

Human Breast Carcinoma Cells Express Type II IL-4 Receptors and Are Sensitive to Antitumor Activity of a Chimeric IL-4-*Pseudomonas* Exotoxin Fusion Protein in vitro and in vivo

P. Leland,¹ J. Taguchi,¹ S. R. Husain,¹ R. J. Kreitman,² I. Pastan,² and R. K. Puri¹

¹Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, U.S.A.

²Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

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Abstract

Background: Human breast carcinoma cell lines express high-affinity interleukin-4 receptors (IL-4R). We examined the expression and structure of these receptors on primary and cultured breast carcinoma cell lines and normal breast epithelial cells. We also tested the antitumor activity in vitro and in vivo of a fusion protein comprised of circular permuted IL-4 and truncated *Pseudomonas* exotoxin, termed IL-4(38-37)-PE38KDEL.

Materials and Methods: Eight different primary cell cultures and cell lines of human breast carcinomas were examined for the expression of IL-4R by radiolabeled binding, reverse transcription polymerase chain reaction (RT-PCR) and Northern analyses, and subunit structure by crosslinking studies. The antitumor activity of IL-4 toxin was tested in vitro by cytotoxicity assays and in vivo in a xenograft model in immunodeficient animals.

Results: ¹²⁵I-IL-4 specifically bound to primary cell cultures and cell lines with a Kd ranging between

0.2 and 1 nM. Breast tumor cells were found to express IL-4R β and IL-13R α' chains, but not IL-2R γ_c chain. These cells were highly sensitive to the cytotoxic effect of IL-4(38-37)-PE38KDEL. The IC₅₀ (concentration inhibiting protein synthesis by 50%) ranged between approximately 0.005–1.5 nM. A normal breast epithelial cell culture was not sensitive to the cytotoxic activity of IL-4(38-37)-PE38KDEL. MDA-MB231 human breast carcinoma cell line formed a rapidly growing tumor in nude mice. Intratumor and intraperitoneal administration of IL-4(38-37)-PE38KDEL caused a dose dependent regression of established tumors. A control toxin, anti-Tac(Fv)-PE38KDEL, targeted to the IL-2 receptor α chain did not cause regression of these tumors.

Conclusions: These results suggest that IL-4(38-37)-PE38KDEL may be a useful agent for targeting of IL-4 receptor positive human breast carcinomas and further studies should be performed to explore fully its potential.

Introduction

Breast cancer is the most common malignancy in women, resulting in the second most frequent

cause of cancer death among women in the United States (1). Recent studies have focused on the development of new potent anti-cancer agents for the treatment of breast cancer refractory to contemporary chemotherapy drugs. Targeted toxins in which ligand or specific antibody is fused to a toxin comprise one such form of anticancer drug. Identification of novel tumor-associated antigens or receptors on human breast

Address correspondence and reprint requests to: R. K. Puri, Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, National Institutes of Health, Bethesda, Maryland, U.S.A. Phone: 301-827-0471; Fax: 301-827-0449; E-mail: PURI@CBER.FDA.GOV

cancer cells may help generate targeted anti-breast cancer agents. Recently, various fusion proteins have been produced that are designed to target human breast cancers. For example, Heregulin-*Pseudomonas* exotoxin, which the ligand Heregulin binds to ErbB-2, ErbB-3 and ErbB-4 receptors, is connected to a truncated form of *Pseudomonas* exotoxin (PE). This cytotoxin is highly cytotoxic in vitro and in vivo to breast cancer cells that overexpress ErbB-4 or ErbB-2 plus ErbB-3 receptors (2). A recombinant epidermal growth factor (EGF) Genistein conjugate, in which EGF was conjugated to soybean-derived protein tyrosine kinase inhibitor, was targeted to an EGF-receptor and was found to be cytotoxic to the EGF-receptor positive breast cancer cells (3). A recombinant, humanized monoclonal anti-Her2 antibody (Herceptin) was able to significantly inhibit growth of breast cancer in an animal model and in the clinic (4). This antibody synergized with paclitaxel when mediating anti-tumor activity against breast tumor xenograft models. Herceptin was recently licensed by the U.S. Food and Drug Administration (FDA) for the treatment of breast cancer. These studies demonstrate that these classes of biotherapeutics can provide an additional mode of breast cancer therapy, although their clinical benefits have yet to be completely explored. It is possible that additional breast tumor-associated receptors or antigens will be identified that may provide new targets for breast cancer therapy.

We and others have identified that human breast cancer cell lines express elevated levels of the receptor for an immune regulatory cytokine, interleukin-4 (IL-4) (5-7). Although the functional significance of this receptor on breast cancer cell lines is not clear, IL-4 can inhibit proliferation of these cells in vitro and induce apoptosis (5-7). It is not known whether these receptors are overexpressed in situ in breast carcinomas. We reported that IL-4 receptors were expressed in situ in renal cell carcinoma and AIDS-associated Kaposi's sarcoma (8,9). Thus, it is likely that breast carcinoma may also express IL-4 receptors in vivo, because breast cancer cell lines express receptors in high numbers. We also found that a variety of solid cancer cells overexpress high-affinity IL-4 receptors (IL-4R) (10-12). These receptors are functional because IL-4 is able to cause signal transduction, inhibit growth, upregulate major histocompatibility (MHC) antigens and intercellular adhesion molecule-1 (ICAM-1) on cancer cells (10-19). IL-4R also are ex-

pressed, although in low numbers, in normal immune cells such as T cells; B cells; monocytes; other blood cells, such as eosinophils, basophils, and fibroblasts; and endothelial cells (10,11). The significance of the overexpression of IL-4R on epithelial cancer cells and the similarities and differences between IL-4R in cancer cells and immune cells is not completely clear.

IL-4 receptors have been shown to be comprised of a 140 kDa protein originally termed IL-4R α (20). Because of similarities in extracellular domains (WSXWS motif and four cysteine residues at a fixed location) and long intracellular domains between the IL-4R α and β chains of receptors for IL-3, IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF), we have recently proposed to rename this chain IL-4R β (18,21). This recommendation also was based on its similarity with the IL-2R β chain, which like IL-4R p140, binds IL-2, but does not transmit a signal on its own (22). The second subunit of the IL-4R system was shown to be a component of the IL-2 receptor system, the γ chain (22,23). Because IL-2R γ chain also was shown to be a component of IL-7, IL-9, and IL-15 receptor systems (24-26), it was named γ_c . Thus, the IL-4R β chain and γ_c form type I IL-4 receptors. Recently, we demonstrated by reconstitution experiments that a 60-70 kDa protein form of interleukin-13 receptor (IL-13R) can substitute for γ_c when mediating IL-4 signaling and, thus, this chain forms a third subunit of the IL-4R system (IL-13R α' also termed as IL-13R α_1) (18,21,27,28). Consequently, IL-4R β and IL-13R α' chains form type II IL-4 receptors. Whether all three chains form an IL-4R complex in cancer cells is not known. It is also not known whether breast cancer cells express Type I or Type II IL-4 receptors. The differences in subunit structure between IL-4R in cancer cells and normal immune cells are also not completely known. We demonstrated that the γ_c chain expressed in immune cells was not expressed on human solid cancer cell lines (11,18,29). Instead, these cells expressed the IL-13R α' (or α_1) chain along with the IL-4R β chain (29,30). Further studies on the structure and function of IL-4R on cancer cells are ongoing. Regardless of differences in IL-4R between normal and cancer cells, we have been able to exploit the overexpression of IL-4R on cancer cells by targeting them with a cytotoxic chimeric protein comprised of IL-4 and PE (31-42).

