Advances in Brief

Receptor for Interleukin 13 on AIDS-associated Kaposi's Sarcoma Cells Serves as a New Target for a Potent *Pseudomonas* Exotoxin-based Chimeric Toxin Protein[1]

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Abstract

AIDS-associated Kaposi's sarcoma (AIDS-KS), the most common malignant complication of human immunodeficiency virus infection, is characterized by neoplastic proliferation of mesenchymal cells. AIDS-KS cells release and respond to an array of cytokines through specific plasma membrane receptors. Specific targeting of potent cytotoxic agents to cell surface receptors/antigens on Kaposi's sarcoma cells may provide effective therapy for this malignancy. We have identified a new target in the form of an interleukin 13 (IL-13) receptor that is overexpressed in the five AIDS-KS cell lines examined. Radiolabeled IL-13 cross-linked to a single protein of about M_r 70,000 in AIDS-KS cells. We utilized a chimeric cytotoxic protein composed of IL-13 and a truncated *Pseudomonas* exotoxin (IL13-PE38QQR), which was found to be specifically and highly cytotoxic to AIDS-KS cells, as determined by protein synthesis inhibition and clonogenic assays. IL13-PE38QQR demonstrated significant antitumor activity in a human epidermoid carcinoma xenograft model. Normal human umbilical vein-derived endothelial, lymphoid, and bone marrow precursor cells expressed low levels of IL-13 receptors, and IL-13 toxin was not cytotoxic to them. Thus, IL-13 receptor on AIDS-KS cells may represent a novel plasma membrane protein(s) that could be utilized to target therapeutic agents.

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Introduction

AIDS-KS[3] is composed predominantly of proliferating spindle-like tumor cells (1, 2). AIDS-KS cells can produce and respond to a variety of potent cytokines and growth factors (3–8). Chief among these are acidic and basic fibroblast growth factor, granulocyte macrophage colony-stimulating factor, platelet-derived growth factor, transforming growth factor β, IL-1, and IL-6 (8–10). The ability of AIDS-KS cells to constitutively release and respond to the array of cytokines suggests a possible cytokine autocrine growth loop in the AIDS-KS etiology (5, 8). The effect of growth factors and cytokines are mediated through their plasma membrane receptors. KS cells have been reported to express receptors for platelet-derived growth factor α and β, IL-6, oncostatin M, and other factors (2).

