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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

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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: ^{125}I-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-4Rβ and IL-13Rα' chains, but not IL-2Rγ_c chain. These cells were highly sensitive to the cytotoxic effect of IL-4(38-37)-PE38KDEL. The IC_{50} (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

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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 moncloclonal 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 antitumor 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 expressed, 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-4Rα (20). Because of similarities in extracellular domains (WSXWS motif and four cysteine residues at a fixed location) and long intracellular domains between the IL-4Rα 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-4Rβ (18,21). This recommendation also was based on its similarity with the IL-2Rβ 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-2Rγ 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-4Rβ 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-13Rα' also termed as IL-13Rα_1) (18,21,27,28). Consequently, IL-4Rβ and IL-13Rα' 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-13Rα' (or α_1) chain along with the IL-4Rβ 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, triiodothionine, 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 streptomicin. 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 μCi 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 μCi/μg. 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 (1×10^6/100 μl) 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 [^{125}I]-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 [^{125}I]-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-4Rβ 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 prehybridyzed for 4 hr at 42°C and hybridized with ^{32}P-labeled cDNA probes of IL-4Rβ, IL-13Rα', 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-4Rβ: 5′ primer, 5′-ATGGGGTGGCTTTGCTCTGGG-3′ and 3′ primer, 5′-ACCTTCCCGAGGAAGTTCGGG-3′; for γ_c, 5′ primer, 5′-CCAGAGGTTCAGTGTTTTGTGTT-3′ and 3′ primer, 5′-CAGGTTTCAGGATTTAGGGTGTA-3′; for IL-13Rα': 5′ primer, 5′ GGAGGATACATCTTGTTTCATGG-3′ and 3′ primer, 5′-GAGCTTCTTACCTATACTCATTTCTTGG-3′. The IL-4Rβ RT-PCR cDNA product was 316 bp; 256 bp; for γ_c and 148 bp for IL-13Rα'. 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 $\times$ 10^6 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 muliplying 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_{50s} 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.

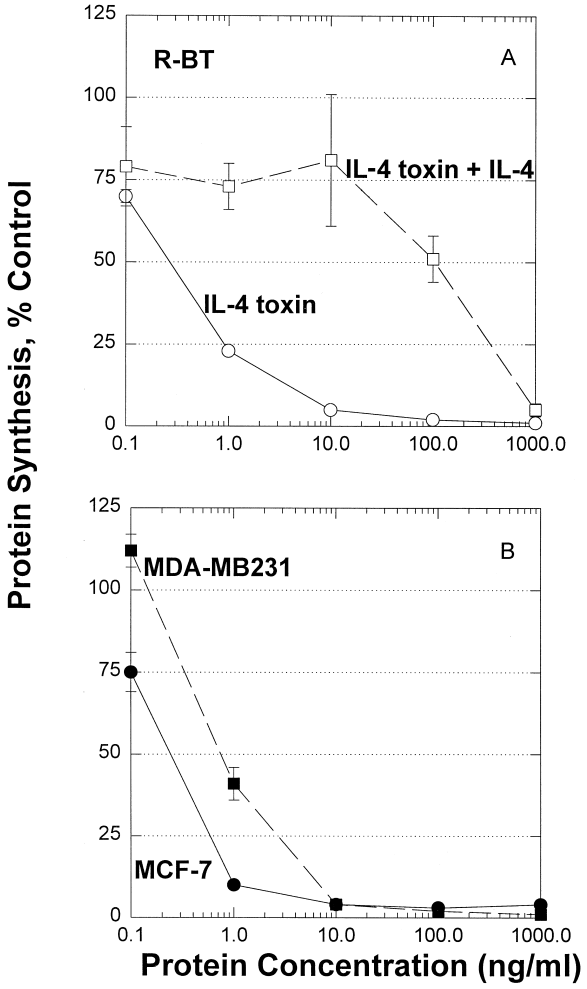

Fig. 1. Cytotoxicity of IL-4(38-37)-PE38KDEL in breast tumors. Ten thousand cells from R-BT primary breast tumor cell culture (A), MCF-7 or MDA-MB231 (B) breast cancer cell lines were incubated with various concentrations of IL-4(38-37)-PE38KDEL. Protein synthesis was measured after 20 hr of culture by incorporation of [^{3}H]-leucine (1 μCi 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 μg/ml of recombinant IL-4 before addition of IL-4(38-37)-PE38KDEL (A). The results are presented as mean ± SD % control of untreated cells from quadruplicate determinations. Mean total counts per minute (cpm) ± SD incorporated in untreated R-BT cells was 11,765 ± 1,138, in MCF-7 cells 53,924 ± 8,835 and in MDA-MB231 cells 113,366 ± 3,261.

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