In the present study, we employed a circularly permuted form of IL-4-toxin [IL-4(38-37)-PE38KDEL], which contained amino acids 38-129 of IL-4 fused via a peptide linker to amino acids 1-37. These are, in turn, fused to amino acids 353-364 and 381-608 of PE, with KDEL at positions 609-612 (37). This IL4-toxin has potent cytotoxic activity against eight different breast cancer cell lines and primary cell cultures. We also investigated the expression and structure of IL-4 receptors in breast cancer cell lines, primary cell cultures and a breast epithelial cell line. We tested the antitumor activity of IL-4(38-37)-PE38KDEL against human breast cancer in vivo in a xenograft model. Our data support further studies on the use of IL-4(38-37)-PE38KDEL for possible treatment of metastatic breast cancer.

Materials and Methods

Recombinant Cytokines and Toxins

Recombinant circularly permuted IL-4-toxin, IL-4(38-37)-PE38KDEL, was produced and purified to >95% homogeneity as described previously (32,33,37,38). Recombinant IL-4 was produced as described (43).

Cell Lines

The primary cultures of human breast carcinoma R-BT, S-BT, and W-BT were established and kindly provided by Dr. Magda Sgagias, Surgery Branch, National Cancer Institute (Bethesda, MD) (44). The breast carcinoma cell lines (MCF-7, BT-20, SK-BR3, ZR-75-1, and MDA-MB231) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Dr. Sgagias also provided one primary epithelial cell culture from normal breast tissue (A-NL). The primary tumor and normal breast cell cultures were cultured in medium comprised of α -minimum essential medium, HAM's F-12, EGF, triiodothyronine, N-2-hydroxy ethylpiperazine-N-2-ethanesulfonic acid (HEPES) ascorbic acid, estradiol, insulin, hydrocortisone, ethanolamine, transferrin, bovine pituitary extract, sodium selenite, glutamine, gentamicin, penicillin, and streptomycin. The breast carcinoma cell lines were cultured in complete media, comprised of RPMI 1640, 10% heat inactivated fetal calf serum (FCS) and gentamycin. These adherent cell lines were routinely passaged every 4–5 days.

Animals

Four-week-old female athymic nude mice (~20 g) were obtained from Frederick Cancer Center Animal Facilities (Frederick, MD). Animals were housed in filter-top cages in a laminar flow hood.

Protein Synthesis Inhibition Assay

The cytotoxic activity of IL-4-toxins was tested as previously described by determining inhibition of protein synthesis (31). Typically, 10^4 breast cancer cells were cultured in leucine-free medium with or without various concentrations of IL-4-toxins for 20–22 hr at 37°C. Then, 1 μ Ci of [3 H]-leucine (NEN Research Products, Wilmington, DE) was added to each well and cells were incubated for an additional 4 hr. Cells were harvested and radioactivity incorporated into cells was measured by a Beta plate counter (Wallac, Gaithersburg, MD).

125 I-IL-4 Binding and Displacement Assay

IL-4 was iodinated with IODOGEN reagent (Pierce, Rockford, IL) according to manufacturer's instructions. The specific activity of radiolabeled IL-4 ranged between 31.5 to 212 μ Ci/ μ g. The IL-4 binding assay was performed by a previously described technique (12,17). Briefly, tumor cells were harvested after brief incubation with versene (Biowhittaker, Walkersville, MD), washed three times in Hanks balanced salt solution and resuspended in binding buffer (RPMI 1640 plus 1 mM HEPES and 0.2% human serum albumin). For the displacement assay, MCF-7 cells ($1 \times 10^6/100 \mu$ l) were incubated at 4°C with 125 I-IL-4 (100–200 pM) with or without increasing concentrations of unlabeled IL-4 or IL-4(38-37)-PE38KDEL. For binding assays, cells were incubated with various concentrations of 125 I-IL-4 with or without 200-fold molar excess of unlabeled IL-4. Following a 2 hr incubation, cell-bound radio-ligand was separated from unbound by centrifugation through a phthalate oil gradient and radioactivity was determined with a gamma counter (Wallac). The number of receptors and binding affinities were determined as previously described (12).

Affinity Cross Linking of 125 I-IL-4 to Its Receptor

MCF-7 and MDA-MB231 cells (5×10^6) were incubated with [125 I]-labeled IL-4 in the pres-

ence or absence of excess unlabeled IL-4 for two hr at 4°C. Bound [¹²⁵I]-IL-4 was cross-linked to IL-4R with disuccinimidyl suberate (DSS) (Pierce Chemical company, Rockford, IL) at a final concentration of 2 mM for 20 min. The cells were then lysed at 4°C with 1% triton X-100 solution containing the following protease inhibitors obtained from Sigma chemical company (St Louis, MO) and Boehringer-Mannheim (Indianapolis, IN): leupeptin (10 µg/ml), trypsin inhibitor (100 µg/ml), pepstatin (10 µg/ml), benzamidine HCl (10 mM), phenanthroline (1 mM) iodoacetamide (20 mM), e-aminocaproic acid (50 mM) and phenyl methyl sulfonic fluoride (PMSF) (1 mM). The resulting lysate was cleared by boiling in sample buffer containing 2-mercaptoethanol and analyzed by electrophoresis through a SDS-PAGE (8%) gel, as previously described (15). The gel was dried and exposed to X-ray film for 7 days to obtain an autoradiograph.

For immunoprecipitation, the [¹²⁵I]-IL-4/IL-4R cross-linked complex was immunoprecipitated from the lysate prepared from MCF-7 cells overnight at 4°C by incubating with protein A sepharose beads that had been preincubated with anti- γ_c or anti-IL-4R β chain antibody. The resulting conjugate was washed twice with solubilizing buffer, diluted with reducing buffer, boiled for 5 min and analyzed by SDS-PAGE, as described above. The gel was dried and autoradiographed.