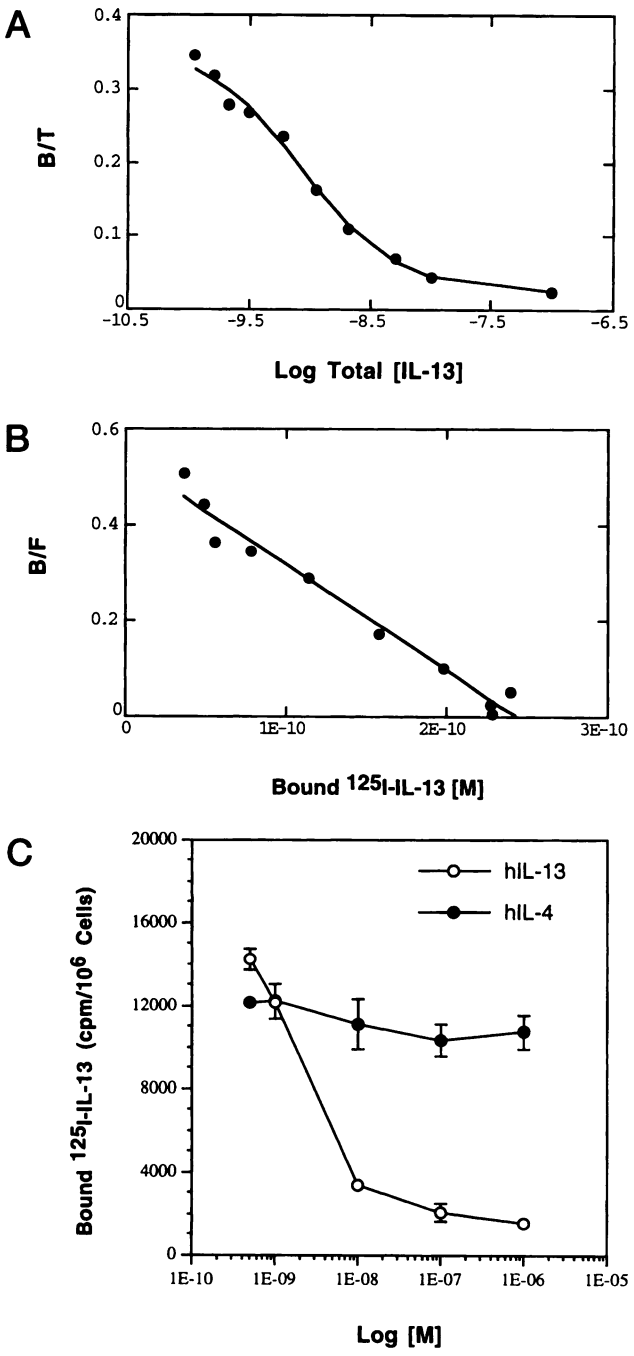
We have observed that AIDS-KS-derived spindle cells express high-affinity IL-4R (11). IL-4, a pleiotropic immunoregulatory cytokine, shares many biological functions with IL-13, another predominantly Th2-derived immunoregulatory cytokine (12, 13). IL-13, like IL-4, has also been shown to suppress HIV-1 replications in human monocyte-derived macrophages (14, 15). Furthermore, we and others have reported recently that the receptors for IL-4 and IL-13 may share a component(s) (16, 17). Because AIDS-KS cells expressed IL-4R, and cytokines play a critical role in the pathogenesis of AIDS-KS, we investigated whether AIDS-KS cells express IL-13R. We further investigated whether IL-13R expressed on AIDS-KS cells can be targeted with a chimeric protein composed of IL-13 and a mutated form of *Pseudomonas* exotoxin (termed IL13-PE38QQR; Refs. 18–20).

Materials and Methods

Cell Culture and Reagents. AIDS-KS-derived spindle cells (KS248, NCB-59, KS54A, KS220B, and ARL-13) were generated from fresh KS tissues obtained from different HIV-positive patients at the University of Southern California School of Medicine hospitals (Los Angeles, CA) in Dr. P. Gill's laboratory, as described previously (21). These cells were grown in 1% gelatin-coated flasks in RPMI supplemented with 15% fetal bovine serum, 2 mM glutamine, essential and nonessential amino acids (1 mM), 1% Nutridoma-HU (Boehringer Mannheim, Indianapolis, IN), penicillin (100 units/ml), and streptomycin (100 μg/ml; BioWhittaker, Walkersville, MD).

Human $CD34^+$ bone marrow precursor cells were obtained from Drs. J. Mule and J. Hirth of the University of Michigan (Ann

[3] The abbreviations used are: KS, Kaposi's sarcoma; AIDS-KS, AIDS-associated KS; IL, interleukin; IL-4R and IL-13R, IL-4 and -13 receptors, respectively; HUVEC, human umbilical vein-derived endothelial cell; PE, *Pseudomonas* exotoxin.



