Targeting of Interleukin-13 Receptor on Human Renal Cell Carcinoma Cells by a Recombinant Chimeric Protein Composed of Interleukin-13 and a Truncated Form of Pseudomonas Exotoxin A (PE38QQR)

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We have previously shown that human renal cell carcinoma (RCC) cells express large numbers of interleukin-13 receptors (IL-13R), a newly described homologous growth factor receptor. To target tumor cells that express IL-13R, we have produced a chimeric protein composed of human IL-13 and a derivative of Pseudomonas exotoxin A, termed PE38QQR. We report here that IL-13-PE38QQR is highly cytotoxic to many human RCC cell lines. IL-13-negative cell lines or cell lines expressing low numbers of IL-13 (<300 sites/cell) that include human bone marrow-derived cells were not susceptible to the cytotoxic effect of IL-13-PE38QQR. The sensitivity of RCC cells to IL-13-PE38QQR correlated positively with the density of IL-13R. The cytotoxic activity of IL13-PE38QQR was competed by an excess of IL-13 in a protein synthesis inhibition assay and confirmed by a clonogenic assay. Even though IL-13 and IL-4 are homologues and IL-4R and IL-13R have been proposed to share a receptor subunit, IL-4 did not compete for the cytotoxicity mediated by IL-13-toxin on RCC. IL-13-PE38QQR competes for [125I]-IL-13 binding sites on RCC cells, although at a lower affinity than the wild-type recombinant cytokine. Human T-cell, B-cell, and monocytic cell lines are unresponsive to the cytotoxic action of IL13-PE38QQR. Thus, our results indicate that IL13-PE38QQR is highly cytotoxic to human RCC cells, although it is not cytotoxic to a variety of normal hematopoietic cells. IL13-PE38QQR should be further investigated preclinically for the treatment of human RCCs. This is a US government work. There are no restrictions on its use.

INTERLEUKIN-13 (IL-13), a pleiotropic immune regulatory cytokine, is produced predominantly by Th2 lymphocytes and also by CD8+ T lymphocytes and some other cells.1,2 The gene for IL-13 is closely linked to the IL-4 gene.3 Also, the amino acid sequence for IL-13 protein has 30% identity to IL-4 protein.4 IL-13 shares many biologic properties with IL-4 on many different target cells.1,3 Compared with IL-4, IL-13 has not been found to have any direct effect on the growth and differentiation of T cells.5 Similarly to IL-4, IL-13 inhibits the production of inflammatory cytokines6,7 and upregulates major histocompatibility complex (MHC) class II and CD23 expression on monocytes.8 In B cells, IL-13 enhances proliferative responses to anti-IgM and anti-CD40 antibodies,9,10 upregulates MHC class II and CD23 expression,10,11 and induces anti-CD40-dependent IgE class switch and IgG and IgM synthesis.10,12

We have previously reported that human renal cell carcinoma (RCC) cells express a large number of IL-13.13 IL-13 crosslinks to a predominant ~70 kD protein, whereas IL-4 crosslinked to two major proteins of 140 kD and 70 kD in RCC cells.13,14 Because IL-13 binding protein is similar in size to the smaller protein of the IL-4R complex and IL-13 competed for the binding of radiolabeled IL-4, it is suggested that IL-13R system shares a component of the IL-4R system. However, IL-4 did not compete for the binding of radiolabeled IL-13 on RCC cells. In addition, antibody to IL-4R p140 did not immunoprecipitate IL-13.13 These studies suggest that the structure of the IL-13R system is complex and that further studies are required to unravel its structure. Because RCC cells express high levels of IL-13R, we were interested in targeting these cancer cells with cytotoxins. The Pseudomonas exotoxin (PE)-based cytotoxins have been used to target cancer cells.15 We have recently produced a recombinant chimeric protein composed of IL-13 and a derivative of PE, PE38QQR.16 PE is a three-domain protein17; domain Ia binds to PE receptors, domain II catalyzes the translocation of the toxin into the cytosol, and domain III shuts off protein synthesis and eventually kills the cells by ADP ribosylation of elongation factor 2.15 PE38QQR is deprived of (1) domain Ia, (2) a portion of domain Ib, and (3) has 2 lysine residues in domain III mutated to glutamine.18 IL-13--based chimeric toxin was cytotoxic to several cell lines derived from various adenocarcinoma.16 In this study, we have investigated the action of IL13-PE38QQR on human RCC cells. We report here that RCC cells are highly sensitive to the cytotoxic effect of IL13-PE38QQR and that some of the cell lines are susceptible to uniquely low concentrations of the chimeric protein. This cytotoxicity was specific because recombinant IL-13 competed for the cytotoxic effect of IL13-PE38QQR and that some of the cell lines are susceptible to uniquely low concentrations of the chimeric protein. This cytotoxicity was specific because recombinant IL-13 competed for the cytotoxic effect of IL13-PE38QQR. Furthermore, IL13-PE38QQR was not cytotoxic to human T-cell, B-cell, and monocytic cell lines and fresh bone marrow-derived cells.