Northern Analysis for IL-4R Subunits

Total RNA was isolated using TRIZOL reagent (GIBCO BRL, Gaithersburg, MD). Equal amounts of total RNA were electrophoresed through a 0.8% agarose/formaldehyde denaturing gel, transferred to a nylon membrane (S&S Nytran; Schleicher and Schuell, Keene, NH) by capillary action and immobilized by ultraviolet crosslinking (Stratagene, Inc., La Jolla, CA). The membrane was then prehybridized for 4 hr at 42°C and hybridized with ³²P-labeled cDNA probes of IL-4R β , IL-13R α' , and γ_c at 42°C overnight. The membranes were subsequently exposed to X-AR film (Eastman Kodak Co. Rochester, NY) to obtain an autoradiogram.

RT-PCR Analysis

RT-PCR analysis was performed as previously described (40). Total RNA was isolated from

cell lines using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer's instructions. The concentration and purity of total RNA was determined by spectrophotometric analysis. One µg of total RNA was used in the RT-PCR assay. RT-PCR conditions were as follows: 95°C for 5 min, 1 cycle; 95°C for 1 min; 72°C for 1 min; 72°C for 1 min, 30–35 cycles; and 72°C for 10 min for the final primer extension sequence. RT-PCR primers for IL-4R β : 5' primer, 5'-ATGGGGTGGCTTTGCTCTGGG-3' and 3' primer, 5'-ACCTTCCCGAGGAAGTTCGGG-3'; for γ_c , 5' primer, 5'-CCAGAGGTTTCAGTGTTCGTGTT-3' and 3' primer, 5'-CAGGTTTCAGG-ATTTAGGGTGTA-3'; for IL-13R α' : 5' primer, 5' GGAGGATACATCTTGTTCATGG-3' and 3' primer, 5'-GAGCTTCTTACCTATACTCATTCTTGG-3'. The IL-4R β RT-PCR cDNA product was 316 bp; 256 bp; for γ_c and 148 bp for IL-13R α' . A 100 bp DNA ladder (GIBCO BRL Life Technologies Inc., Gaithersburg, MD) was used as a base pair reference marker.

Antitumor Activity of IL-4-Toxin in Nude Mice Implanted with Human Breast Tumor

Human breast tumor nodules were established in nude mice by subcutaneous injection of 3-4 × 10⁶ MDA-MB231 cells in 100 µl of phosphate-buffered saline (PBS) containing 0.2% human serum albumin (HSA) into the abdomen on day 0. Palpable tumors developed within 3–5 days. Tumor size was calculated by multiplying two perpendicular diameters.

Two routes for administration of IL-4 (38-37)-PE38KDEL were employed: intraperitoneal (i.p.) and intratumoral (i.t.). The mice were i.p.-injected with 100 µl excipient or 50, 100 or 150 µg/kg twice daily for 5 consecutive days. Another group of mice was slowly i.t.-injected (20 µl) with excipient or an IL-4(38-37)-PE38KDEL dose of 250 µg/kg/dose on days 8, 10 and 12. Each injection was placed into a different area of the tumor. An additional group of mice was i.t.-injected with chimeric toxin at a dose of 750 µg/kg/dose on days 8, 10 and 12, followed by reinjection with 500 µg/kg/dose on days 22, 24 and 26. A third group of animals were i.t.-injected with 750 µg/kg/dose on days 22, 24 and 26, followed by reinjection with 500 µg/kg/dose on days 36, 38, and 40.

Statistics

The significance of differences in mean tumor sizes among treatment groups was analyzed by unpaired Student's *t*-test. All *p*-values are presented as two-sided analysis.

Results

Cytotoxicity of IL-4-toxins Against Breast Carcinoma Cell Lines and Primary Cell Cultures

IL-4-toxins, including IL-4(38-37)-PE38KDEL, have been shown to have cytotoxic activity against cell lines that express IL-4 receptors (IL-4R) (32–42). However, it is not known whether primary cell cultures of human breast carcinoma express IL-4R and if they do, whether these cells and breast cancer cell lines are susceptible to the cytotoxic activity of IL-4-toxins. We tested four primary cell cultures of breast carcinoma generated from four patients undergoing surgical resection for their cancer, as previously described (44). Three of four primary cell cultures were sensitive to the cytotoxic activity of circular permuted IL-4-toxin and one of these three was extremely sensitive to IL-4(38-37)-PE38KDEL (Fig. 1A and Table 1). The IC_{50} (the concentration of toxin causing inhibition of protein synthesis in target cells by 50%) ranged between 0.2 to 240 ng/ml (4 pM to 4.8 nM). The cytotoxic activity of IL-4(38-37)-PE38KDEL was specific, as an excess of recombinant IL-4 neutralized the cytotoxic activity of IL-4-toxin to primary breast carcinoma cell culture R-BT (Fig. 1A).

Like primary cell cultures, breast carcinoma cell lines were also very sensitive to the cytotoxic activity of IL-4-toxin. Protein synthesis was inhibited in a concentration-dependent manner against four of five breast cancer cell lines examined. The IC_{50} s for IL-4(38-37)-PE38KDEL ranged between 0.4 ng/ml to 75 ng/ml (8 pM to 1.5 nM) (Fig. 1B and Table 1).

Inhibition of ^{125}I -IL-4 Binding by IL-4-toxins on MCF-7 Breast Carcinoma Cell Line

To determine the binding affinity of IL-4(38-37)-PE38KDEL to breast cancer cells, we performed displacement assays where [^{125}I]-IL-4 binding was inhibited by either unlabeled IL-4 or IL-4 toxin. As we reported in other cancer cell lines, IL-4(38-37)-PE38KDEL displaced ^{125}I -IL-4 at a similar concentration as unlabeled IL-4 on MCF-7 cell line (Fig. 2) (39,40). The EC_{50} (protein

concentration required for 50% inhibition of ^{125}I -IL-4 binding) for IL-4(38-37)-PE38KDEL was ~0.5 nM and for IL-4 it was ~0.4 nM. These data suggested that IL-4(38-37)-PE38KDEL bound to IL-4R with similar affinity to IL-4 and circular permutation or fusion of PE did not modify its binding affinity to breast cancer cells.