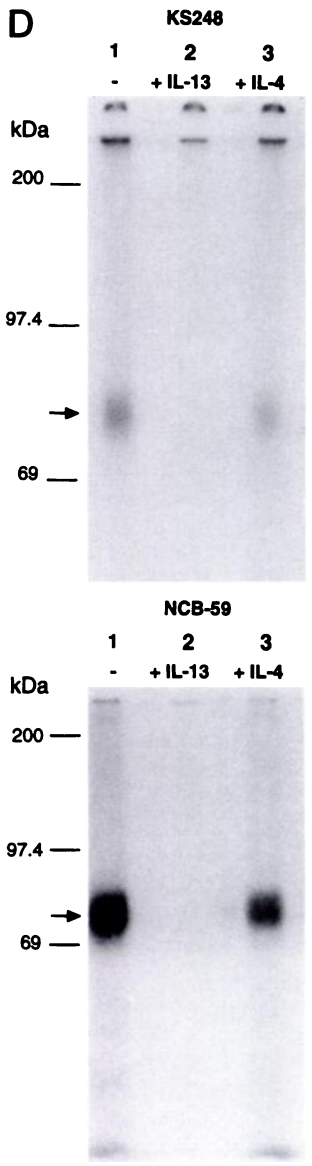


Fig. 1 Expression of IL-13R on AIDS-KS cells. The binding of ^{125}I-labeled IL-13 was performed as described in "Materials and Methods." The data shown here are for the KS248 cell line and are representative of multiple experiments for each AIDS-KS cell line. Mean number of binding sites and receptor affinity for each cell line are summarized in Table 1. The displacement curve (*A*) and Scatchard analysis (*B*) were generated from the binding data using the LIGAND program (26). *C*, competitive binding assay on NCB-59. Data are expressed as actual ^{125}I-labeled IL-13 bound (cpm) to cells in the presence of an increasing concentration of either IL-13 or IL-4. The points are the mean ± SD of duplicate determinations, and the experiment was repeated for this and the KS248 cell line with similar results. NCB-59 cells (1×10^6) incorporated 14,893 ± 188 (cpm ± SD) in the absence of IL-13 or IL-4. *D*, IL-13R subunit structure on AIDS-KS cells. Cross-linking of IL-13R on KS248 and NCB-59 was performed as described in "Materials and Methods." The SDS-polyacrylamide gel was dried and exposed to X-ray film for 10 days. The positions of standard molecular weight markers in thousands are shown on the *left*. *Arrows*, the specific IL-13R band.

Arbor, MI). The cells were activated in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum in the presence of IL-3 (10 ng/ml), IL-6 (10 ng/ml), granulocyte macrophage colony-stimulating factor (10 ng/ml), and stem cell factor (50 ng/ml) overnight prior to the incubation with IL13-PE38QQR.

Recombinant human IL-4 was provided kindly by Schering Corp. (Kenilworth, NJ). IL-13 was expressed in *Escherichia coli* and purified by one of us (W. D.). Prior to use, we compared IL-13 produced by us and the one obtained from Dr. A. Minty (Sanofi Elf Bio Recherches, Laberge, France). IL-13 from both sources were found to be identical in biological activity (*i.e.*, support of TF-1-cell proliferation) as well as in their ability to displace ^{125}I-labeled IL-13 from a cell surface. A polyclonal rabbit antibody (P7) to the p140 form of the human IL-4R was a kind gift from the Immunex Corp. (Seattle, WA).

Radioreceptor Binding. Recombinant human IL-13 was labeled with ^{125}I (Amersham Corp.) by using IODO-GEN reagent (Pierce Chemical Co.) according to the manufacturer's instructions. The specific activity of radiolabeled IL-13 ranged from 20 to 147 μCi/μg protein. The binding of ^{125}I-labeled IL-13 was measured by the method described previously (16). Briefly, AIDS-KS cells (0.5×10^6) were incubated at 4°C for

Table 1 IL-13R on human AIDS-KS and normal cell lines and cytotoxic activity of IL13-PE38QQR

IL13-PE38QQR (474 amino acid protein) is composed of IL-13 (114 *N*-terminal amino acids) and domain II and domain III of PE molecule (18). Cytotoxicity of IL13-PE38QQR was performed as described in "Materials and Methods."

Cell type	IL-13 binding sites/cell[a]	K_d (pM)	IC_{50} (ng/ml)[b] (mean ± SD)	Ref. No.
AIDS-KS				
KS-248	14452 ± 288	365 ± 121	3 ± 1	
NCB-59	7962 ± 35	525 ± 241	8 ± 1	
KS220B	5,774[c]	124	15 ± 9	
KS54A	5651 ± 934	484 ± 79	137 ± 40	
ARL-13	2413 ± 643	428 ± 64	270 ± 50	
Normal cells[d]				
HUVEC (endothelial)	<200[e]	-	>1000	
$CD34^+$ (activated bone marrow)[e]	ND[f]		>1000	
H9 T cells (T lymphocytes)	UD[g]		>1000	16, 20
DH (EBV B cells)	UD		>1000	16, 20
U937 (promonocytic)	UD		>1000	16, 20

[a] The radioreceptor binding assay for all the cell lines was performed the same way as mentioned in the legend to Fig. 1*A*. The binding sites per cell and receptor affinity (K_d) for each cell type were calculated from binding data.