MATERIALS AND METHODS

Recombinant cytokines and toxins. Recombinant IL-13 was produced by one of the authors (W.D.). This Escherichia coli-overex-
pressed and then purified protein was found to be mitogenic to human TF-1 cell line13 and competed for the binding of radiolabeled IL-13, as did IL-13 provided by Sanofi Elf Bio Recherches (Laberge, France; a kind gift of Dr A. Minty). Recombinant IL-4 was provided by Schering-Plough Research Institute (Kenilworth, NJ). Recombinant IL13-PE38QQR was produced and purified to greater than 95% homogeneity, as described previously.16

Cell lines. RCC cell lines were generated from tumor specimens obtained from Wayne State University School of Medicine (Detroit, MI; kindly provided by Dr G. Hillman) or the Surgery Branch of the National Cancer Institute (Bethesda, MD; kindly provided by Drs G. Weiss and M. Linehan), as previously described.19 Single-cell suspensions of these tumors were cultured in complete medium composed of Dulbecco’s minimum essential medium (DMEM) supplemented with 3 mmol/L glutamine, 50 μg/mL Gentamycin, and 10% heat-inactivated fetal calf serum (FCS) obtained from Biowhittaker (Walkersville, MD).

Human T-cell lymphoma (H9) and promonocytic cell lines (U937) were obtained from American Type Culture Collection (ATCC; Rockville, MD). The Epstein-Barr virus (EBV)-transformed human B-cell line (DH), BL-41, and JD-38 Burkitt’s lymphoma cell lines were obtained from Dr Giovanna Tosato (Food and Drug Administration, Bethesda, MD). All of these cell lines were cultured in complete media (RPMI 1640 supplemented with 3 mmol/L glutamine, 50 μg/mL Gentamycin, and 10% heat-inactivated FCS).

Human peripheral blood lymphocytes (PBL) were isolated from heparinized blood (buffy coats) obtained from normal donors at the Transfusion Unit of the National Institutes of Health (Bethesda, MD). PBL were isolated over Ficoll Hypaque (lymphocyte separation medium [LSM]; Organon Teknika Corp, Durham, NC) gradient. Lymphocytes were cultured in a 5% CO2 incubator in RPMI 1640 (Biowhittaker) medium supplemented with 10% FCS and other ingredients listed above. PBL at a density of 2 × 10⁶ cells/mL, 2 mL/well in 24-well plates with phytohemagglutinin (PHA; 5 μg/mL).
were cultured for 3 days at 37°C. Cells were harvested and 2 × 10^4 activated cells were used for cytotoxicity assay. Recombinant IL-2 (rIL-2; 20 IU/mL) was added to cells to maintain growth and viability.

**Protein synthesis inhibition assay.** The cytotoxic activity of IL13-PE38QQR was assessed as previously described. Typically, 1 × 10^4 RCC tumor cells or other cells were cultured in leucine-free medium with or without various concentrations of IL13-PE38QQR for 20 to 22 hours at 37°C. Then, 1 μCi of (H)Leucine (NEN Research Products, Wilmington, DE) was added to each well and incubated for an additional 4 hours. Cells were harvested and radioactivity incorporated into cells was measured by a Beta plate counter (Wallac-LKB, Gaithersburg, MD).

**Clonogenic assay.** The cytotoxicity of IL13-PE38QQR was also tested using a colony-forming assay. Five to six hundred PM-RCC, WS-RCC, MA-RCC, and MA-RCC, washed three times in Hank’s Balanced Salt Solution, and resuspended in binding buffer (RPMI 1640 plus 1 mmol/L HEPES and 0.2% human serum albumin). For IL-13 displacement assay, RCC (1 × 10^4/100 μL) cells were incubated at 4°C with [125I]-IL13 (100 to 200 pmol/L) with or without increasing concentrations of unlabeled IL-13 or IL13-PE38QQR. After 2 hours of incubation, cell bound radioligand was separated from unbound by centrifugation through a phthalate oil gradient and radioactivity was determined with a gamma counter (Wallac).

