IL-13 Receptor-Targeted Cytotoxin Cancer Therapy Leads to Complete Eradication of Tumors with the Aid of Phagocytic Cells in Nude Mice Model of Human Cancer[1]

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Tumor-directed therapeutic approaches require unique or overexpressed specific Ag or receptor as a target to achieve selective tumor killing. However, heterogeneous expression of these targets on tumor cells limits the efficacy of this form of therapy. In this study, we forced abundant expression of IL-13Rα2 chain by plasmid-mediated gene transfer in head and neck, as well as prostate tumors to provide a potential target. This was followed by successfully treating xenograft tumor-bearing nude mice with IL-13R-directed cytotoxin (IL13-PE38QQR). Although we did not observe an indirect cytotoxic bystander effect conveyed to nontransduced tumor cells in vitro, our approach in vivo led to a complete regression of established tumors transfected with IL-13Rα2 chain in most animals. We found that the tumor eradication was achieved in part by infiltration of macrophages and NK cells, assessed by immunohistochemistry. Moreover, head and neck tumors xenografted in macrophage-depleted nude mice were less sensitive to the antitumor effect of IL-13 cytotoxin. Because we did not observe vector-related toxicity in any vital organs, our novel combination strategy of gene transfer of IL-13Rα2 chain and receptor-directed cytotoxin therapy may be a useful approach for the treatment of localized cancer. ***The Journal of Immunology,*** **2002, 169: 7119–7126.**

In the past decade, numerous efforts have been made to target human cancer with tumor-directed immunotoxins and cytotoxins. The success of this approach depends on the expression of unique or overexpression of specific Ags or receptors on the tumor cell surface compared with normal tissues (1–5). Because of the heterogeneous nature of human cancer, it is not always possible to expect that these Ags or receptors will also be overexpressed in vivo. Although antitumor activity in preclinical animal models of human solid tumors has been impressive, these results have not translated into successful clinical studies (6). Therefore, additional approaches are needed to enhance expression level of desired Ag or receptor in vivo in solid tumors.

Over the last 7 years, we have identified the IL-13R as a specific tumor cell surface target for receptor-directed cytotoxin therapy. To target IL-13Rs on human solid cancer cells, we produced a recombinant agent that binds to IL-13R on tumor cells (7). This molecule is a chimeric protein composed of IL-13 and a truncated form of a powerful bacterial toxin called *Pseudomonas* exotoxin (fusion protein termed IL13-PE38QQR or IL-13 cytotoxin). We have shown that this toxin is highly cytotoxic to IL-13R-positive tumor cells in vitro and in vivo in animal models of human tumors (8–16). Although tumor cells derived from glioblastoma, AIDS-associated Kaposi's sarcoma, renal cell carcinoma, and head and neck cancer express high levels of receptor for IL-13, tumor cells derived from breast, colon, ovarian, prostate, and lung cancer express a modest or low level of IL-13R, which may not be sufficient for the receptor-targeted cytotoxin therapy (8–16). To overcome this limitation, we hypothesized that tumor cells coaxed to express more IL-13R might be more susceptible to the effects of IL-13R-targeted cytotoxin. To achieve this goal, we used the IL-13R subunit IL-13Rα2 chain as a possible target for this strategy, as the IL-13Rα2 chain is known to bind IL-13 with highest binding affinity and is internalized after binding to its ligand (17–20). Using cDNA transfection of several tumor cell lines, we have demonstrated that gene transfer of IL-13Rα2 chain can sensitize tumor cells to the cytotoxic effect of IL-13 cytotoxin (21). More recently, we demonstrated that treatment with IL-13 cytotoxin mediates complete regression of IL-13Rα2 chain-transfected tumors established in immunodeficient mouse models of human cancer (12, 15).

In the current study, to explore this approach further as well as more closely mimic an actual clinical situation, we intratumorally injected IL-13Rα2 chain-encoding plasmid into tumors established from human head and neck (A253) and prostate (DU145) cancer cell lines by s.c. implantation in nude mice. These mice were treated systemically or intratumorally with IL-13 cytotoxin. We evaluated gene transfer efficiency of IL-13Rα2 chain in vivo, vector migration by determining expression in vital organs, and the mechanism responsible for antitumor activity resulting from the combination of gene transfer of IL-13Rα2 chain, followed by IL-13 cytotoxin therapy. Based on our observation of a remarkable antitumor activity, we propose that this approach could be applied to a variety of solid tumors in vivo and perhaps in the clinic for the treatment of localized tumors that either do not constitutively express, express low levels, or express heterogeneous levels of IL-13Rα2 chain.

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Materials and Methods

Cell culture, reagents, and plasmids

The human A253 and DU145 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured, as previously described (12, 15). rIL13-PE38QQR was produced and purified in our laboratory (7, 16). cDNA encoding human IL-13Rα2 chain (17, 22) was cloned into the VR1020 mammalian expression vector (a kind gift from Vical, San Diego, CA) using *Pst*I and *Bgl*II sites, and the sequences of the flanking regions of the junctions were verified by direct sequencing (ABI Prism 310; PerkinElmer, Wellesley, MA). Gene expression of IL-13Rα2 cDNA in the VR1020 vector is driven by the CMV promotor (23). The resulting construct was expanded in *Escherichia coli* and purified using endotoxin-free EndoFree Mega kit (Qiagen, Valencia, CA).

Protein synthesis inhibition assay

The in vitro cytotoxic activity of IL13-PE38QQR was measured by the inhibition of protein synthesis (11). All assays were performed in quadruplicate, and the concentration of IL-13 cytotoxin at which 50% inhibition of protein synthesis occurred was calculated (IC_{50}).