[b] IC_{50}, the concentration of IL-13 toxin at which 50% inhibition of protein synthesis is observed compared to untreated cells; this was determined as described in "Materials and Methods." The mean IC_{50} for individual tumors is shown and was determined from two to five experiments for five AIDS-KS cell lines.

[c] Binding sites determined using a single saturating concentration of ^{125}I-labeled IL-13 from a single experiment.

[d] K_d values could not be calculated.

[e] $CD34^+$ bone marrow precursor cells were activated as described in "Materials and Methods."

[f] ND, not done.

[g] UD, undetectable.

2 h with ^{125}I-labeled IL-13 (500 pM) with or without increasing concentrations (10 pM–200 nM) of unlabeled IL-13. The duplicate samples of the cells associated with ^{125}I-labeled IL-13 were separated from free ^{125}I-labeled IL-13 by centrifugation through a cushion of phthalate oils. The cell pellets were counted in a gamma counter (Wallac, Gaithersburg, MD).

For the displacement assay, NCB-59 cells (0.5×10^6) were incubated with ^{125}I-labeled IL-13 (500 pM) with or without increasing concentration (up to 1000 nM) of IL-13 or IL-4, as described above.

Affinity Cross-Linking Studies. Cells (3×10^6) were labeled with ^{125}I-labeled IL-13 in the presence or absence of excess unlabeled IL-13 or IL-4 for 2 h at 4°C. The bound ^{125}I-labeled IL-13 was cross-linked to its receptor with disuccinimidyl suberate (Pierce Chemical Co.) at a final concentration of 2 mM for 45 min, as described elsewhere (16).

Protein Synthesis Inhibition Assay. Protein synthesis was determined by the incorporation of [^{3}H]leucine into AIDS-KS tumor cells. The cytotoxicity of the chimeric toxin IL13-PE38QQR on various KS cells was tested as reported previously (18–20). In brief, 1×10^4 cells per well were cultured overnight in 96-well flat-bottomed microtiter plates. Medium was aspirated and replaced by 200 μl of leucine-free medium (Biofluids, Inc., Rockville, MD) with or without various concentrations of IL13-PE38QQR (up to 1000 ng/ml). For blocking experiments, cells were preincubated with IL-13 or IL-4 (2 μg/ml) for 45 min at 37°C prior to the addition of IL-13 toxin to the cells. Cells were incubated additionally for 20–24 h at 37°C, and then 1 μCi of [^{3}H]leucine (DuPont NEN Research Products, Boston, MA) was added to each well and cultured for an additional 4 h. The cell-associated radioactivity was measured with a Beta plate counter (Wallac, Gaithersburg, MD).

Clonogenic Assay. Five hundred KS248, NCB-59, and HUVECs were plated in triplicate in gelatinized 100-mm Petri dishes, and the next day, IL-13 or IL13 toxin at various concentrations or control medium was added. The plates were incubated for 10 days. The medium was then removed, and colonies were fixed and stained with 0.25% crystal violet (Sigma Chemical Co.) in 25% ethyl alcohol. The colonies containing more than 50 cells were scored.

Antitumor Activity of IL13-PE38QQR. Human epidermoid carcinoma cells (3×10^6) obtained from American Type Culture Collection (Rockville, MD) were injected s.c. into 4–6-week-old female nude mice (Charles River Breeding Laboratories). After 7 days, the tumors were palpable and treated with IL13-PE38QQR (1.0 μg) or control excipient (0.2% human albumin serum in PBS) i.p. every day for 3 days. Tumors were measured, and volumes were calculated based on the following formula: Volume = Length × $(\text{Width})^2 \times 0.4$.

