Internalization Property of Interleukin-4 Receptor α Chain Increases Cytotoxic Effect of Interleukin-4 Receptor-targeted Cytotoxin in Cancer Cells

Koji Kawakami, Mariko Kawakami, Pamela Leland, and Raj K. Puri
Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

ABSTRACT

Although the receptor for interleukin-4 (IL-4R) is highly expressed on solid human cancer cells, its significance and internalization function is still unclear. To address these issues, we reconstituted Chinese hamster ovarian (CHO-K1) cells with various components of the IL-4R by transient transfection and performed internalization assays using radiolabeled IL-4. Radiolabeled IL-4 internalized through the IL-4Rα chain in a time-dependent manner. When the IL-4Rα chain was cotransfected with the IL-13Rα1 or γc chain, the IL-4 internalization level was identical to IL-4Rα transfectants, suggesting that the IL-4Rα chain plays a major role in IL-4 internalization. These results were confirmed by determining the cytotoxicity of a chimeric protein composed of IL-4 and a mutated form of Pseudomonas exotoxin [IL4(38–37)-PE38KDEL] in CHO-K1 cells transfected with increasing concentrations of IL-4Rα cDNA. To use the internalization property of the IL-4Rα chain in the context of IL-4R-targeted cytotoxin therapy, we transiently transfected pancreatic and brain tumor cells with IL-4Rα chain. Surprisingly, these tumor cells acquired 4–75-fold higher binding activity to IL-4 compared with control cells. Consequently, the cytotoxic activity of IL-4 toxin to these cancer cells was enhanced 5–13-fold compared with control cells as assessed by protein synthesis inhibition and clonogenic assays. Taken together, a combination approach that involves transfer of the IL-4Rα gene and IL-4R-targeted cytotoxin therapy may serve as a novel approach for cancer therapy.

INTRODUCTION

Targeting of cell surface antigens or receptors expressed on tumor cells is a modern and effective approach for cancer therapy (1–5). In the last decade, many investigators have focused on the identification of tumor-specific targets and approaches that use these targets for cancer therapy. In this context, we have identified the IL-4Rα as a specific tumor cell surface target for novel cytotoxic agents (5).

IL-4R has been shown to be overexpressed in a wide variety of murine and human tumor cells in vitro and in vivo (6–9). It was found that the IL-4R system could exist in three different types. Type I IL-4Rs consist of a major Mr 140,000 protein (IL-4Rα, also known as IL-4Rβ) and the IL-2Rγ chain (γc). Type II receptors are composed of IL-4Rα and IL-13Rα1 (also known as IL-13Rα1) chains. In type III IL-4R, all three chains may form a functional IL-4R complex (10–14). The significance of expression of IL-4Rs on solid tumor cells is unrecognized; however, we and others have observed that solid human tumors, including malignant melanoma, breast carcinoma, ovarian carcinoma, mesothelioma, glioblastoma, renal cell carcinoma, head and neck carcinoma, and AIDS-associated Kaposi’s sarcoma, express IL-4R (6–9, 15–24).

We and others have investigated the mechanism of IL-4R internalization after binding to ligand (25–28). Among the IL-4R components, IL-4Rα chain seems to play a premier role in ligand binding (14), and a recent study showed that IL-4Rα and γc chains are responsible for ligand-induced internalization in human T cells (28). However, we have reported that the IL-4R system in solid tumor cells is composed of IL-4Rα and IL-13Rα1 chains (type II IL-4R; Refs. 11–14, 22, 24). This observation suggests that in solid tumor cells, internalization of IL-4-IL-4R could occur through IL-4Rα and IL-13Rα1 chains or IL-4Rα chain alone. To study this phenomenon, we reconstituted Chinese hamster ovary (CHO-K1) cells by transient transfection with various chains of IL-4R and examined the internalization characteristics of these transfectants. We found that the IL-4Rα chain by itself can internalize after binding to 125I-IL-4.