Animal studies

Athymic nude mice 4 wk old (~20 g in body weight) were purchased from Frederick Cancer Center Animal Facilities (Frederick, MD). Animal care was in accordance with the guidelines of National Institutes of Health Animal Research Advisory Committee. Human head and neck and prostate tumor models were established in the nude mice by s.c. injection of 5×10^6 A253 or DU145 cells in 150 μl PBS into the flank. These mice with established tumors were injected intratumorally with 25 μg of IL-13Rα2 cDNA-encoding vector mixed with 20 mM *N*-(1-[2,3-dioleoyloxy]propyl)-*N*,*N*,*N*-trimethylammonium chloride:cholesterol (1:1 molar ratio) liposome (Sigma-Aldrich, St. Louis, MO) (24, 25). For antitumor activity, mice were injected with IL13-PE38QQR or excipient either i.p. (500 μl/mouse) or intratumorally (30 μl/tumor), and tumors were carefully measured by Vernier calipers. Tumor size was calculated by multiplying length and width of the tumor on a given day. The statistical significance of tumor regression was calculated by Student's *t* test. All statistical tests were two sided.

Reverse-transcriptase PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY), and RT-PCR was performed as described (26).

Immunohistochemistry

Immunohistochemistry was performed using the Vector ABC peroxidase kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Subcutaneous tumor samples were harvested 3 days after the IL13-PE38QQR treatment (day 12) and fixed with 10% Formalin (paraffin-embedded sections) or snap frozen with OCT compound (frozen sections). Paraffin-embedded sections were deparaffinized by xylene treatment and washed with alcohol (100–50%) and PBS. Slides were incubated with Abs against murine macrophage (F4/80; Caltag Laboratories, Burlingame, CA), NK cells (NK1.1; Caltag Laboratories), or inducible NO synthase (iNOS,[4] M19; Santa Cruz Biotechnology, Santa Cruz, CA) (0.4–1 μg/ml) or isotype control for 18 h at 4°C. Slides were then developed using diaminobenzidine substrate-biotinylated peroxidase reagent (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich). Immunohistochemical assays were performed two to three times independently with similar results, and slides were assessed by two investigators (K. Kawakami and M. Kawakami).

For immunofluorescent assays, frozen sections were stained with anti-human mAb for IL-13Rα2 (Diaclone, Besancon, France) or costained with Abs for macrophages and iNOS. Slides were fixed in acetone at −20°C for 5 min and air dried. Nonspecific binding was blocked by treatment with 10% serum for 1 h, followed by incubation with Abs or isotype control. Sections were subsequently incubated for 1 h with secondary Abs that had either tetramethylrhodamine isothiocyanate or FITC tags. After three washes with PBS, slides were dried and layered with Vectashield antifluorescence fading mounting medium (Vector Laboratories) and a covership. The slides were viewed in an Olympus IX70 fluorescence microscope using appropriate filters (Olympus Optical, Melville, NY). Images were compiled from sets of three consecutive single optical sections using SPOT INSIGHT V 3.2 software (Diagnostic Instruments, Sterling Heights, MI).

[4] Abbreviations used in this paper: iNOS, inducible NO synthase; b.i.d., twice per day; q.d., once per day.

Macrophage depletion

Macrophage depletion in animals was performed, as described previously (27, 28). Carrageenan (type II; Sigma-Aldrich) was dissolved in sterile PBS at 5 mg/ml. The solution was heated to 56°C to ensure complete solubilization. Mice were treated by i.p. injection of 200 μl (1 mg) of carrageenan on days 3, 7, and 14 after the tumor implantation. Control mice received 200 μl of sterile PBS.

Results

Cytotoxic effect of IL-13 cytotoxin correlates with the expression of IL-13Rα2 chain in vitro

We stably transfected head and neck (A253) and prostate cancer (DU145) cell lines with IL-13Rα2 chain. Expression level was confirmed by RT-PCR and radiolabeled IL-13-binding studies (12, 15). Using cloned tumor cells, we determined the sensitivity of IL-13 cytotoxin in vitro and also determined whether cells in the process of dying as a consequence of exposure to IL-13 cytotoxin can mediate bystander cytotoxic effect to non-IL-13Rα2-transfected tumor cells. Tumor cells transfected with IL-13Rα2 chain were mixed with vector-only-transfected (mock control) cells in various ratios, and the cytotoxic activity of IL-13 cytotoxin was evaluated by protein synthesis inhibition assays (Fig. 1*A*). As the concentration of IL-13Rα2 chain-positive cells increased, the cytotoxic effect of IL-13 cytotoxin increased. The IC_{50} (IL-13 cytotoxin concentration causing 50% inhibition of protein synthesis) correlated positively with the level of IL-13Rα2 expression. Highest cytotoxicity of IL-13 cytotoxin was observed when 100% cells expressed IL-13Rα2 chain.

To confirm these observations, we mixed IL-13Rα2 chain-positive and IL-13Rα2 chain-negative cell mixtures in different ratios,

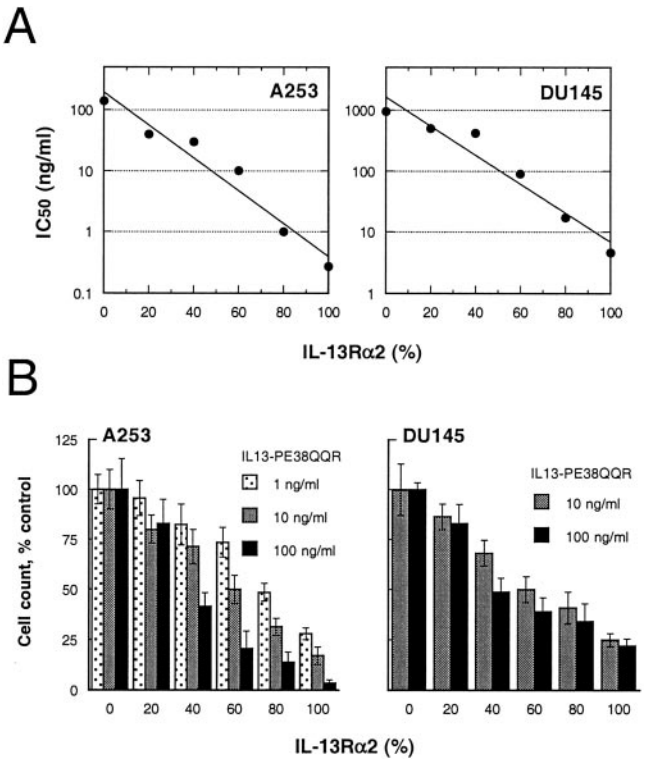

FIGURE 1. In vitro relationship between IL-13Rα2 chain expression in tumor cells and the cytotoxic effect of IL-13 cytotoxin. IL-13Rα2-positive A253 and DU145 cells were mixed with vector-transfected IL-13Rα2-negative tumor cells in different ratios, and cytotoxicity was determined as described in *Materials and Methods*. *A*, IC_{50} calculated from protein synthesis inhibition assays. *B*, Cell viability after 5 days of IL-13 cytotoxin treatment.