Results and Discussion

AIDS-KS Cells Express High-Affinity IL-13R

We found that all five KS cell lines examined expressed large numbers (2,400–14,500 sites/cell) of high-affinity (K_d = 124–525 pM) IL-13R (Fig. 1, *A* and *B*; Table 1). In contrast, normal HUVECs that have been shown to respond to IL-13 (22) expressed low numbers of IL-13R (<200 sites/cell; Table 1). Similarly, resting human T cells, EBV-immortalized B cells, and U937 promonocytic cell line did not show detectable levels of IL-13R (Table 1). To examine the interaction of IL-13R with IL-4 that has been proposed previously (16, 17), we examined whether IL-4 could compete for the binding of IL-13. It has been observed that in certain cell lines, IL-4 competes for the binding of IL-13 (16, 19, 20, 23). However,

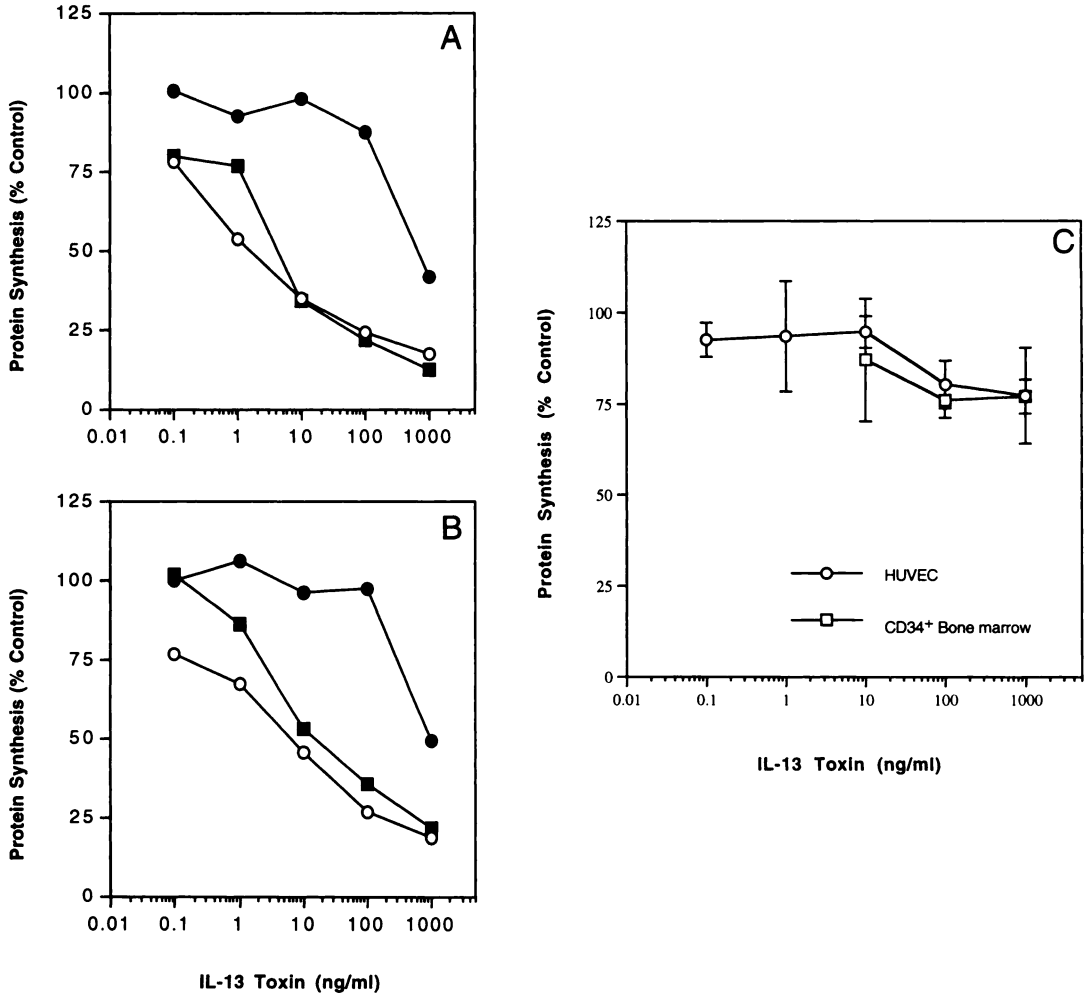


Fig. 2 Cytotoxicity of IL13-PE38QQR on AIDS-KS, endothelial, and $CD34^+$ bone marrow precursor cells. KS248 (*A*) and NCB-59 (*B*) cells were cultured with various concentrations of IL13-PE38QQR (0–1000 ng/ml) with or without IL-13 or IL-4 (2 μg/ml), as discussed in "Materials and Methods." The data were obtained from the mean of quadruplicate determinations, and the assay was repeated several times. The concentration of IL13-PE38QQR at which 50% inhibition of protein synthesis (IC_{50}) occurred was calculated. ○, IL13-PE38QQR; ●, IL13-PE38QQR+IL-13; ■, IL13-PE38QQR+IL-4. *C*, HUVECs (○) and $CD34^+$ bone marrow precursor cells (□) were also cultured with various concentrations of IL13-PE38QQR, and cytotoxicity was determined as described above. Data are means; *bars*, SD.

as shown in Fig. 1*C*, IL-13 completely inhibited for the binding of ^{125}I-labeled IL-13, whereas IL-4 modestly inhibited the binding of IL-13 on two AIDS-KS cell lines examined. This suggests that IL-13R does not interact significantly with IL-4 in AIDS-KS cells, and that IL-13R expression on AIDS-KS cells may be independent of IL-4R expression.

Subunit Structure of IL-13R on AIDS-KS

