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

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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 ovary (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-4R chain in a time-dependent manner. When the IL-4R chain was cotransfected with the IL-13Rα1 or γc chain, the IL-4 internalization level was identical to IL-4R transfectants, suggesting that the IL-4R 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 [IL-4(38–37)-PE38KDEL] in CHO-K1 cells transfected with increasing concentrations of IL-4R cDNA. To use the internalization property of the IL-4R chain in the context of IL-4R-targeted cytotoxin therapy, we transiently transfected pancreatic and brain tumor cells with IL-4R transfectants, suggesting that the IL-4R chain plays a major role in IL-4 internalization after binding to ligand (14). Among the IL-4R components, IL-4R chain seems to play a major role in IL-4 internalization after binding to ligand (25–28). Among the IL-4R components, IL-4R chain seems to play a major role in IL-4 internalization after binding to ligand (25–28). Among the IL-4R components, IL-4R chain seems to play a major role in IL-4 internalization after binding to ligand (25–28). Among the IL-4R components, IL-4R chain seems to play a major role in IL-4 internalization after binding to ligand (25–28).
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 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 was established results.


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 m 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 γc chains (kindly provided by Dr. W. Leonard of the National Institutes of Health, Bethesda, MD) were cloned into pME18S mammalian expression vector, which is driven by SV40 promoter. cDNAs for different receptor chains 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 × 105/100-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-4R 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 × 106 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
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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 μM each of deoxynucleotide triphosphates, 1 unit/μl RNase inhibitor, 2.5 μM random hexamer, and 2.5 units/μl AmpliTaq Gold DNA polymerase (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.

RT-PCR Analysis. To detect the mRNA expression of the 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 μM 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.

Radioreceptor 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 IL-4 (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 IL-4 (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 with various chains of IL-4R showed different degrees of internalization.

Fig. 1. Internalization of ¹²⁵I-IL-4 in CHO-K1 cells. Two days after transfection with various IL-4R chains, CHO-K1 cells were preincubated in binding buffer containing 0.2% chloroquine at 37°C, followed by incubation with 0.5 nM ¹²⁵I-IL-4 at 4°C for 2 h. The temperature was then increased to 37°C, and internalization assays were performed. Data are expressed as a percentage of total IL-4 bound at time 0. □, surface-bound IL-4 on the cells; ●, internalized IL-4. Values are the means of two independent experiments. When not shown, SDs (bars) are smaller than the symbol.
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 IC50 (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 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 IL-4Rα Chain.** 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,
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 IC50 of IL-4 toxin in the IL-4Rα gene-transfected cells were calculated to be 5–13-fold lower than vector-only transfected 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-4Rα-transfected cells (IC50 = 500 ng/ml in control versus 45 ng/ml in IL-4Rα-transfected COLO587 cells; Fig. 5B). Because IL-13R 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 only (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 + IL-4.
are known to be sensitive to both IL-4 toxin and IL-13-PE38QQR, were used as positive control. These data further confirm that IL-4 toxin-induced cytotoxicity is IL-4Rα 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-2Rγ (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
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Fig. 7 In vitro inhibition of SU.86.86 cell growth as assessed by clonogenic assay. SU.86.86 cells transfected with vector only (control) or IL-4Rα chain (500 cells/group) were allowed to adhere in Petri dishes, and the medium was replaced with medium containing various concentrations (0–100 ng/ml) of IL4(38–37)-PE38KDEL. Cells were cultured for 10 days, and colonies consisting of at least 50 cells were scored after staining with crystal violet (A). B, results are expressed as percentage of colonies formed by treated cells compared with untreated cells. Control cells formed 335 ± 19 colonies, and IL-4Rα chain-transfected cells formed 366 ± 14 colonies. Data are means of triplicate determinations; bars, SD.

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 antitherapeutic drugs acyclovir or ganciclovir (51, 52). In another approach, the cytotoxic deaminase gene 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 down-regulated 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 hematological 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 I/II 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 indicated 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.

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