To target IL-4Rs on human solid cancer cells, we have produced a recombinant agent that binds to IL-4R on tumor cells (5, 8). This molecule is a chimeric protein composed of circular permuted IL-4 and a truncated form of a powerful bacterial toxin called Pseudomonas exotoxin [IL4(38–37)-PE38KDEL], and we have shown that this toxin is highly cytotoxic to IL-4R-

Received 6/6/01; revised 10/3/01; accepted 10/3/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 These studies were conducted as part of a collaboration between the Food and Drug Administration and Neurocrine Biosciences Inc. under a Cooperative Research and Development Agreement (CRADA).
2 To whom requests for reprints should be addressed, at Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, NIH Building 29B, Room 2NN10, 29 Lincoln Drive MSC 4555, Bethesda, MD 20892. Phone: (301) 827-0471; Fax: (301) 827-0449; E-mail: puri@cber.fda.gov.

The abbreviations used are: IL, interleukin; IL-4R and IL-13R, interleukin-4 and interleukin-13 receptor, respectively; γc, common γ chain; PE, Pseudomonas exotoxin A; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR.
positive tumor cells in vitro (8, 16, 20–24) and in vivo (23, 24, 29, 30). On the basis of these preclinical developments, IL-4 toxin is being tested in the clinic. In our initial study, IL4(38–37)-PE38KDEL was infused over a 4–8-day period into recurrent malignant high-grade glioma by one to three stereotactically placed catheters; in six of nine patients, IL-4 toxin mediated extensive necrosis of tumor without systemic toxicity (31). Because in this Phase I clinical trial the numbers of patients were small, no correlation between dose and tumor response could be determined. However, ongoing Phase I/II clinical trial supported these observations and demonstrated an advantage of this form of therapy. Again, no dose-dependent tumor response was clearly observed.4 The long-term effect of this form of therapy is being evaluated and may not be apparent until a Phase III controlled clinical trial is undertaken. However, results to date indicate that IL-4 toxin can be safely given to patients with glioblastoma multiforme and that not all patients seem to respond to IL-4 toxin therapy. Although in tissue culture 100% of glioma samples express IL-4Rα mRNA, it is possible that the level of surface expression of IL-4Rα protein is different in different samples. In vitro data suggest that the cytotoxicity of IL-4 toxin depends on the number of IL-4 binding sites/cell: the higher the receptor numbers, the higher the sensitivity to IL-4 toxin. Therefore, it would be of interest to determine whether the IL-4Rα chain can increase sensitivity to IL-4 toxin. An increase in sensitivity may help patients with cancer by gene transfer of this chain followed by IL4(38–37)-PE38KDEL therapy. A new Phase I clinical trial for the therapy of renal cell and breast cancer therapy began recently. In this trial, IL-4 toxin will be administered i.v. every alternate days for 3 injections. Although we did not test the IL-4R expression level in fresh or fixed tumors, theoretically, a higher expression level of IL-4Rα chain may be desirable to achieve maximum effects of systemic treatment.

In addition, although IL-4 toxin is highly cytotoxic to cancer cells that express IL-4Rα, its cytotoxic efficacy is limited in cell types that express no or low levels of this receptor. On the basis of previous studies that have shown the importance of correlation between receptor number on the cell surface and efficacy of receptor-targeted toxins, we hypothesized that transfer of the IL-4Rα gene into these cells may increase the sensitivity to IL-4 toxin. Because in clinical trials not all patients’ tumors seem to respond, it is possible that not all cells in a tumor sample express IL-4Rα chain or that a majority of tumor cells express lower levels of IL-4Rα chain. Therefore, patients may benefit further by transfer of gene encoding this chain, which may sensitize all cancer cells to IL-4 toxin therapy. To demonstrate this, human pancreatic (SU.86.86 and COLO587) and primary brain tumor (BT10 and BT12) cell lines were transfected with IL-4Rα chain, and cytotoxicity assays were performed. IL-4Rα transfectants dramatically enhanced the sensitivity to the cytotoxic effect of IL-4R-targeted cytotoxin in vitro.

**MATERIALS AND METHODS**

**Recombinant Cytokine and Toxin.** Recombinant human IL-4 and IL-13 were produced and purified in our laboratory (39). Recombinant IL-4 toxin IL4(38–37)-PE38KDEL, containing the circularly permuted IL-4 mutant in which amino acids 38–129 were linked to amino acids 1–37 via a GGNGG linker and then fused to truncated toxin PE38KDEL, consisting of amino acids 253–364 and 381–608 of PE, followed by KDEL, was expressed in _Escherichia coli_ and purified by a modified procedure as described previously and provided by Neurocrine Biosciences Inc. (San Diego, CA; Refs. 21, 23). Recombinant IL13-PE38QQR was produced and purified in our laboratory (40, 41).