The subunit structure of IL-13R was investigated by cross-linking studies. As shown in Fig. 1*D*, ^{125}I-labeled IL-13 cross-linked to one major protein migrating as a single band at M_r ~83,000 on two AIDS-KS cell lines, KS248 and NCB-59 (Fig. 1*D*, *Lane 1, top* and *bottom*). After subtracting the molecular weight of IL-13 (M_r 13,000), the size of the major protein was estimated as M_r ~70,000. This cross-linked protein was specific IL-13 binding protein, given that no cross-linking was observed in the presence of a 500-fold molar excess of unlabeled IL-13 (Fig. 1*D*, *Lane 2, top* and *bottom*). Similar to the binding studies, IL-4 only partially displaced ^{125}I-labeled IL-13 cross-linking to M_r ~70,000 protein (Fig. 1*D*, *Lane 3, top* and *bottom*). These data suggest that IL-13R on AIDS-KS cells are composed predominantly of a single M_r ~70,000 protein that binds the IL with high affinity.

IL13-PE38QQR Is Highly Cytotoxic to AIDS-KS Cells

Protein Synthesis Inhibition Assay. We have recently produced a chimeric protein composed of IL-13 and a truncated form of *Pseudomonas* exotoxin (IL13-PE38QQR), which was found to be potently cytotoxic to IL-13R-positive solid tumor cells (18–20). The PE part of this chimeric protein is a two-domain protein: domain II catalyzes the translocation of the toxin into the cytosol, and domain III shuts off the protein synthesis by ADP ribosylating the elongation factor II that ultimately kills cells. Because, as shown above, AIDS-KS cells overexpress IL-13R, we

Table 2 In vitro inhibition of AIDS-KS cell growth by IL-13 and IL13-PE38QQR in a clonogenic assay

Results are expressed as percentage of colonies formed in treated cells compared to untreated cells. KS248, NCB-59, and HUVEC cells formed 88 ± 6, 210 ± 6, 66 ± 5 colonies (mean ± SD), respectively. The IC_{50} by clonogenic assay was calculated to be approximately 1.2 and 7.0 ng/ml for KS248 and NCB-59 cell lines, respectively; however, HUVECs were not sensitive at all (IC_{50} not achieved).