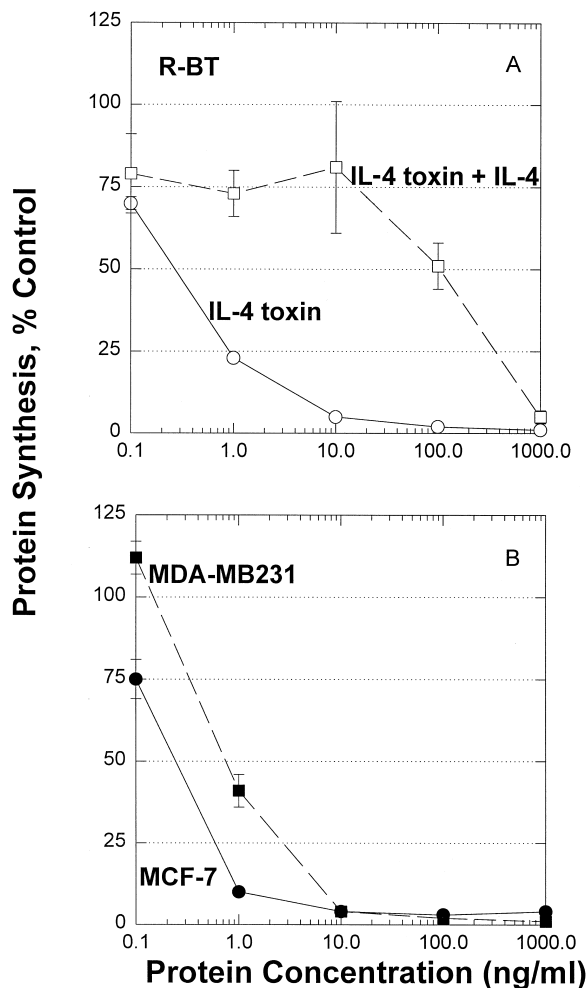


Fig. 1. Cytotoxicity of IL-4(38-37)-PE38KDEL in breast tumors. Ten thousand cells from R-BT primary breast tumor cell culture (A), MCF-7 or MDA-MB231 (B) breast cancer cell lines were incubated with various concentrations of IL-4(38-37)-PE38KDEL. Protein synthesis was measured after 20 hr of culture by incorporation of [3H]-leucine (1 μ Ci for an additional 4 hr), as described in the "Materials and Methods" section. For competition experiments, R-BT cells were preincubated for 45 min with 2 μ g/ml of recombinant IL-4 before addition of IL-4(38-37)-PE38KDEL (A). The results are presented as mean \pm SD % control of untreated cells from quadruplicate determinations. Mean total counts per minute (cpm) \pm SD incorporated in untreated R-BT cells was 11,765 \pm 1,138, in MCF-7 cells 53,924 \pm 8,835 and in MDA-MB231 cells 113,366 \pm 3,261.

Table 1. IL-4R expression and cytotoxicity of IL-4(38-37)-PE38KDEL on human breast carcinoma cell lines and primary cell cultures

Cells	IL-4(38-37)-PE38KDEL IC ₅₀ ^a (ng/ml)	IL-4R Expression ^d (binding sites/cell)
<i>Primary breast tumor cell cultures:</i>		
R-BT	0.2 ± 0.03 ^b	954 ± 59
S-BT	5 ± 3	ND ^e
W-BT	240 ^c	ND
K-BT	1000	ND
<i>Breast cancer cell lines:</i>		
MCF-7	0.4 ± 0.13	2263 ± 157
BT-20	75 ± 7	1703 ± 144
SK-BR3	<1000	ND
ZR75-1	7 ± 5	4687 ± 118
MDA-MB231	1.8 ± 1.6	4598 ± 167

For cytotoxicity assays, 1×10^4 cells were cultured with IL-4-toxins for 20 hr at 37°C, pulsed with 1 μ Ci of [³H]-leucine and further incubated for 4 hr. Cells were harvested and counted as described under Materials and Methods.

^aIC₅₀, the concentration of IL-4-toxin at which 50% inhibition of protein synthesis is observed, compared with untreated cells.

^bThe values are presented as mean ± SEM of five experiments performed in quadruplicate.

^cSingle experiment performed in quadruplicate.

^dSingle saturating concentration of ¹²⁵I-IL-4 was used to calculate binding sites. Results are shown as mean ± SD.

^eND = not done

IL-4R Expression on Breast Carcinoma Cell Lines and Primary Cultures

We previously reported that human breast carcinoma cell lines express high-affinity IL-4R (5). In binding studies, we found that a primary cell culture of breast carcinoma (R-BT) also bound to radiolabeled IL-4 in a concentration-dependent manner. Displacement analysis revealed that these receptors were of high affinity (~1 nM). MDA-MB231 and MCF-7 breast cancer cell lines also expressed high-affinity IL-4R (~0.2 nM) (Fig. 3). The number of binding sites/cell was calculated by single point binding assay and found to vary in different cell types (Table 1).

Crosslinking of ¹²⁵I-IL-4 to Breast Carcinoma Cells

The structure of IL-4R on two different breast carcinoma cell lines was examined next. This was performed by crosslinking radiolabeled IL-4 to surface IL-4R, followed by SDS-PAGE under reducing conditions (Fig. 4A). [¹²⁵I]-IL-4 crosslinked to two prominent proteins at approximately 155 kDa and 85 kDa on both breast cancer cell lines (lanes 1 and 4). In addition,

[¹²⁵I]-IL-4 crosslinked to one protein of about 40 kDa. None of these bands was observed when crosslinking was performed in the presence of 200-fold molar excess of IL-4, indicating that the observed bands were involved in specific [¹²⁵I]-IL-4 binding (Fig. 4A, lanes 2 and 5). Assuming a molecular weight of 15 kDa for hIL-4 and subtracting it from the kDa values indicated on the gel, the molecular weights of the [¹²⁵I]-IL-4 binding proteins were estimated at about 140 kDa, 70 kDa, and 25 kDa, respectively. We reported that the IL-4 receptor shares two chains with IL-13 receptors in various cell lines (21,27). To investigate whether the IL-4 receptor may be related to IL-13R in breast cancer cell lines, we competed for binding of [¹²⁵I]-IL-4 by IL-13 on the MCF-7 cell line. As shown in Fig. 4B, like IL-4, IL-13 also displaced the binding of radiolabeled IL-4 for both bands.

To confirm the identity of [¹²⁵I]-IL-4-IL-4R cross-linked complexes, immunoprecipitation with anti-IL-4R antibody was performed before electrophoresis on SDS-gel. As shown in Fig. 4A (lanes 3 and 6), all three prominent bands were immunoprecipitated, indicating