**Cell Lines.** Chinese hamster ovary (CHO-K1) and human pancreatic cancer cell lines (SU.86.86 and COLO587) were purchased from the American Type Culture Collection (Manassas, VA). Human glioblastoma cell lines (BT10 and BT12) were established at the Cleveland Clinic (Cleveland, OH) as described previously (42, 43). Cells were cultured in AMEM (CHO-K1) or RPMI 1640 (SU.86.86 and COLO587) containing 10% FBS (BioWhittaker Inc., Walkersville, MD), 1 mM HEPES, 1 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker Inc.). Human glioblastoma cell lines were cultured in astrocyte growth medium (Clonetics-BioWhittaker Inc.) containing 5% FBS.

**Transient Transfection.** cDNA encoding human IL-4Rα (kindly provided by Dr. M. Widmer of Immunex Corp., Seattle, WA), IL-13Rα1, and Yc chain targets were inserted in the vector, using the _XhoI_ and _XbaI_ restriction sites, and the sequences of flanking regions of junctions were verified by direct sequencing (14, 44–47). Plasmid DNA (12 μg/100-mm culture dish) was transfected into semi-confluent cells by GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s instructions. Briefly, cells (2 × 10^6/100-mm dish) were incubated with the DNA-GenePORTER mixture for 5 h in DMEM (BioWhittaker). DMEM containing 20% FBS was then added, and incubation was continued. Twenty-four h after transfection, the medium was changed to DMEM with 10% FBS, and cells were incubated for an additional 24 h. Approximately 48 h after the start of transfection, cells were trypsinized and experiments were performed.

**Internalization Assays.** Internalization assays were performed as described previously (27, 41). CHO-K1 cells transfected with various chains of the IL-4Rα were incubated in binding buffer containing 0.2 nM chloroquine at 37°C for 5 min to prevent degradation of internalized 125I-IL-4. The cells were then washed, and 2 × 10^5 cells were incubated with 0.5 nM 125I-IL-4 at 4°C for 2 h. After the free 125I-IL-4 was removed, cell pellets were resuspended in 2 ml of binding buffer and incubated at 37°C. At various time intervals, two duplicate sets of 50-μl aliquots were taken. One set was incubated with

---

Transfer of IL-4Rα Chain Gene Increases Cytotoxic Effect of IL-4R-targeted Cytotoxin

Elmer Corp., Norwalk, CT). A 10–μl aliquot of the reverse transcription reaction was amplified in a 100-μl final volume of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 50 mM KCl, 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp.), 0.1 μg of specific primers for IL-4Rα chain (11). The PCR product (10 μl) was run on 2% agarose gel for UV analysis.

RT-PCR Analysis. To detect the mRNA expression of IL-4Rα chain in transfected cancer cells, we isolated total RNA using TRIZOL reagent (Life Technologies, Inc., Grand Island, NY) and then performed RT-PCR analysis. Two μg of total RNA were incubated for 30 min at 42°C in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 50 mM KCl, 1 mM each of deoxynucleotide triphosphates, 1 unit/μl RNase inhibitor, 2.5 μM random hexamer, and 2.5 units/μl Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Corp., Norwalk, CT). A 10-μl aliquot of the reverse transcription reaction was amplified in a 100-μl final volume of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 50 mM KCl, 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp.), and 0.1 μg of specific primers for IL-4Rα chain (11). The PCR product (10 μl) was run on 2% agarose gel for UV analysis.

Radioactive Binding Assays. Recombinant human IL-4 was labeled with 125I (Amersham Corp., Arlington Heights, IL), using IODO-GEN reagent (Pierce, Rockford, IL). The specific activity of the radiolabeled cytokine was estimated to be 20.4 μCi/μg (IL-4) of protein. For binding experiments, 5 × 10⁵ cells in 100 μl of binding buffer (RPMI 1640 containing 0.2% human serum albumin and 10 mM HEPES) were incubated with 200 pm 125I-IL-4 with or without various concentrations (10 pm to 100 nm) of unlabeled IL-4 or IL-13 at 4°C for 2 h. Cell-bound 125I-IL-4 was separated from unbound by centrifugation through a phthalate oil gradient, and radioactivity was determined with a gamma counter (Wallac, Gaithersburg, MD).