incubated with IL-13 cytotoxin for 5 days, and then counted viable cells by trypan blue staining (Fig. 1*B*). At a concentration of 10 ng/ml, IL-13 cytotoxin was enough to kill >75% of both A253 and DU145 tumor cells when 100% of tumor cells were positive for IL-13Rα2 chain. Cytotoxicity of both tumor cell lines decreased proportionally when IL-13Rα2-transfected cells were mixed with nontransfected tumor cells. These results indicate that the cytotoxicity of IL-13 cytotoxin depends on IL-13Rα2 chain expression and that IL-13Rα2 chain-positive dying tumor cells do not mediate bystander cytotoxic effect to nontransduced tumor cells.

Antitumor effect of IL-13 cytotoxin on head and neck tumors with heterogeneous expression of IL-13Rα2 chain in vivo

Because cytotoxic activity of IL-13 cytotoxin in vitro correlated with IL-13Rα2 chain expression in tumor cells, we investigated to what degree of heterogeneity of IL-13Rα2 expression is required in tumors to reveal optimal antitumor activity of systemic IL-13 cytotoxin treatment in animals. For this assessment, A253 tumor cells transfected with IL-13Rα2 chain were mixed with vector-only-transfected (mock control) cells in various ratios, and 5×10^6 cells were injected s.c. in nude mice. Mice were then i.p. injected with IL-13 cytotoxin (50 μg/kg, twice per day (b.i.d.) for 5 days) from day 4 through 8. As shown in Fig. 2, in all the animal groups except for 0%, IL-13Rα2-expressing tumor (only mock control cells injected)-bearing mice A253 tumor growth was inhibited during IL-13 cytotoxin administration. After the treatment period, tumors gradually grew again; however, mean size of 25% IL-13Rα2-expressing tumors (74 mm^2) was significantly smaller than 0% IL-13Rα2 control tumors (182 mm^2) ($p < 0.0005$) by day 35. IL-13 cytotoxin showed antitumor activity against A253 tumors in IL-13Rα2 expression level-dependent manner, and complete disappearance of tumor was observed in one of six 75% IL-13Rα2-expressing tumor-bearing mice and four of six 100% IL-13Rα2-expressing tumor-bearing mice by day 35. These results suggest that the correlation between IL-13Rα2 expression and sensitivity to IL-13 cytotoxin is not linear, and that heterogeneous expression of IL-13Rα2 chain (at low levels) on tumor cells can still successfully sensitize them to the cytotoxic effect of IL-13 cytotoxin in vivo.

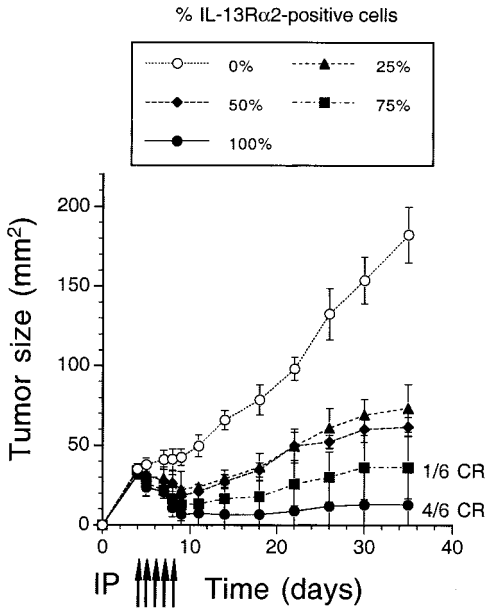

FIGURE 2. Antitumor activity of IL-13 cytotoxin in tumors expressing heterogeneous levels of IL-13Rα2 chain in vivo. A253 tumor cells transfected with IL-13Rα2 chain were mixed with vector-only-transfected (mock control) cells in various ratios, and injected s.c. in nude mice. Mice were then i.p. injected with IL-13 cytotoxin (50 μg/kg b.i.d. for 5 days) from days 4 through 8. Arrows in *x*-axis indicate the days of injection of IL-13 cytotoxin.

Persistence of IL-13Rα2 chain expression in tumors in vivo after intratumoral vector administration

To achieve gene expression of IL-13Rα2 chain in vivo, A253 and DU145 tumors rapidly growing in the flank of the nude mice were intratumorally injected with plasmid vector-encoding IL-13Rα2 chain cDNA. Plasmids were mixed with liposomes for optimal gene transfer in vivo and injected on days 4, 5, and 6 after tumor implantation. Tumors were subsequently resected at various time points after plasmid injections and subjected to RT-PCR and immunofluorescence microscopy analyses for IL-13Rα2 gene expression. IL-13Rα2 chain mRNA was found to be expressed continuously at high levels until day 18 in both tumor models (Fig. 3*A*). The level of expression was decreased on day 21 posttumor implantation, and by day 27 no detectable signal could be observed. These results were confirmed by immunofluorescence microscopy in A253 tumor sites using mAb to IL-13Rα2 chain (Fig.