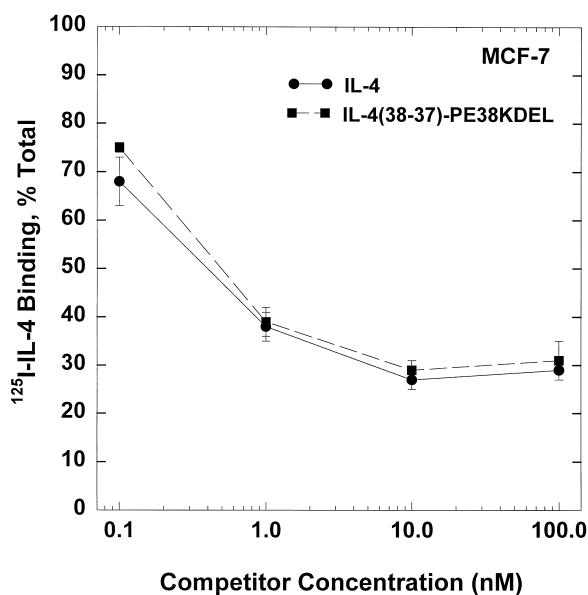


Fig. 2. Displacement of ¹²⁵I-IL-4 binding by IL4 and IL-4(38-37)-PE38KDEL. MCF-7 breast cancer cells were incubated at 4°C with 100 pM [¹²⁵I]-IL-4 and various concentrations of either IL-4 or IL-4(38-37)-PE38KDEL. After 2 hr, cells were centrifuged through a mixture of phthalate oils and cell pellets were counted in a gamma counter. The data points shown are mean ± SD of duplicate determinations. A total of 1,491 ± 137 cpm bound to 1 × 10⁶ MCF-7 cells. The SDs are shown where deviations are larger than the size of point symbols.

that all these proteins formed an IL-4R complex on breast carcinoma cells.

RT-PCR and Northern Analysis of IL-4R Subunits on Breast Carcinoma Cells

We next examined the expression of IL-4Rβ, IL-13Rα' and γ_c chains, all of which can form IL-4R complexes in different cell types. As shown in Figure 5, RT-PCR products of 316 bp for IL-4Rβ and 148 bp for IL-13Rα' chain were expressed in all of the studied breast carcinoma cell lines and primary cell cultures. In contrast, most breast cancer cell lines did not express the 255 bp γ_c product, but it was detected in R-BT primary breast tumor cell culture (Fig. 5, lane 4).

To confirm whether the γ_c chain was expressed in breast cancer cell lines and primary cell cultures, we performed Northern analysis to examine mRNA for IL-4Rβ, IL-13Rα' and γ_c chains. As shown in Fig. 6, IL-4Rβ and IL-13Rα' mRNA was abundantly expressed in all breast cancer cell lines and primary breast cancer cell cultures, however, mRNA for the γ_c chain was not expressed in any cell lines.

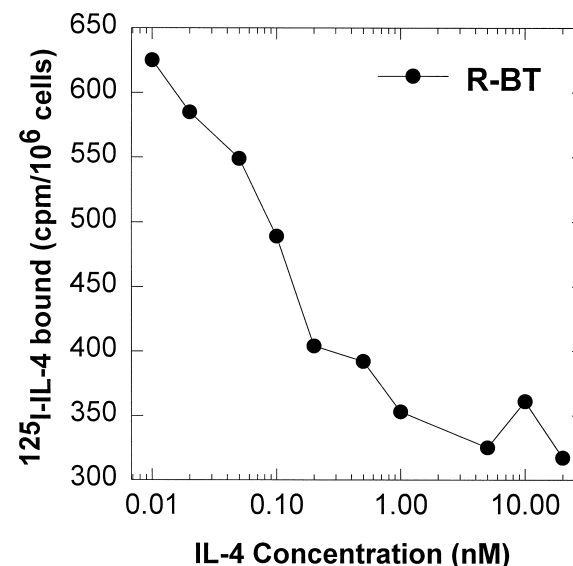
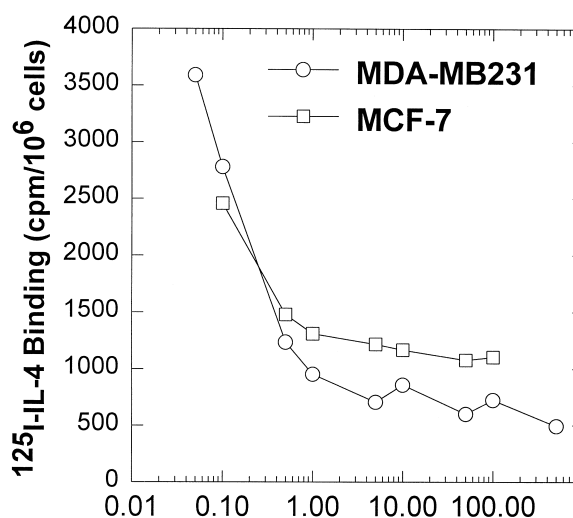


Fig. 3. Displacement analysis of ¹²⁵I-IL-4 binding to breast cancer cells. This was performed on MDA-MB231 and MCF-7 cell lines (upper panel) and R-BT breast cancer primary cell culture (lower panel). Specific binding data was utilized to generate the Scatchard curves. Typically, for these experiments, a single cell suspension of tumor cells was incubated for 2 hr with increasing concentrations of unlabeled excess IL-4 in the presence of a fixed concentration (100-200 pM) of ¹²⁵I-IL-4 at 4°C. Bound radioactivity was determined as described in the materials and methods section.

These data supported our crosslinking studies and further demonstrated that IL-4Rβ and IL-13Rα' chains formed an IL-4R complex on breast carcinoma cells and γ_c did not form a complex, as seen in immune cells. These data indicated that breast tumor cells expressed type II IL-4 receptors.

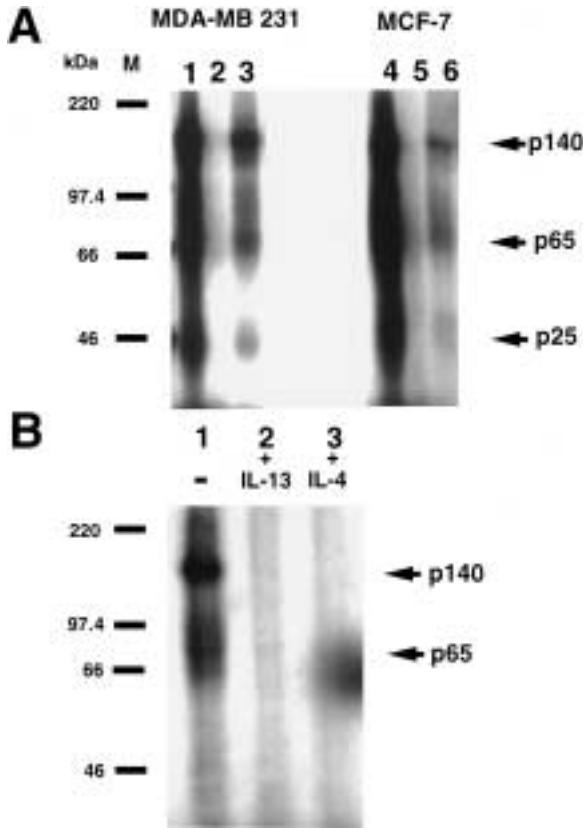


Fig. 4. Crosslinking of ¹²⁵I-IL-4 to IL-4 receptors on breast carcinoma cells. (A) MDA-MD231 and MCF-7 cells (5×10^6) were incubated with ¹²⁵I-IL-4 in the absence (lanes 1 and 4) or presence of excess unlabeled IL-4 (lanes 2 and 5) for 2 hr at 4°C. In both these cell lines ¹²⁵I-IL-4 crosslinked receptors were immunoprecipitated with anti-IL-4 receptor antibody (M-7) (lanes 3 and 6). (B) MCF-7 cells were also crosslinked with radiolabeled IL-4 in the absence (lane 1) and presence of excess of IL-13 (lane 2) or IL-4 (lane 3). Bound ¹²⁵I-IL-4 was cross-linked to IL-4R with disuccinimidyl suberate (DSS). The cells were then lysed at 4°C with modified RIPA buffer. The resulting lysate was analyzed by electrophoresis through an SDS-PAGE (7%) gel. The gel was dried and exposed to an X-ray film for 7 days at -80°C. The molecular weight markers are shown on the left. The positions of different receptor chains are indicated. The dark area in lane 3 (B) represents autoradiography exposure artifact.