Protein Synthesis Inhibition Assay. The cytotoxic activity of IL-4 toxin was tested as described previously (8). Typically, 10⁶ cells were cultured in leucine-free medium with or without various concentrations of IL4(38–37)-PE38KDEL for 20–22 h at 37°C, after which 1 μCi of [³H]leucine (NEN Research Products, Boston, MA) was added to each well and incubated for an additional 4 h. Cells were harvested, and radioactivity incorporated into cells was measured by a beta plate counter (Wallac).

Clonogenic Assay. The in vitro cytotoxic activity of IL4(38–37)-PE38KDEL against SU.86.86 cells transfected with IL-4Rα chain or vector only (mock control) was also determined by a colony-forming assay. The cells were plated in triplicate in 100-cm² Petri dishes with 7 ml of RPMI 1640 containing 20% FBS and were allowed to attach for 20–22 h. The number of cells/plate was chosen such that >100 colonies were obtained in the control group. The cells were exposed to different concentrations of IL-4 toxin (0–100 ng/ml) for 10 days at 37°C in a humidified incubator. The cells were washed, fixed, and stained with crystal violet (0.25% in 25% alcohol). Colonies consisting of >50 cells were scored. The percentage of colony survival was determined from the number of colonies formed in the control and treated groups.

RESULTS

Internalization of 125I-IL-4 in CHO-K1 Cells Reconstituted with Various Chains of IL-4R. We first reconstituted CHO-K1 cells with various chains of the IL-4R and performed internalization assays. As shown in Fig. 1A, cells transfected

![Graph showing internalization of 125I-IL-4 in CHO-K1 cells reconstituted with various chains of IL-4R.](clincancerres.aacrjournals.org)
with vector only (mock) control did not show internalization of radiolabeled IL-4 into the cells. When cells were transfected with IL-4Rα chain cDNA, the internalization of ligand was increased to a high level (Fig. 1B; 44% at 120 min). However, when CHO-K1 cells were transfected with IL-4Rα and γc (type I IL-4R; Fig. 1C), IL-4Rα and IL-13Rα1 (type II IL-4R; Fig. 1D), or all three chains (type III IL-4R; Fig. 1E), the internalization level was almost identical (40–44% at 120 min) compared with cells transfected with IL-4Rα alone (Fig. 1A). Although solid tumor cells express type II IL-4R, which is composed of IL-4Rα and IL-13Rα1 chains, cells that express the IL-4Rα chain internalize the ligand only as well as type II IL-4R-expressing cells.

**IL-4 Toxin Is Cytotoxic to CHO-K1 Cells Transfected with IL-4Rα Chain in a Gene Dose-dependent Manner.** To further confirm the results obtained by internalization assays, we assessed the cytotoxicity of recombinant IL4(38–37)-PE38KDEL, which targets IL-4R. IL4(38–37)-PE38KDEL binds to IL-4R and is internalized by endocytosis, subsequently causing cell death through the inhibition of new protein synthesis. Thus, cytotoxicity observed in transfected cells indicates receptor internalization (8, 16, 20–24, 41). CHO-K1 cells were transfected with various amounts of IL-4Rα chain (0–12 μg) cDNA, and the sensitivity to IL4(38–37)-PE38KDEL was determined. As shown in Fig. 2, CHO-K1 cells transfected with vector only or a small amount (0–3 μg) of IL-4Rα cDNA were slightly sensitive to the cytotoxic effect of IL4(38–37)-PE38KDEL. However, when CHO-K1 cells were transfected with higher concentrations (6–12 μg) of IL-4Rα cDNA, sensitivity to IL4(38–37)-PE38KDEL was increased in a gene dose-dependent manner. The IC_{50} (IL-4 toxin concentration causing 50% inhibition of protein synthesis) were 540 ng/ml (6 μg of IL-4Rα cDNA), 300 ng/ml (9 μg of IL-4Rα cDNA), and 80 ng/ml (12 μg of IL-4Rα cDNA), respectively. These data suggest that the internalization of IL-4 increases as the IL-4Rα chain expression level increases, and consequently, the cytotoxicity of IL4(38–37)-PE38KDEL increases as the IL-4Rα chain expression level in the target cell increases.