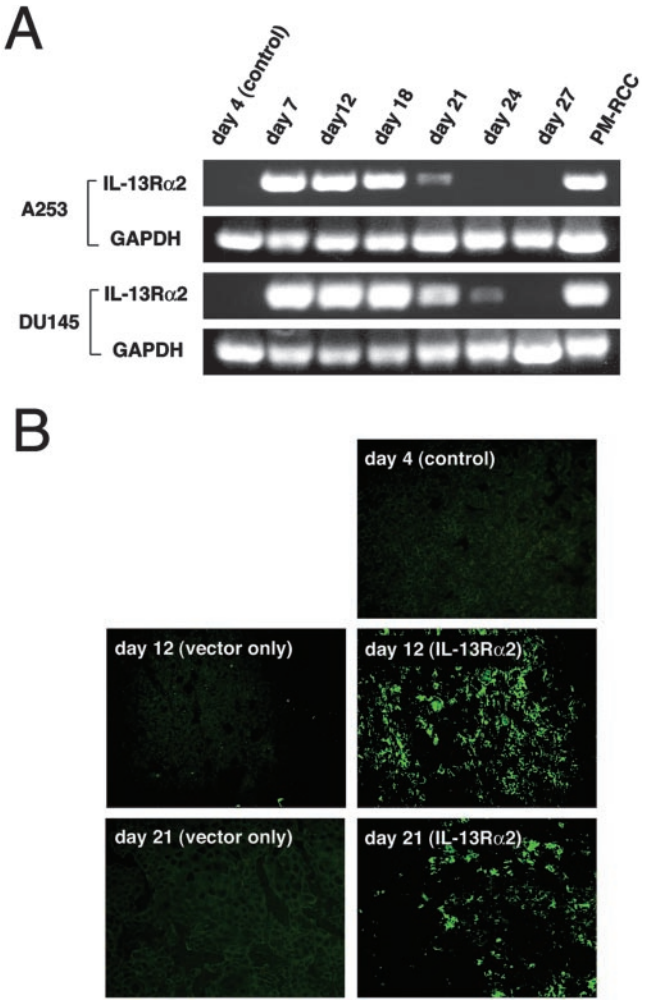

FIGURE 3. IL-13Rα2 chain expression in head and neck, and prostate tumor tissues after intratumoral injections of IL-13Rα2-encoding plasmid. *A*, RT-PCR using total RNA from tumors and PM-RCC cell line for a positive control. *B*, Immunofluorescence assay in A253 tumors using mAb to IL-13Rα2 chain (×100). The photomicrographs shown are representative pictures obtained from multiple tumor samples from several animals.

3*B*). After three injections of IL-13Rα2 cDNA on days 4, 5, and 6, intense protein expression was observed on day 12, decreasing by day 21, and finally disappearing on day 27 (data not shown). Tumor slides from several animal experiments demonstrated that mean IL-13Rα2 expression level in tumors was 20–30% on day 12 and 5–10% on day 21. These results indicate that the expression of IL-13Rα2 in the gene-transferred tumor site was maintained for 12–15 days after three injections of plasmid vector.

IL-13Rα2 chain-targeted antitumor activity of IL-13 cytotoxin

Once we were able to express consistent level of IL-13Rα2 chain in s.c. growing solid tumors, we tested the antitumor activity of IL-13 cytotoxin in an A253 human head and neck tumor xenograft model. First, we developed tumors in both the right and left flanks of nude mice. Then animals received either a vector-only injection (right flank) or an injection of IL-13Rα2 chain plasmid (left flank) intratumorally on days 4, 5, and 6. Subsequently, these animals were treated from days 5 through 9 with IL-13 cytotoxin using either an i.p. (25 or 50 μg/kg b.i.d. for 5 days) or intratumoral (100 or 250 μg/kg once per day (q.d.) for 5 days) route of administration (Fig. 4*A*). Tumors injected with vector only (right flank) were insensitive to IL-13 cytotoxin treatment and grew rapidly. By day 36, tumor growth in control (excipient-injected) and IL-13 cytotoxin-treated animals did not show any significant difference. In contrast, tumors injected with IL-13Rα2 chain plasmid (left flank) started to regress during the treatment period in both i.p. and intratumoral treated animals. Although in mice treated i.p. complete regression of tumors could not be achieved, the mean size of tumors measured on day 36 was significantly reduced (72 mm^2 in 25 μg/kg, and 54 mm^2 in 50 μg/kg groups, respectively) compared with the control (174 mm^2) ($p < 0.0005$). In contrast, intratumoral administration of IL-13 cytotoxin resulted in dose-dependent antitumor activity associated with complete regression of tumors. In the 250 μg/kg dose group of mice, tumors were completely not palpable by day 16. On day 28, tumors recurred in two of the six mice; however, their size remained significantly smaller ($p < 0.0005$) than control mice, and four of the six (250 μg/kg) mice remained completely tumor free in their left flanks out to the end of the experiment on day 36. Results for a representative mouse from each group are depicted (Fig. 4*B*).

We also used another tumor model (DU145 prostate tumor) for in vivo gene transfer of IL-13Rα2 chain, followed by IL-13 cytotoxin treatment using a protocol similar to the one for the A253 tumor model (Fig. 4*C*). Again, tumors targeted with vector only (right flank) did not respond to the IL-13 cytotoxin treatment and grew as aggressively as tumors implanted in mice treated with excipient. For as long as we followed these animals (day 50), mean tumor size in control (excipient only) and treated groups failed to exhibit any difference. In contrast, tumors modified to express IL-13Rα2 chain by plasmid injection (left flank) responded extremely well to the antitumor effect of IL-13 cytotoxin. After i.p. administration (50 μg/kg b.i.d. × 5 days), the rapid growth of IL-13Rα2-targeted tumors was arrested in all mice, and by the last day of the experiment (day 50), mean size of tumors was significantly smaller compared with control (163 mm^2 vs 38 mm^2, $p < 0.0005$). In addition, one of five test animals showed complete disappearance of an established tumor. In mice treated intratumorally, a much better tumor response was observed. Tumors in three of six (100 μg/kg) and five of six (250 μg/kg) mice completely regressed by day 12, and had not recurred by day 50. Overall, the mean tumor size in IL-13 cytotoxin-treated mice remained significantly smaller ($p < 0.0005$) compared with excipient-treated animal throughout the experiment.