Antitumor activity of IL-4-toxin

To determine the antitumor activity of IL-4-toxin, we established a breast tumor model in nude mice and then tested various routes of IL-4 toxin administration. First, we investigated whether intratumoral treatment would lead to regression of established breast cancer. MDA-MB 231 tumor cells were implanted subcutaneously in immunodeficient animals. When

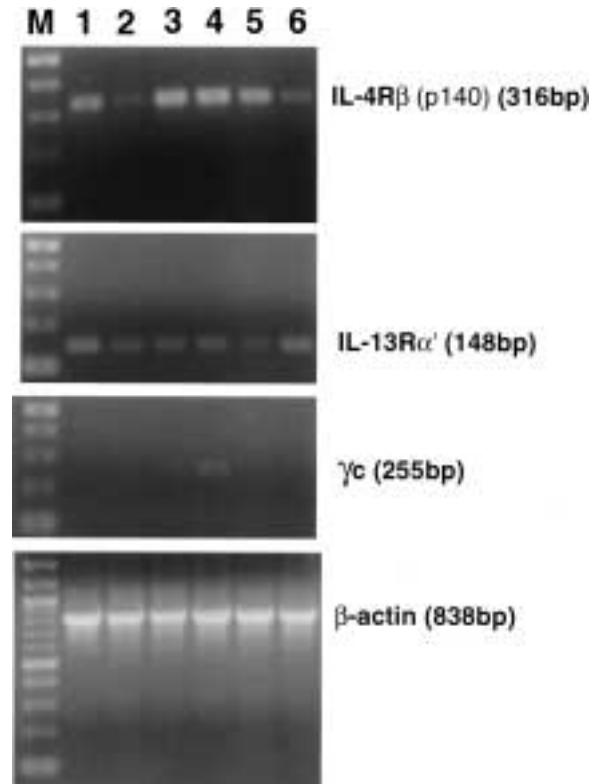


Fig. 5. RT-PCR analysis of different receptor chains. The polymerase chain reaction (PCR) mixture containing specific primers was amplified as described in "Materials and Methods." The numbered lanes represent RNA from BT-20 (1), MCF-7 (2), MDA-MB231 (3), R-BT (4), SK-BR3 (5) and ZR-75-1 (6) breast tumor cell lines. The polymerase chain reaction (PCR) conditions for β-actin were similar to the conditions used for IL-4R chains.

the tumors reached a mean size of 18–25 mm³ (50 mm³), the mice were injected i.t. with varying doses of IL-4 toxin, as specified in "Materials and Methods." Anti-tac-immunotoxin, which binds to the IL-2 receptor α chain, served as a negative control since breast cancer cells may not express IL-2 receptors. The treatment began 8 days after tumor implantation and these animals received two additional injections on days 10 and 12. IL-4-toxin treatment caused a dose-dependent regression of breast tumors (Fig. 7A), as the antitumor effect was more pronounced at the higher dose (750 μg/kg). Although the tumors began to grow again 10 days after the last dose of toxin, the growth rate of tumors in treatment groups was significantly slower, compared with the control group. For example, on day 45 after intratumor administration of IL-4-toxin, the lowest dose (250 μg/kg) caused a significant inhi-

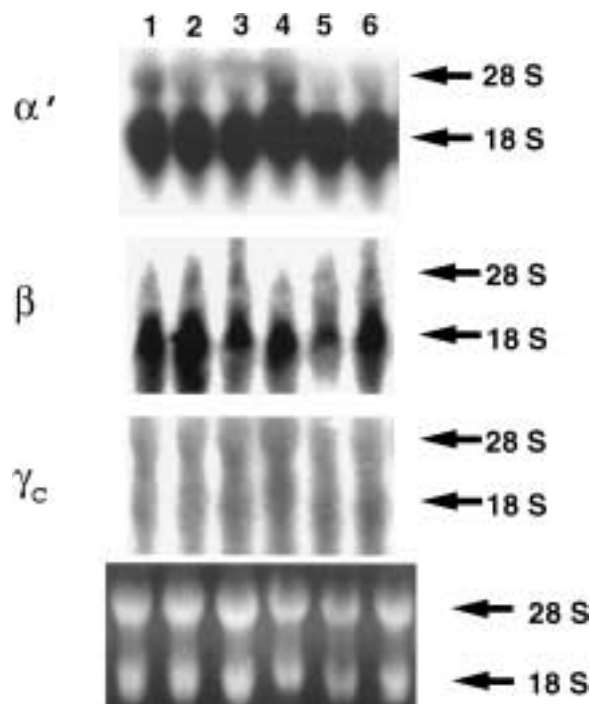


Fig. 6. Northern analysis of IL-4R subunits on breast cancer cells. Total RNA from these cells was electrophoresed (10–20 $\mu\text{g}/\text{lane}$) and hybridized with a ^{32}P -labeled cDNA probe for different IL-4R chains. Autoradiography was performed for 4 hr for IL-13R α' , 8 day for IL-4R β chain and 3–8 days exposure for γ_c . The numbers at the top of the gel represent RNA from BT20 (1), R-BT (2), SK-BR3 (3), ZR-75-1 (4), MCF-7 (5), and MDA-MB231 (6) breast cancer cell lines.

bition of tumor growth, compared with the control animals treated with excipient ($p < 0.008$) or with anti-Tac immunotoxin ($p < 0.0009$).

Animals that received the highest dose (750 $\mu\text{g}/\text{kg}$) of IL-4-toxin on days 8, 10 and 12 after tumor implantation received additional intratumor doses (500 $\mu\text{g}/\text{kg}$) of IL-4-toxin on days 22, 24 and 26. These mice again showed further decrease in tumor size, however, these tumors continued to grow until the end of the experiment. The tumor sizes in this group were significantly lower, compared with the control, anti-Tac-immunotoxin group or lower-dose treated groups ($p < 0.0005$ vs. control, $p < 0.0001$ vs. anti-Tac-immunotoxin and $p < 0.05$ vs. 250 $\mu\text{g}/\text{kg}$). The control mice were sacrificed and the experiment was terminated on day 59, because of heavy tumor burden in accordance with the guidelines of NIH Animal Research Advisory Committee.