**Expression of IL-4Rα Chain mRNA Increases in Cancer Cells after Transfection of IL-4Rα cDNA.** Four cancer cell lines (two pancreatic cancer cell lines, SU.86.86 and COLO587; and two glioblastoma cell lines, BT10 and BT12) were examined for IL-4Rα chain mRNA expression by RT-PCR. As shown in Fig. 3, we found that low-level expression of IL-4Rα chain was observed in three of these four cancer cell lines (COLO587 cells did not seem to express mRNA for this chain). After gene transfer, IL-4Rα-transfected cell lines showed ample mRNA expression. PM-RCC cells, which express IL-4Rα chain mRNA, served as a positive control, and glyceraldehyde-3-phosphate dehydrogenase mRNA served as an internal control.

**Binding of Radiolabeled IL-4 to Pancreatic Cancer Cells Increases after Transfection with IL-4Rα Chain.** We then determined the expression of IL-4R on pancreatic cancer cell lines by 125I-IL-4 binding assays. Two pancreatic cancer cell lines that express no or low levels of mRNA for the primary IL-4-binding component, IL-4Rα chain, showed limited binding to 125I-IL-4 (Fig. 4). However, when these cells were transfected with IL-4Rα chain, the binding activity of 125I-IL-4 was dramatically increased. An excess of unlabeled IL-4 inhibited the binding of 125I-IL-4, indicating specificity. Because these cancer cells expressed IL-4R with shared IL-13Rα1 chain (type II IL-4R; Refs. 11, 22, 48), we also tested whether IL-13 displaced the binding of 125I-IL-4. As expected, IL-13 showed partial displacement of 125I-IL-4 binding in SU.86.86 and COLO587 cell lines. From these experiments, we also calculated the number of IL-4-binding sites on cell lines transfected with IL-4Rα or vector alone (mock control). As shown in Table 1, after transfection with IL-4Rα chain, IL-4-binding sites increased 4–75-fold compared with control cells.

**IL-4Rα Chain Gene-transfected Cancer Cells Demonstrate Increased Sensitivity to IL4(38–37)-PE38KDEL.** We have developed a chimeric fusion protein, IL-4 toxin [IL4(38–37)-PE38KDEL], that targets IL-4R on the surface of cancer cells. Although IL-4 toxin has been proven to have very high antitumor activity against IL-4R-expressing cancer cells,
Transfer of IL-4Rα Chain Gene Increases Cytotoxic Effect of IL-4R-targeted Cytotoxin

the activity of this protein is limited against cancer cells that express no or low levels of IL-4R. In an attempt to overcome this problem, we introduced the IL-4Rα chain gene into cancer cells and examined the cytotoxic activity of IL-4 toxin toward these cells by protein synthesis inhibition assay. As shown in Fig. 5, cancer cells expressing different levels of IL-4R demonstrated different sensitivity to IL-4 toxin (Fig. 5, left panels). When these cancer cells were transfected with the IL-4Rα chain gene, sensitivity to IL-4 toxin was highly enhanced (Fig. 5, right panels). The IC_{50} of IL-4 toxin in the IL-4Rαα-transfected cells were calculated to be 5–13-fold lower than vector-only transfectant control cells (Table 1). Interestingly, COLO587 cells that did not express IL-4Rα chain mRNA also showed modest sensitivity to IL-4 toxin. This sensitivity was dramatically increased in IL-4Rαα-transfected cells (IC_{50} = 500 ng/ml in control versus 45 ng/ml in IL-4Rαα-transfected COLO587 cells; Fig. 5B). Because IL-13Rα has been shown to share two chains with the IL-4R system, we also investigated whether IL-13 displaced the cytotoxic activity of IL-4 toxin. Similar to binding studies, cytotoxicity mediated by IL-4 toxin was neutralized efficiently by excess IL-4 and partially by IL-13. These results indicated that transfer of the IL-4Rα chain gene allows continued formation of correct IL-4R and IL-13R systems because these cells express IL-13Rα1 chain, which is necessary for the assembly of type II IL-4R and IL-13R.

IL-13-PE38QQR Is Not Cytotoxic to IL-4Rα Chain Gene-transfected Cancer Cells. We also examined the cytotoxicity of an irrelevant cytotoxin, IL-13-PE38QQR (40), in SU.86.86 cancer cells transfected with vector alone (mock control) or IL-4Rα chain. SU.86.86 (A), COLO587 (B), BT10 (C), and BT12 (D) cells were cultured with various concentrations of IL4(38-37)-PE38KDEL (0–1000 ng/ml) with or without IL-4 or IL-13 (2 μg/ml). The results are represented as means ± SD (bars) of quadruplicate determinations, and the assay was repeated three times. ○, IL4(38-37)-PE38KDEL; □, IL4(38-37)-PE38KDEL + IL-13; and Δ, IL4(38-37)-PE38KDEL + IL4.