Proteins	Colonies ± SD (% control)		
	KS248	NCB-59	HUVEC
IL-13 (ng/ml)			
1	90 ± 6	95 ± 5	95 ± 11
10	79 ± 3	82 ± 1	98 ± 8
100	76 ± 1	79 ± 2	101 ± 7
1000	65 ± 1	77 ± 2	98 ± 5
IL13-PE38QQR (ng/ml)			
1	51 ± 2	81 ± 3	93 ± 4
5	22 ± 4	51 ± 3	101 ± 2
50	9 ± 2	26 ± 3	102 ± 6
100	3 ± 1	18 ± 2	96 ± 7
200	0	0	94 ± 3

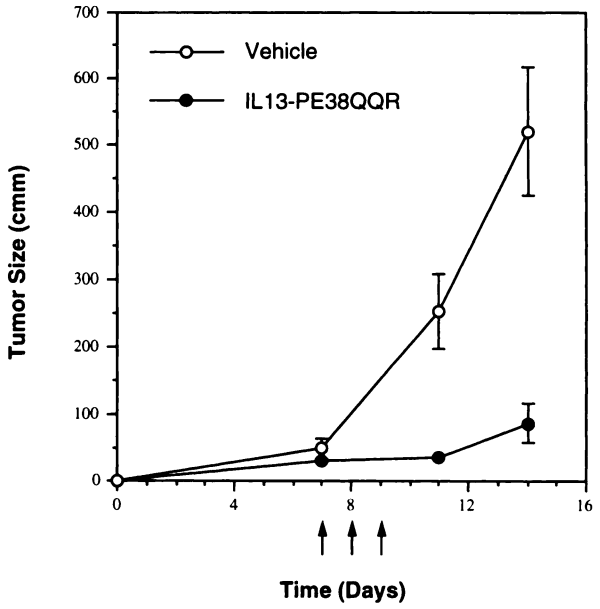


Fig. 3 Antitumor activity of IL13-PE38QQR. Nude female mice (five in each group) were injected s.c. with A431 (3×10^6 cells) on day 0, and on days 7, 8, and 9 (*arrows*), they received either control excipient (○) or 1.0 μg IL13-PE38QQR (●). *Bars*, SD.

next examined whether IL-13R on these cells could be targeted by IL-13 toxin. By utilizing IL13-PE38QQR, we observed that all five cultures of AIDS-KS cell lines were highly sensitive to the cytotoxic effect of IL-13 toxin as determined by protein synthesis inhibition assay (data shown for two cell lines, KS248 and NCB-59; Fig. 2, *A* and *B*). The 50% inhibition in protein synthesis (IC_{50}) in five AIDS-KS cell lines was achieved between 3 and 270 ng/ml (60–5400 pM) of the chimeric toxin (Table 1). The cytotoxic activity of IL13-PE38QQR was blocked completely by an excess of IL-13 but not blocked significantly by an excess of IL-4, indicating that cytotoxicity mediated by IL-13 toxin is specific, and IL-13Rs on AIDS-KS cells are distinct from IL-4R and do not interact with IL-4 (Fig. 2, *A* and *B*). There are several other types of cells in which the toxic effect of IL13-PE38QQR could not be competed with IL-4. We demonstrated recently that IL-4 did not compete with the cytotoxic effect of IL13-PE38QQR in human renal cell carcinoma cells (20) and human glioma cells (19). In addition, we have also shown that IL-4 cannot compete for IL-13 binding in human renal cell carcinoma cells (16). Thus, these results agree with our previous observations.

The cytotoxicity of IL13-PE38QQR was also tested on normal HUVECs, resting and activated $CD34^+$ bone marrow precursor cells, EBV-immortalized B cells, resting T cells, and monocytic cell lines. Consistent with the expression of low numbers of IL-13R (Table 1), IL13-PE38QQR was not cytotoxic ($IC_{50} > 1000$ ng/ml) to these cells (Fig. 2*C*; Table 1).