IL-13 cytotoxin shows extremely potent antitumor activity to large tumors targeted with IL-13Rα2 chain

To further determine whether our combination approach using IL-13Rα2 chain gene transfer followed by IL-13 cytotoxin therapy is effective in much larger tumors, we implanted A253 tumor cells in the right and left flanks of nude mice, letting them grow until reaching 80 mm^2 in either side by day 18. Tumors were then injected with vector only (right flank) or IL-13Rα2 chain-encoding vector (left flank) on days 18, 19, and 20, followed by either i.p. (50 μg/kg b.i.d. × 7 days) or intratumoral (250 μg/kg q.d. × 7 days) administration of IL-13 cytotoxin (Fig. 5). Regardless of the route of administration, only IL-13Rα2-targeted tumors (left flank) regressed dramatically during the treatment period. In the i.p. injected group, only IL-13Rα2-targeted tumors showed significant decrease in size compared with excipient-injected control (179 mm^2 vs 52 mm^2, $p < 0.0005$) on day 36. In animals injected

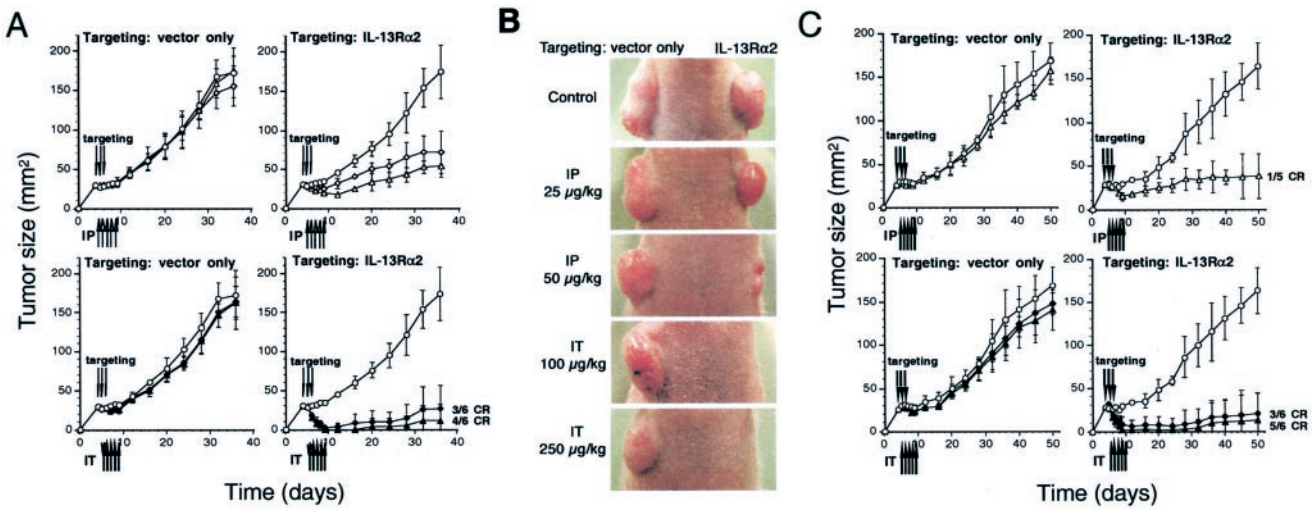

FIGURE 4. Antitumor activity of IL-13 cytotoxin on head and neck and prostate tumor xenograft models after intratumoral administration of plasmid for IL-13Rα2 chain. A253 (*A*) or DU145 (*C*) tumors were xenografted in both flanks of nude mice ($n = 6$). Tumors in the left flank were injected with IL-13Rα2 plasmid, while right flank with vector only by intratumoral injection on days 4, 5, and 6, followed by IL-13 cytotoxin administration either i.p. (IP) or intratumorally (IT) on days as indicated by arrows; symbols, excipient only (○); IP, 25 (◇) or 50 μg/kg (△); and IT, 100 (◆) or 250 μg/kg (▲). *B*, Pictures shown are of A253 tumor-bearing mice on day 36.

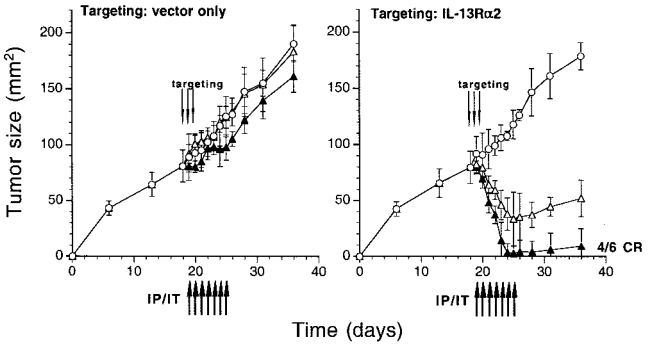

FIGURE 5. Antitumor activity of IL-13 cytotoxin toward large head and neck tumor xenografts in nude mice. When A253 tumors reached 80 mm^2 in size, they ($n = 6$) were injected intratumorally with IL-13Rα2 chain plasmid (left flank) or vector only (right flank) on days 18, 19, and 20, and treated with IL-13 cytotoxin either i.p. (IP) or intratumorally (IT) on days indicated by arrows; symbols, excipient only (○); IP, 50 μg/kg (△); and IT, 250 μg/kg (▲).

intratumorally with IL-13 cytotoxin, five of the six showed complete regression of tumors by the end of the IL-13 cytotoxin injection (day 25). Although one tumor recurred later, four of the six mice remained tumor free in their left flanks in a long-term (day 90) followup.