Table 1. IL-4R binding sites and cytotoxicity of IL-4 toxin to cancer cell lines and IL-4Rαα transfectants.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IL-4R binding sites (sites/cell), mean ± SD</th>
<th>IC_{50} (ng/ml), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SU.86.86</td>
<td>680 ± 50</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>COLO587</td>
<td>2,700 ± 130</td>
<td>0.26 ± 0.2</td>
</tr>
<tr>
<td>BT10</td>
<td>13 ± 2.0</td>
<td>500 ± 75</td>
</tr>
<tr>
<td>BT12</td>
<td>ND</td>
<td>4.7 ± 30</td>
</tr>
<tr>
<td></td>
<td>10 ± 0.9</td>
<td>0.95 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.9 ± 1.4</td>
</tr>
</tbody>
</table>

\*IC_{50}, concentration of IL-4 toxin at which 50% inhibition of protein synthesis is observed compared with untreated cells.

\* Cells transfected with vector only served as control.

\* ND, not done.
are known to be sensitive to both IL-4 toxin and IL13-PE38QQR, were used as positive control. These data further confirm that IL-4 toxin-induced cytotoxicity is IL-4R specific.

To further determine the specificity of IL-4Rα-mediated cytotoxicity, we previously transfected CHO-K1 cells with an irrelevant chain (IL-13Rα2) and then tested their susceptibility to IL-4 toxin (41). No susceptibility to IL-4 toxin was observed; however, as expected, a cytotoxin comprising IL-13 and PE (IL13-PE38QQR) was cytotoxic to these cells (41). These data suggest that an irrelevant chain does not sensitize cells to the cytotoxic activity of IL-4 toxin. Only IL-4Rα chain sensitizes cells to the cytotoxic effect of IL-4 toxin.

Inhibition of Colony Formation of SU.86.86 Cells by IL4(38–37)-PE38KDEL Is Enhanced after IL-4Rα Chain Gene Transfer. To further confirm our observation that transfer of the IL-4Rα chain gene increases the sensitivity of cancer cells to IL-4 toxin, we performed a colony formation assay with the SU.86.86 cell line. After transient transfection with IL-4Rα or vector (mock control) cDNA, cells were plated in Petri dishes and incubated with various concentrations of IL-4 toxin. After 10 days of culture, colonies were stained and counted. As shown in Fig. 7A, in cells transfected with the IL-4Rα chain gene, colony formation was strongly inhibited compared with control cells. The IC50 was estimated to be more than 10 times lower in IL-4Rα chain transfectants compared with control cells (Fig. 7B). This result further suggests that transfer of the IL-4Rα chain gene increases the sensitivity of cancer cells to IL-4 toxin.

DISCUSSION

In this study, we demonstrate that the IL-4Rα chain undergoes internalization into cells after binding to its ligand, IL-4. Introduction of IL-2Rγ or IL-13Rα1 chain, two accessory molecules for functional IL-4R, did not further improve internalization, indicating that IL-4Rα does not require accessory chains for internalization. We further confirmed our findings by colony formation assay.

We and others have previously demonstrated that the IL-4Rα chain is a major binding component in the IL-4R system (13, 14, 28, 42), but this chain by itself does not mediate signal transduction. For signal transduction, the IL-4Rα chain must form a complex with IL-2Rγc (type I IL-4R) or IL-13Rα1 (type II IL-4R) chains (12–14, 49). Friedrich et al. (28) have reported that the IL-4Rα chain can mediate independent ligand internalization in human T cells; however, γc is required to slow down rapid dissociation of the IL-4 and IL-4Rα complex. In our study, however, the γc chain seemed to have no effect on IL-4 internalization and dissociation in CHO-K1 cells. These different results could be attributable to the different type of cells used in reconstitution studies. Because we previously have shown that IL-4 toxin was not cytotoxic to CHO-K1 cells transfected with γc chain alone (41), IL-4Rα chain is thought to be responsible for ligand binding and internalization.