An additional five animals were also i.t. injected with IL-4(38-37)-PE38KDEL when tumors had almost doubled in size and reached 36 mm^2 . These animals received 750 $\mu\text{g}/\text{kg}$ of IL-4-toxin on days 22, 24 and 26, followed with 500 $\mu\text{g}/\text{kg}$ of IL-4-toxin on days 36, 38 and 40. The growth of these large tumors also was slowed, compared with control tumors (data not shown). These animals were sacrificed on day 52 due to large tumors.

In another experiment, cohorts of five nude animals bearing subcutaneous human breast cancer xenografts were given intraperitoneal injections with escalating doses of IL-4(38-37)-PE38KDEL. These injections were given 2 times a day for 5 days. As shown in Fig. 7B, IL-4(38-37)-PE38KDEL caused regression of established breast cancer nodules in a dose-dependent manner. At the highest dose (150 $\mu\text{g}/\text{kg}/\text{dose}$), one of the five animals showed complete regression of the established tumor. The size of the tumor in the remaining four animals continued to be significantly smaller, compared with any other groups, including controls ($p < 0.05$ control vs. 100 $\mu\text{g}/\text{kg}$ dose; $p < 0.006$ control vs. 150 $\mu\text{g}/\text{kg}$ dose). The tumors continued to grow in control animals, eventually reaching about 65 mm^2 . All animals were sacrificed due to large tumor burdens.

Sensitivity of Breast Tumor Cultures after in vivo Passage and IL-4-toxin Therapy

To determine whether breast tumor cells acquired resistance after in vivo passage or after IL-4-toxin therapy, tumors were excised from control and IL-4-toxin-treated animals. Single cell suspensions were prepared by enzyme digestion and the resulting cells were passaged at least once before cytotoxicity of IL-4(38-37)-PE38KDEL was determined. The sensitivity to IL-4(38-37)-PE38KDEL, as well as the IC_{50} , was similar on cells derived from control animals and IL-4-toxin treated animals. The IC_{50} was also similar to that observed in cells not injected into animals (not shown).

Discussion

We have demonstrated that human breast cancer cells are highly sensitive to the cytotoxic activity of a IL-4 receptor targeted chimeric toxin comprised of IL-4 and a mutated form of PE. Recombinant IL-4(38-37)-PE38KDEL was also

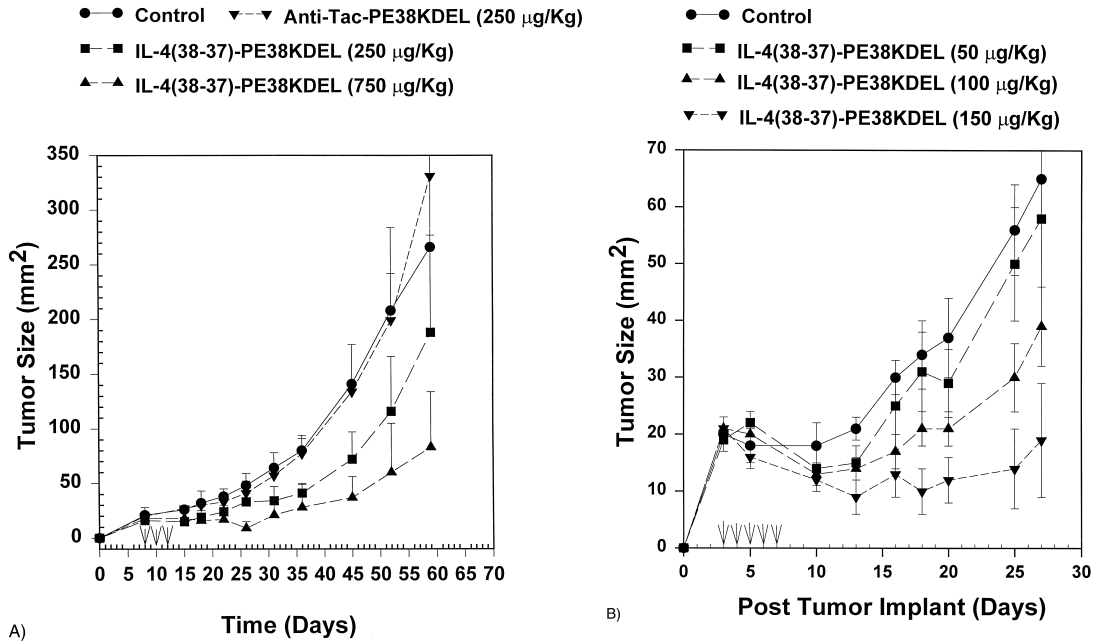


Fig. 7. Antitumor activity of IL-4(38-37)-PE38KDEL. Four- to six-week-old nude mice were injected subcutaneously with $3-4 \times 10^6$ MDA-MB231 breast tumor cells on day 0. (A) Animals were injected i.t. on days 8, 10 and 12, with varying doses of IL-4(38-37)-PE38KDEL or a fixed dose of anti-Tac-PE38KDEL in 20 μ l volume in a different area of the tumor on each day of injection. The mice that received 750 μ g/kg of IL4(38-37)-PE38KDEL were re-injected i.t. with a dose of 500 μ g/kg on days 22, 24 and 26. The legend represents the dosage and number of injections

on alternate days. Anti-Tac-immunotoxin (anti-Tac-PE38KDEL) binds to IL-2 receptor α chain and served as a negative control. The data presented are mean \pm SD of five animals in each group. (B) Cohorts of five animals were i.p. administered with doses of 50, 100, or 150 μ g/kg IL-4(38-37)-PE38KDEL 2 times a day for 5 days, beginning on day 3. In the control animals injected with excipient, the tumor continued to grow to a size of ~ 65 mm² by day 27, when all animals were sacrificed. The data presented are mean \pm SEM of 5 animals in each group.

active in vivo and caused regression of established breast tumors in a xenograft model. Intraperitoneal and intratumoral administration of IL-4(38-37)-PE38KDEL caused significant antitumor activity in a breast tumor model without any significant systemic toxicity.

Breast cancer cell lines and primary cell cultures express high-affinity receptors for IL-4 on their cell surface. The receptor numbers vary with the cell lines. There does not appear to be a correlation between sensitivity to IL-4-toxin and number of IL-4 receptors. R-BT cells are very sensitive to the cytotoxic activity of the IL-4-toxin, even though they express the lowest number of IL-4 receptors. A normal breast epithelial cell culture was not sensitive to the cytotoxic activity of IL-4-toxins, indicating that normal breast epithelial cells may not express or express very low levels of IL-4R. IL-4(38-37)-PE38KDEL appears non-cytotoxic to resting normal human lymphoid cells, indicating that this agent could be useful for breast cancer therapy without affecting normal cells.

