] −, not detectable on Western blot; +/−, detectable only after extended exposure on Western blot or immunoprecipitation plus Western blot; +, relative expression level on Western blot; ND, not done; E2, estrogen.

(43, 44), or HRG (34, 45, 46). Despite much evidence that HRG/NDF can bind and activate ErbB-3 receptor in transfected cells or keratinocytes that express only the ErbB-3 receptor (47), Siegall *et al.* (45) have recently found that cells expressing both ErbB-2 and ErbB-3 are not killed by the HRGβ2-PE40 fusion protein. However, Jeschke *et al.* (46) reported that the cytotoxicity of their HRG-PE40 correlated with either ErbB-3 or ErbB-4 receptor expression. The discrepancy may be due to differences in receptor quantitation or different variants of the same cell lines used. It is also possible that the composition of

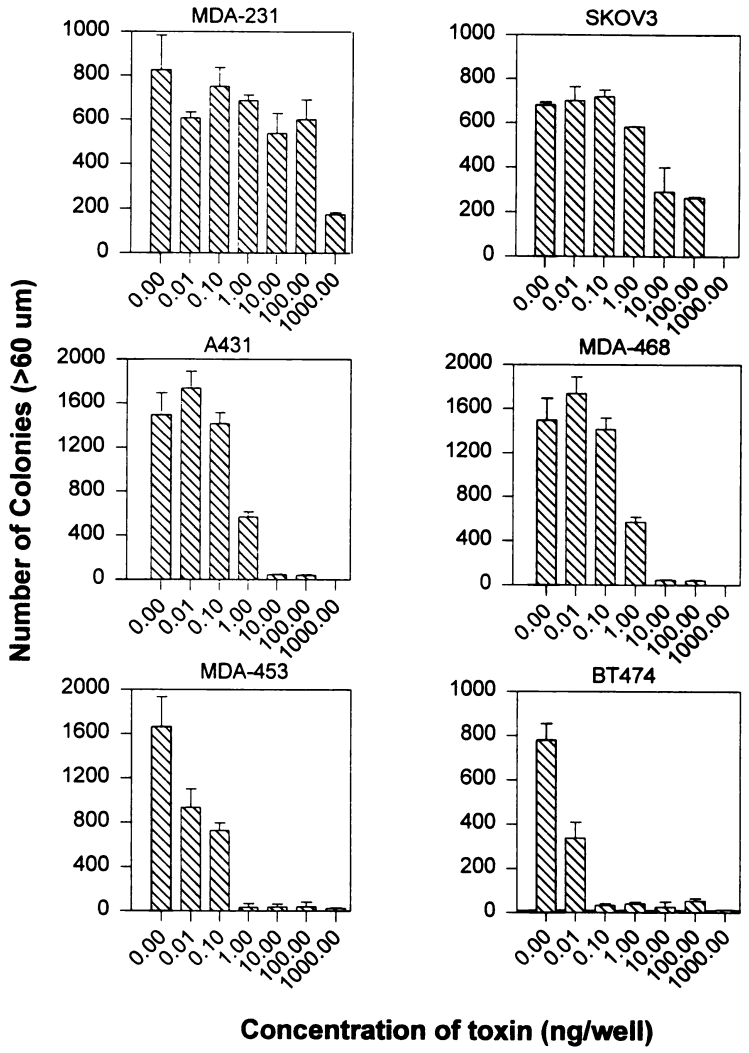

Fig. 2 Inhibition of soft-agar colony formation of various cells by HELβ1-PE38KDEL at various concentrations. Cells were incubated for 2 weeks, and colonies that were >60 μm from three dishes were counted with Bausch and Lomb Image Analyzer. *Columns*, averages of three dishes; *bars*, SD.

the ErbB receptors varies with the different cell lines, leading to the difference in the ratio of the IC_{50}s. We used a series of antibodies specific for each of the ErbB receptors to screen the expression of ErbB receptors by Western blotting, as well as combined immunoprecipitation plus Western blotting (see Fig. 6). Results of the Western blotting analysis for the EGFR, ErbB-2, ErbB-3, and tyrosine phosphorylation status of these receptors are shown in Fig. 3 and are summarized in Table 1. The highly sensitive group of cells (MDA-453, BT-474, MDA-361, and N87) expressed high levels of ErbB-2, ErbB-3, and ErbB-4 receptors. BT474 and N87 express low levels of the EGFR, which are detectable only after extended exposure. Cells showing intermediate response to the HRG-PE toxin killing (MDA-468 and A431) express high levels of EGFR and moderate levels of ErbB-3. The MCF-7 cells express low to moderate levels of all four receptors. Among the three resistant cell lines, MDA-231 express moderate levels of EGFR and low amounts of the ErbB-2, but levels of the ErbB-3 and ErbB-4 are not detectable even after extended exposure. MDA-231 cells also express the ligand HRG/NDF. MDA-453/LCC6 cells, which are resistant to killing, express only moderate levels of ErbB-3, and the ErbB-3 is activated upon stimulation with the chimeric toxin (see below). SKOV3 cells (also resistant) express high levels of ErbB-2 but not ErbB-3 or ErbB-4.

Because most of the human cancer cell lines express more than one ErbB receptor family member and differences in heterodimerization of these receptors may alter the binding affinity and/or endocytosis, it is difficult to exactly correlate the receptor expression and the killing efficacy of the HRG-PE toxins. We, therefore, tested HEL-PE toxin killing on 32D cells transfected with EGFR, erB-2 (48), erbB-3, erbB-4, or erbB-2 plus erbB-3 (49). None of the ErbB receptors were detectable in the parental 32D cells (49). The wild-type and EGFR-, erbB-2-, or erbB-3-transfected 32D cells are resistant to the HRG-PE killing, with

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