Various investigators have studied and concluded that sensitivity to immunotoxins depends on the number of receptors and the rate of internalization (32–38). However, for the IL-4R system, it was not clear whether the IL-4Rα chain can internalize by itself after binding to ligand or whether it needs additional accessory receptor molecules for internalization. In addition, it was not known whether the internalized form of the IL-4Rα chain could internalize enough molecules of IL-4 toxin for cytotoxicity in target cells. In the present study, we showed that the IL-4Rα chain by itself can internalize after binding to its ligand. This is an important observation because it would be very difficult to reconstitute an internalized form of IL-4R if more than one chain of the IL-4R system were needed for the internalization.

Because we found that the IL-4Rα chain plays a major role in ligand binding and internalization, we are interested in optimizing IL-4R-directed cancer therapy. We have demonstrated that various human solid cancer cells express high levels of IL-4R and that these IL-4Rs are composed of IL-4Rα and IL-13Rα1 chains (type II IL-4R; Refs. 6–9, 15–24). Although a IL-4R-targeted cytotoxin, IL4(38–37)-PE38KDEL, is highly cytotoxic to IL-4R-expressing cancer cells, its antitumor effect...
is limited in cells that express no or low levels of IL-4Rα chain (22, 24). High-level antigen expression on tumor cells is shown to be a critical factor for antigen-targeted therapeutics such as monoclonal antibodies or cytotoxins (32–38). To sensitize cancer cells to the cytotoxic effect of IL-4 toxin, we transiently transfected these cells with the IL-4Rα chain. After transient transfection of IL-4Rα chain in pancreatic and brain cancer cell lines, the binding of IL-4 and sensitivity to the IL-4R-targeted cytotoxin IL4(38–37)-PE38KDEL were dramatically increased. These transfected cancer cells acquired 4–75-fold higher binding activity for IL-4 compared with control cells, and the cytotoxic activity of IL-4 toxin was enhanced 5–13-fold compared with control cells as assessed by protein synthesis inhibition and clonogenic assays. Because clonogenicity in vitro correlates with in vivo malignant phenotype in xenografts, our findings predict that the antitumor activity of IL-4 toxin will be improved in vivo in animal models of human cancer transfected with IL-4Rα chain (50). Thus, a combination approach that involves IL-4Rα gene transfer and IL-4R cytotoxin therapy may serve as a novel approach for cancer therapy.

It is of interest to note that COLO587 cells, which do not express mRNA for IL-4Rα chain, also showed modest sensitivity to IL-4 toxin. The reason for this modest sensitivity is not clear. COLO587 cells express mRNA for IL-13Rα1, and this chain can form complex with IL-4 and IL-4Rα chain. It is therefore possible that IL-4 toxin can internalize after binding to IL-13Rα1 chain. Alternatively, an unknown protein may be involved in IL-4 toxin-induced cytotoxicity.

Sensitization of cancer cells to a particular cancer therapeutic agent is a modern strategy for cancer therapy. In these approaches, target genes are introduced into cancer cells, followed by therapeutic irradiation or chemotherapy using the prodrug. One such approach has been vigorously tested in cancer preclinical models and in clinical trials. The HSV-tk gene is transfected into cancer cells, which sensitizes these cells to the cytotoxic effect of the antitherapeutics acyclovir or ganciclovir (51, 52). In another approach, the cytotoxic deaminase is introduced into cancer cells by plasmid or viral vector-mediated gene transfer. Cells that express cytosine deaminase convert 5-fluorocytosine, a fungicidal and bacterial drug, to 5-fluorouracil, which is then phosphorylated and subsequently inhibits gene transcription, resulting in cell death (53, 54). Apoptosis-inducing tumor suppressor genes, such as wild-type p53, are also used to sensitize cancer cells to radiotherapy (55). Our strategy, which combines IL-4Rα gene transfer and IL-4R-targeted cytotoxin, is similar to the prodrug approaches, and further investigation and development of this approach in vitro and in vivo may reveal its usefulness in clinical trials.