The structure of IL-4R on breast cancer cell lines was assessed by affinity crosslinking studies. Radiolabeled IL-4 crosslinked to two prominent proteins migrating at 140 and 65 kDa and none of these bands were observed when crosslinking was performed in the presence of an excess of unlabeled IL-4. The 140 and 65 kDa molecular mass of these cross-linked proteins corresponded to IL-4R β and IL-13R α' chain, respectively, as per our previous nomenclature (18). IL-13R α' has been shown to be a novel and essential component of the IL-4 receptor system in cells that do not express IL-2R γ_c (27). Breast cancer cells did not express IL-2R γ_c chain, as antibody to IL-2R γ_c did not immunoprecipitate IL-4 receptor components in crosslinking studies (not shown). The lack of γ_c chain expression was confirmed by RT-PCR and Northern analysis, while mRNA for IL-4R β and IL-13R α' was abundantly expressed. These results indicate that breast carcinoma cells express type II IL-4 receptors (18,29).

Our data confirm previous results demonstrating that IL-4(38-37)-PE38KDEL has significant antitumor activity in vitro and in vivo (37-42). We have reported that IL-4(38-37)-PE38KDEL can cause complete regression of established human epidermoid carcinoma and glioma tumors in nude mouse models (38,42). Based on those studies, it was assumed that breast cancer cell lines that expressed IL-4R would also be sensitive to the cytotoxic activity of IL-4(38-37)-PE38KDEL. However, expression of IL-4 receptors on primary breast tumor cultures and sensitivity of these cultures and tumor cell lines to IL4-toxins had not been demonstrated. Our current studies confirm previous assumptions and demonstrate that IL-4 receptor positive breast cancer cells are generally sensitive to IL-4(38-37)-PE38KDEL. Since five of nine cell lines and primary cell cultures were highly sensitive, it is believed that IL-4(38-37)-PE38KDEL may have a major role in the management of patients with breast cancer.

In vitro sensitivity to IL-4(38-37)-PE38KDEL has been shown to generally correlate with in vivo antitumor activity against epidermoid and brain tumor models (38,42). However, it was not known whether in vitro sensitivity of breast cancer cells to IL-4(38-37)-PE38KDEL would correlate with in vivo antitumor activity. Although we observed significant antitumor activity against breast tumors when injected i.t., complete regression of the tumor was not observed in this model, as seen in human glioma models, even though equal doses of IL-4(38-37)-PE38KDEL were administered via the same route. In addition, systemic (intraperitoneal) administration of IL-4(38-37)-PE38KDEL required more frequent injections to cause significant regression of tumors. The reason for different sensitivity to IL-4-toxin is not clear. MDA-MB231 cells used for in vivo studies were as sensitive as U251 glioma cells to IL-4-toxin (IC_{50} for both cell lines ranged between 2-4 ng/ml). It is possible that trafficking of IL-4(38-37)-PE38KDEL to breast cancer nodules is more restricted, compared with U251 glioma nodules. This is likely because single cell suspensions from treated nodules maintained their sensitivity to IL-4(38-37)-PE38KDEL. It is known that tumor nodules often develop physiological barriers restricting the ability of many macromolecules to home to tumor cells (45). Enhancement of the permeability of tumor vasculature may increase sensitivity of these tumors to targeted toxins.

In contrast to i.p. and i.t. routes, administration of IL-4(38-37)-PE38KDEL by the i.v. route did not demonstrate significant antitumor activity in the breast tumor model (data not shown). This observation is in contrast to that observed in the brain tumor model, in which IL-4(38-37)-PE38KDEL demonstrated significant antitumor activity when injected by the i.v. route (42). Due to short serum half-life ($t_{1/2} = 10$ min) (38) of IL-4(38-37)-PE38KDEL, i.v. administered drug is not available in sufficient quantity to mediate antitumor activity in the breast tumor model. Any manipulation that increases serum half-life may enhance antitumor activity of IL-4-toxin. One way to achieve this would be to administer IL-4(38-37)-PE38KDEL by continuous infusion using a 5-day cycle. Alternatively, co-administration of agents that can increase vascular permeability for a short period of time may improve drug availability to tumors. In this regard, IL-2 a T cell-derived cytokine, has been shown to increase capillary permeability in various models and in patients undergoing IL-2 therapy for cancer (46). Concurrent administration of IL-2 may not only increase drug trafficking, it may also improve antitumor activity against breast cancer through host immune activation. These hypotheses must be tested in preclinical models.

Other immunotoxins targeted to breast cancer have been tested in preclinical models and in the clinic. A B3 antibody that recognizes carbohydrate antigen (Le^y) on many human solid cancers when attached to various mutated forms of PE (B3-PE, B3-Lys-PE40 or B3-Lys-PE38) has shown significant antitumor activity against breast cancer and other tumor models (47,48). Similarly, another antibody (BR96) targeted to the Le^y antigen when chemically conjugated to doxorubicin or conjugated or fused to PE has shown significant antitumor activity against breast cancer models (49,50). In a phase I clinical trial, B3-LysPE38 also showed some antitumor activity against breast cancer (48). IL-4(38-37)-PE38KDEL appears to be similar in activity to B3-LysPE38, BR96-doxorubicin (BR96-dox) or BR96 sFV-PE40 in breast cancer model, as shown in the current study. However, it is noteworthy that the size of IL-4(38-37)-PE38KDEL is considerably smaller than B3-PE, B3-Lys-PE38, BR96-dox or BR96 sFV-PE40. Furthermore, in a clinical trial, B3-LysPE38 caused vascular leak syndrome, presumably through the activation and

lysis of endothelium and other normal tissues. Since smaller molecules may have better tumor penetration and IL-4(38-37)-PE38KDEL has less reactivity to normal tissues, it is likely that IL-4(38-37)-PE38KDEL would be better tolerated with improved antitumor activity. Additional comparative studies are needed to address this issue. Furthermore, since all these agents target different antigens or receptors on breast cancer cells, it is possible that a combination therapy with two of these agents may have synergistic antitumor activities against breast cancer.

In conclusion, circular permuted IL-4-toxin, IL-4(38-37)-PE38KDEL, may be a useful therapeutic agent for the treatment of human breast cancer because a majority of human breast carcinoma cell cultures and lines expressed high levels of IL-4R and were sensitive to its cytotoxic activity. Further *in vivo* studies should be performed to explore the role of IL-4(38-37)-PE38KDEL in breast cancer models.

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