Infiltration of immune cells into tumor site after IL-13Rα2 plasmid and IL-13 cytotoxin treatment

To investigate the mechanism of antitumor response by approach of in vivo gene transfer, followed by a targeted cytotoxin therapy, we examined treated tumors for cellular infiltration. Three days after the completion of combination therapy, A253 and DU145 tumors were resected and examined histologically. In IL-13Rα2-targeted A253 tumors treated with 100 μg/kg (q.d. × 5 days) of intratumoral IL-13 cytotoxin administration, several necrotic areas were observed in sections of dying tumor. These areas were surrounded by fibrotic elements (Fig. 6, *A* and *B*). Interestingly, a number of phagocytes, most resembling monocytes morphologically, were observed in necrotic areas and in remaining viable tumor cell area. In contrast, in A253 tumors injected with vector only, no cell infiltration was observed (data not shown). We also observed similar phenomenon in IL-13Rα2-targeted DU145 tumors after 50 μg/kg (b.i.d. × 5 days) of IL-13 cytotoxin administered i.p. (data not shown).

To determine whether inflammatory cells had infiltrated at the area between remaining tumor cells and necrotic region, we performed immunohistochemical staining of sections same as in Fig. 6, *A* and *B* (A253 tumors). We found moderate numbers of macrophage marker (F4/80)-positive cells (Fig. 6*C*), NK marker (NK1.1)-positive cells (Fig. 6*D*), and iNOS marker (M19)-positive cells (Fig. 6*E*). Although some cancer cells were positive for iNOS, other infiltrating cells also appeared to be positive for iNOS. To determine whether some of these iNOS-positive cells were macrophages, we costained tumor sections with F4/80 (green) and M19 (red) Abs and examined the sections by fluorescence microscopy. As shown in Fig. 6*F*, A253 tumors that received the IL-13Rα2 gene followed by IL-13 cytotoxin treatment demonstrated colocalization of macrophage and iNOS stains. However, staining for either macrophages or iNOS could not be detected in untreated A253 tumors (data not shown).

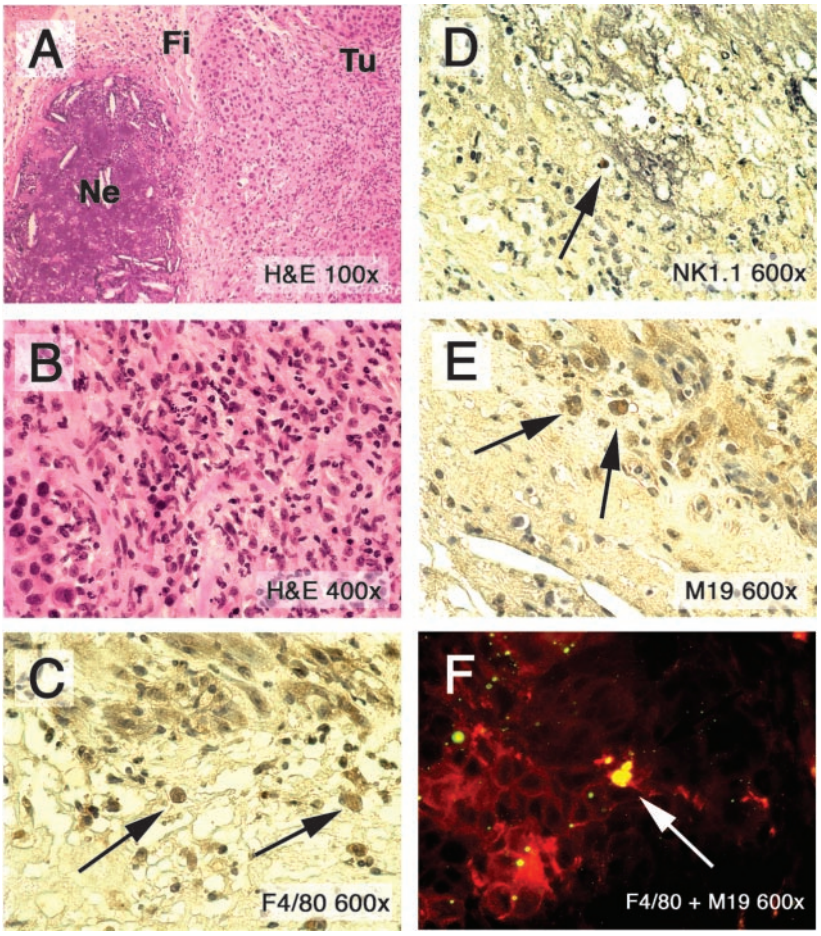

FIGURE 6. Histological examination of tumors injected with IL-13Rα2 plasmid, followed by IL-13 cytotoxin therapy. Established A253 head and neck tumors were injected with plasmid for IL-13Rα2 gene, followed by IL-13 cytotoxin intratumorally (100 μg/kg dose). Tumors were resected 3 days after the completion of IL-13 cytotoxin injections. H&E sections (*A*, ×100; *B*, ×400) and immunohistochemistry results using Abs to F4/80 (*C*), NK1.1 (*D*), or M19 (*E*) are shown (*C–E*, ×600). *F*, Colocalization of double stainings for F4/80 (green) and M19 (red) is assessed using immunofluorescence microscopy (×600). Fi, fibrosis; Ne, necrotic tissues; Tu, tumor cells.

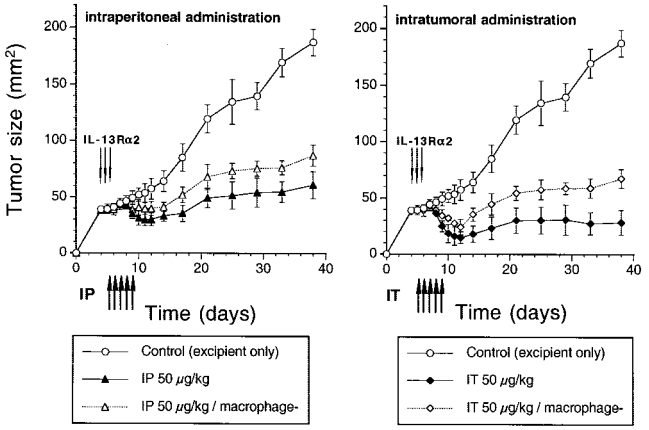

FIGURE 7. In vivo IL-13Rα2 gene-transferred tumors in macrophage-depleted animals showed less sensitivity to IL-13 cytotoxin. Mice injected with A253 tumor cells s.c. (day 0) were then injected with carrageenan (type II) on days 3, 7, and 14. IL-13Rα2 cDNA injection and IL-13 cytotoxin treatment (50 μg/kg dose, i.p. or intratumorally) was performed following the schedule mentioned in the legend to Fig. 4.