Clonogenic Assay. *In vitro* clonogenic assays were also performed in the presence of IL-13 or IL13-PE38QQR to examine the effect of these proteins on the proliferation of AIDS-KS cells. IL-13 by itself induced a modest inhibition of proliferation of AIDS-KS cells, indicating that IL-13R on AIDS-KS cells are functional (Table 2). On the other hand, IL-13 did not have any effect on the colony formation of endothelial cells. IL13-PE38QQR caused marked inhibition of colony formation in two AIDS-KS cell lines examined in a concentration-dependent manner (Table 2). Because clonogenicity *in vitro* correlates well with the *in vivo* malignant phenotype in xenografts (24, 25), our data suggest the antitumor activity of IL13-PE38QQR. Complete inhibition of colony formation was observed at 200 ng/ml (4.0 nM) IL-13 toxin. The IC_{50} of IL13-PE38QQR by clonogenic assay corroborated well with the IC_{50} determined by protein synthesis inhibition experiments. Similar to the protein synthesis assay, there was no effect of IL-13 toxin on colony formation of the endothelial cell line.

Next, we wished to examine the antitumor activity of IL13-PE38QQR *in vivo* against AIDS-KS. Unfortunately, three of the AIDS-KS cell lines implanted s.c. into nude, nude/beige, and nude irradiated mice did not develop into tumors consistently. Thus, *in vivo* antitumor activity of IL-13 toxin could not be evaluated against AIDS-KS. However, the antitumor activity of IL13-PE38QQR was evaluated by utilizing a human epidermoid carcinoma xenograft model. As shown in Fig. 3, IL13-PE38QQR-treated mice showed pronounced antitumor activity, and the tumor size did not increase significantly. Control mice showed a continued increase in tumor size. These results demonstrate that IL13-PE38QQR also exerts *in vivo* antitumor activity.

To the best of our knowledge, this is the first demonstration of the expression of a large number of high-affinity IL-13R on AIDS-KS cells that are internalized after binding to the ligand. AIDS-KS cells are extremely sensitive to IL13-PE38QQR, and this cytotoxic activity is extremely specific, because an excess of recombinant IL-13 completely blocked the protein synthesis inhibition mediated by chimeric toxin. IL-4 did not compete significantly for the IL-13 binding as well as the cytotoxicity of IL-13 toxin. This indicates that the IL-13Rs on AIDS-KS cells do not exhibit any significant commonality with IL-4R, the characteristic that was attributed to many normal cells and some adenocarcinomas (17, 18, 23).

Furthermore, we observed that there was a positive correlation between the level of IL-13R expression and sensitivity to the

IL13-PE38QQR, except in KS54A cells. It is possible that the differential internalization rate, intracellular processing of the toxin, or both play a role. IL13-PE38QQR was only found to be cytotoxic to AIDS-KS cell lines and not to normal endothelial cells. In addition, human T and B cells, monocytes, and resting or growth factor-activated bone marrow cells were not sensitive to the cytotoxic effect of IL13-PE38QQR (20). The insensitivity of normal cells to IL13-PE38QQR is most probably due to the lack of or low number of IL-13R expression on these cells.

In conclusion, we have demonstrated that five out of five AIDS-KS cell lines express a large number of IL-13 receptors, and AIDS-KS cells are highly sensitive to a chimeric protein composed of IL-13 and *Pseudomonas* exotoxin. We have also demonstrated that IL13-PE38QQR has a significant antitumor activity against human cancer. Additional studies are ongoing to examine the expression of IL-13R in additional KS samples *in vitro* and *in vivo*. The specific probes or antibodies to IL-13R is currently not available. The availability of these reagents will help us address whether IL-13R on AIDS-KS cells constitutes an adequate model for KS therapy. Efforts are under way to grow KS cells in immunodeficient animals to evaluate the antitumor activity of IL13-PE38QQR.

Thus, IL-13 receptors may serve as a unique target for the delivery of cytotoxic agents such as *Pseudomonas* exotoxin or, alternatively, IL-13R-directed gene therapy using growth modulatory genes, including tumor suppressor genes.

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