We have recently found that not all tumor cells in a glioma sample express detectable levels of IL-4Rα chain (56). In addition, in one completed and other ongoing Phase I/II clinical trials, we have observed that not all glioma tumors respond to IL-4 toxin therapy. Thus, it is possible that transfer of the IL-4Rα chain gene will sensitize cancer cells, allowing better antitumor activity of the toxin. Because glioblastoma is an intracranial disease, it is technically feasible to force expression of the IL-4Rα chain in vivo by various techniques, including plasmid-mediated gene transfer followed by IL-4 toxin therapy. In addition, we have reported that human breast cancer cells express IL-4R and that IL-4 toxin can mediate antitumor activity in an animal model of human breast cancer. However, complete responses were not seen. There may be many reasons for the low antitumor activity in this cancer model. It is possible that IL-4 toxin was not able to bind to the tumor target in sufficient concentrations or that, alternatively, IL-4 receptors were downregulated in vivo. Thus, in both situations direct transfer of the IL-4Rα chain gene may sensitize these cells to low doses of IL-4 toxin for better antitumor activity. Furthermore, additional localized tumors, such as pancreatic tumors, gastric cancer, head and neck cancer, ovarian cancer, non-small cell lung cancer, and mesothelioma tumors, may also show heterogeneity in IL-4R expression. These tumors can be injected with plasmid vector carrying IL-4Rα chain by various standard techniques followed by immunotoxin therapy for optimal antitumor activity.

We have previously demonstrated that normal resting T cells, B cells, and monocytes and resting or activated bone marrow precursor (CD34) cells express low levels of IL-4R and that IL-4 toxin is not cytotoxic to these cells in tissue culture (IC50 >1000 ng/ml; which is more than 1000-fold higher than that seen in glioma cell lines; Ref. 20). These immune cells seem to express IL-4Rα chain and IL-2Rγ chain, which form a
functional, type I IL-4R complex (49). However, when T cells are activated, IL-4 toxin becomes cytotoxic to these cells because activation of T cells up-regulates IL-4R (57). However, in the context of the clinical situation, it is not expected that a large number of T cells will be activated in vivo and that deletion of some of these cells may not have deleterious effects in cancer patients. Normal endothelial and fibroblast cells also express IL-4Rα chain, and it forms a complex with IL-13Rα1 chain, forming a functional type II IL-4R (11, 13, 49). We have reported that normal brain and skin, and diseased kidney tissue samples seem to express IL-4Rα mRNA and protein (42, 58, 59); however, it is not clear to what extent this chain is expressed on the cell surface. Similarly, IL-4Rα chain was found to be expressed on many vital organs; however, the extent of surface expression of this chain is not known. Lack of availability of normal human tissue samples free of infiltrating lymphoid, endothelial, and fibroblast cells makes it difficult to determine the true expression of IL-4R in normal tissues. However, to determine the toxicity to normal vital organs, we administered IL-4-toxin i.v. to cynomolgous monkeys because human IL-4 binds to monkey cells (23) and any toxicity in monkeys will reflect human situation. Monkeys received i.v. injections of 50 and 200 μg/kg doses of IL-4 toxin for 3 alternate days, and serum chemistry and hematomatological tests were performed at various time points. Systemic administration of IL-4 toxin did not show any toxicities in other vital organs in these monkeys except for an elevation of hepatic transaminases. These data indicate that hepatocytes express IL-4R or that IL-4 toxin is metabolized in the liver, causing nonspecific toxicity as is usually seen with many immunotoxins (23, 38). On the basis of these and other preclinical studies, Phase II/III clinical trials are being undertaken for glioblastoma and other cancer therapy.

In conclusion, this is the first demonstration that tumor cells that do not express or express low levels of IL-4R can be sensitized to the cytotoxic effect of IL-4R-targeted cytotoxin therapy after transfer of the IL-4Rα chain gene. On the basis of studies in monkeys, which indicate that many vital organs do not seem to be sensitive to IL-4 toxin and that the IL-4R gene can be locally delivered for many cancers, our strategy, which combines cytokine receptor gene transfer and cytotoxin therapy, may serve as a powerful cancer therapeutic approach.

ACKNOWLEDGMENTS

We thank Dr. S. Rafat Husain for helpful suggestions and Dr. Bharat H. Joshi (both from Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, FDA) for providing radiolabeled IL-4.

REFERENCES


Internalization Property of Interleukin-4 Receptor α Chain Increases Cytotoxic Effect of Interleukin-4 Receptor-targeted Cytotoxin in Cancer Cells

Koji Kawakami, Mariko Kawakami, Pamela Leland, et al.

Clin Cancer Res 2002;8:258-266.

Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/1/258

Cited articles  This article cites 55 articles, 33 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/1/258.full#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/8/1/258.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.