In vivo IL-13Rα2 gene-transferred tumors showed less sensitivity to IL-13 cytotoxin in macrophage-depleted animals

We observed that some F4/80-positive phagocytes infiltrated within the dying tumors. To assess whether these infiltrated phagocytes mount an impact on the tumor regression mechanism during the cancer therapy approach or were recruited as a result of cell death, we evaluated the antitumor activity of IL-13 cytotoxin after IL-13Rα2 gene transfer in macrophage-depleted nude mice. Mice injected with A253 tumor cells s.c. (day 0) were then injected with carrageenan (type II) on days 3, 7, and 14. IL-13Rα2 cDNA injection and IL-13 cytotoxin treatment (50 μg/kg dose, i.p. or intratumorally) was performed following the schedule mentioned in Fig. 4. As shown in Fig. 7, during the IL-13 cytotoxin treatment period, tumor regression in both macrophage-depleted and not-depleted groups were observed. However, the extent of tumor regression in macrophage-depleted mice was less pronounced compared with not-depleted mice. By the end of the experiment (day 38), mean tumor size in macrophage-depleted mice was significantly larger (87 mm^2) compared with mice without macrophage depleted (61 mm^2) in i.p. IL-13 cytotoxin treatment groups ($p < 0.002$). Intratumoral route was superior in inhibiting tumor growth compared with i.p. route even though doses administered were the same. By day 38, mean tumor size in macrophage-depleted mice was again significantly larger (68 mm^2) compared with mice without macrophage depletion (29 mm^2) in intratumoral treatment groups ($p < 0.0005$). These results confirm that macrophages infiltrating into regressing tumors as a result of IL-13Rα2 gene transfer, followed by IL-13 cytotoxin therapy, play at least some role in antitumor mechanism of this approach.

IL-13Rα2 chain was not detected in any vital organs after intratumoral plasmid administration

To examine whether intratumorally administrated IL-13Rα2 plasmid migrated to distant, nontarget vital organs resulting in IL-13 cytotoxin-mediated organ toxicity, we collected vital organs including liver, lung, kidney, spleen, heart, and blood as well as A253 tumors on day 12 (3 days after the completion of IL-13Rα2 gene transfer and i.p. or intratumoral IL-13 cytotoxin therapy). All tissue specimens were snap frozen. Total RNA and tissue sections were analyzed for IL-13Rα2 expression or any evidence of cellular damage. IL-13Rα2 chain mRNA was not detected by sensitive RT-PCR in any vital organs or blood cells, except in IL-13Rα2 plasmid-injected tumor tissues (Fig. 8*A*). Similarly, coupled with immunofluorescence using mAb to IL-13Rα2 chain, no detectable protein expression was observed in any organs examined. Using this approach, IL-13Rα2 chain protein was only detected in A253 tumors that were administrated with IL-13Rα2 plasmid (Fig. 8*B*). In addition, we did not observe any organ toxicity by histological examination (data not shown).

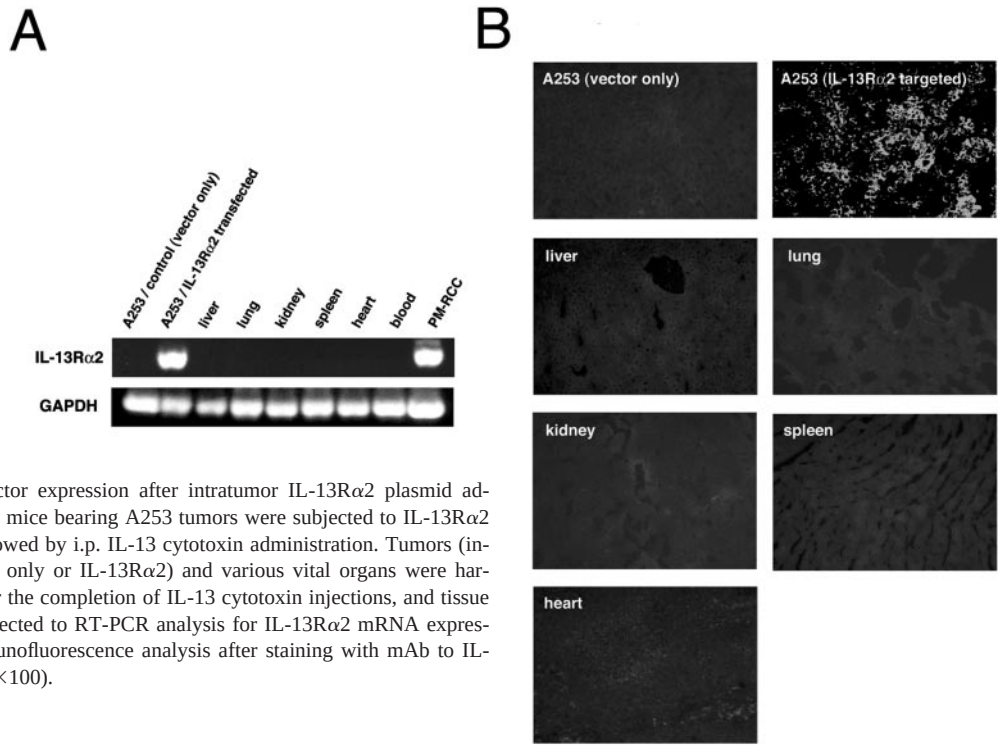

FIGURE 8. Vector expression after intratumor IL-13Rα2 plasmid administration. Nude mice bearing A253 tumors were subjected to IL-13Rα2 gene transfer, followed by i.p. IL-13 cytotoxin administration. Tumors (injected with vector only or IL-13Rα2) and various vital organs were harvested 3 days after the completion of IL-13 cytotoxin injections, and tissue sections were subjected to RT-PCR analysis for IL-13Rα2 mRNA expression (*A*) and immunofluorescence analysis after staining with mAb to IL-13Rα2 chain (*B*, ×100).

Discussion

In this proof-of-concept study, we demonstrate that in vivo intratumoral gene transfer of IL-13Rα2 chain followed by IL-13R-targeted cytotoxin administration represents promising new approach for locoregional cancer therapy. In our preliminary studies, we failed to show desirable and pronounced antitumor activity of IL-13 cytotoxin after one injection of IL-13Rα2-encoding plasmid. Consequently, we decided to perform three injections: three intratumoral injections of IL-13Rα2 chain-encoding vector mixed with liposome were sufficient for target expression lasting for 12–15 days. These observations suggest that one can delay IL-13 cytotoxin administration for a longer period of time, allowing maximum expression of transgene. Furthermore, multiple injections of IL-13 cytotoxin could be performed for optimal antitumor response. The lack of IL-13Rα2 expression or toxicity in any nontarget vital organs suggests IL-13 cytotoxin may eliminate IL-13Rα2-expressing tumors exclusively. To our knowledge, this is the first report involving tumor cells enforced to express an artificial receptor target, followed by therapy comprised of a unique receptor-directed recombinant antitumor agent.

Based on published reports, we assumed that plasmid-mediated gene transfer in vivo may not result in the transgene transduction of every tumor cell. Because the cytotoxic effect of IL-13 cytotoxin is specific to IL-13Rα2 chain expression in tumor cells transfected in vitro, we hypothesized that this combination therapy may not cause a bystander effect or other apoptotic pathway-mediated tumor cell death in vivo (29–31). It seemed that this approach in animal models of human cancer would not result in a desired, robust antitumor effect. However, to our surprise, we found that tumors manipulated to express IL-13Rα2 chain gene responded to IL-13 cytotoxin therapy. Moreover, complete regression of tumors was achieved in many animals. By histological examination, we found that these successful results were obtained because of cellular infiltration of phagocytes and NK cells, in addition to a direct IL-13 cytotoxin effect. Some of these infiltrating phagocytes secreted iNOS. However, whether iNOS-positive macrophages participated in IL-13 cytotoxin-induced tumor regression or these cells were infiltrating as a result of tumor necrosis induced by IL-13 cytotoxin was not clear. To address this important issue, we performed macrophage depletion experiment. Mice injected with A253 tumor cells s.c. were injected with carrageenan (type II) on days 3, 7, and 14. IL-13Rα2 cDNA was then intratumorally injected, followed by IL-13 cytotoxin treatment by two routes. IL-13 cytotoxin by both i.p. and intratumoral routes caused lower antitumor activity when macrophages were depleted before treatment compared with undepleted groups. The difference in mean tumor size between control and macrophage-depleted animals was statistically significant by both routes of IL-13 cytotoxin administration. These results confirmed a role for phagocytes in the tumor regression observed; however, other mechanisms must also be involved. Thus, combination of IL-13Rα2 chain gene transfer and IL-13 cytotoxin treatment seems to enhance host immune response at the tumor site, thereby eliminating residual tumor cells that escaped the cytotoxic effect of IL-13 cytotoxin. In addition, although nude mice do not have a functionally competent immune system, they have B cells, monocytes, and NK cells that may be sufficient to eliminate remaining tumor cells following IL-13Rα2 chain gene injection and IL-13 cytotoxin therapy. Further delineation of the role of the immune response in the mechanism of tumor regression is under investigation in our laboratory.

We also considered a possibility that other mechanism(s) may be involved in the pronounced antitumor effect of IL-13 cytotoxin in less than 100% IL-13Rα2-positive tumors. It is possible that IL-13Rα2 transfection of host endothelial cells or fibroblasts results in an antiangiogenesis/antistromal effect, which by itself may be basis of tumor rejection. To address these issues, we performed a mixing experiment, in which in vitro IL-13Rα2 stable transfected A253 tumor cells were mixed in various ratios with mock vector-transfected control tumor cells (Fig. 2). These mixtures of cells were s.c. injected into nude mice, and then mice were treated with IL-13 cytotoxin by two routes of injection. Tumor-expressing 0% IL-13Rα2 chain continued to grow, forming large nodules; however, tumors expressing various percentages of IL-13Rα2 chain showed statistically significant antitumor response as a result of IL-13 cytotoxin treatment. These results suggest that tumor rejection proceeds when host nontumor cells, e.g., endothelial cells or fibroblasts, are not directly exposed to intralesional plasmid, and that the antitumor effect is not mediated through antiangiogenesis or antistromal effect of plasmid injection.

IL-13Rα2 chain has been shown to be highly expressed on cell lines derived from certain types of solid tumors, including malignant glioma and primary cell cultures derived from glioma tumors. The extent of receptor expression in vivo remains unknown (8, 10, 11, 32, 33). In addition, localized neoplasias such as pancreatic, head and neck, ovarian, and prostate cancer do not express IL-13Rα2 chain, or only limited percentage of tumor cells express IL-13Rα2 chain (12, 15, 16, 21, 26, 34, 35). Therefore, these tumors will not be sensitive to the cytotoxic effect of IL-13 cytotoxin. Moreover, in some situations, IL-13 cytotoxin may not be able to home to tumor target in sufficient concentrations to mediate an antitumor effect even if these tumors express sufficient numbers of IL-13Rs. In these situations, direct gene transfer of IL-13Rα2 chain may sensitize these tumor cells to the antitumor effect of IL-13 cytotoxin at low doses. Our current results support these hypotheses and demonstrate a potent antitumor effect of IL-13 cytotoxin in two tumor models.

In conclusion, we demonstrate that combined forced expression of IL-13Rα2 chain in tumor cells, followed by target-direct cytotoxin therapy yields excellent antitumor activity enlisting the aid of the host's immune system. Because this novel combinational approach elicits complete regression of extremely large tumors in animal models of human head and neck, and prostate cancer without any induction of vital organ toxicity, we believe this strategy could be applied in the clinic against many types of cancer.

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