**RESULTS**

**Cytotoxicity of IL13-PE38QQR.** It was of interest to examine the cytotoxicity of IL13-PE38QQR on RCC cells because we have previously shown that human RCC cells express large numbers of IL-13 receptors. Therefore, 4 primary cell cultures (PM-RCC, WS-RCC, MA-RCC, and HL-RCC) and 1 long-term culture (CAKI-1) of RCC cell lines were tested. RCC cells were sensitive to the cytotoxic activity of IL13-PE38QQR, with IC_{50} ranging from as low as 0.03 ng/mL to 350 ng/mL (0.6 pmol/L to 7 nmol/L; Fig 1 and Table 1). All four primary cultures of RCC cells generated in our laboratory seemed to be more sensitive to IL13-PE38QQR compared with the long-term RCC cell line (CAKI-1). The cytotoxic activity of IL13-PE38QQR was specific and mediated through IL-13R, because excess IL-13 neutralized the cytotoxic activity of IL13-PE38QQR (Fig 1). Thus, RCC cells are killed by IL13-PE38QQR at uniquely low concentrations of the chimeric protein.

**Correlation between IL-13R expression and sensitivity to IL13-PE38QQR.** As previously reported, the primary RCC cell lines, such as PM-RCC, WS-RCC, HL-RCC, and MA-RCC, expressed various numbers of high- to intermediate-affinity IL-13R. However, IL-13 binding characteristics on CAKI-1 RCC cell line were not determined. We therefore performed IL-13 binding studies on these RCC cells using [125I]-IL13. As shown in Table 1, CAKI-1 RCC cell line did not bind radiolabeled IL-13 well and only expressed less than 100 IL-13 binding sites/cell. The sensitivity of these cell lines to IL13-PE38QQR also varied depending on the number of IL-13 binding sites per cell. CAKI-1 RCC cell line expressed the least number of IL-13 binding sites and were least sensitive to IL13-PE38QQR. In contrast, HL-RCC cells, which express 150,000 IL-13 binding sites/cell, were extremely sensitive to the chimeric toxin.

**In vivo passage of MA-RCC does not decrease sensitivity to IL13-PE38QQR.** To determine the antitumor activity of IL13-PE38QQR against human RCC, we attempted to grow human RCC cells as subcutaneous tumors in nude mice, irradiated (300 rads) nude mice, and SCID mice. However, we were not able to consistently grow these RCC cells in any of these immunocompromised mice (data not shown). In some cases, tumors did grow very slowly but became centrally necrotic with a white rim of viable RCC cells.

Therefore, antitumor activity of IL13-PE38QQR could not be evaluated in vivo under these conditions. However, we were able to passage MA-RCC in nude mice and use the passaged tumors to prepare single-cell suspensions. These cells did grow in tissue culture and, after 1 to 3 passages, we examined their sensitivity to IL13-PE38QQR. As shown in Fig 2A, MA-RCC were very sensitive to IL13-PE38QQR and passaging of these RCC cells in vivo twice did not decrease their sensitivity. These data suggest that IL-13R levels do not change by in vivo passage of RCC tumor cells.

**IL13-PE38QQR is not cytotoxic to immune cells, monocytes, bone marrow-derived cells, and Burkitt’s lymphoma cells.** The cytotoxic activity of IL13-PE38QQR was also examined on PHA-activated T cells, a CD4^+ T-cell lymphoma line (H9), normal bone marrow cells, an EBV-transformed B-cell line, 2 Burkitt’s lymphoma cell lines, and a pre monocytic cell line (U937).
Fig 2. Sensitivity of IL13-PE38QQR to MA-RCC after in vivo growth and on immune cells, bone marrow cells, and B-cell lymphoma cell lines. Cytotoxic activity of IL13-PE38QQR was determined against cultured MA-RCC single cell suspension generated from tumors that grew in nude mice (MA-MS1) and MA-MS1 grown again in nude mice (MA-MS2) (A); PHA-activated PBL, T-cell lymphoma (H9), and EBV-transformed cell line (B); promonocytic cell line (U937) and human bone marrow cells (C); and BL-1 and JD-38 Burkitt’s lymphoma B-cell lines (D). PHA-activated T cells (2 x 10⁶ cells) were cultured in the presence of 20 IU/mL IL-2 with IL13-toxin for a total of 24 hours. Bone marrow cells were obtained from a normal donor and 10⁴/well were used in cytotoxic assay. Protein synthesis was determined as described in Fig 1. The data are presented as the mean percentage of control of untreated cells. MA-RCC, MA-MS1, and MA-MS2 incorporated 2,420 ± 2,715, 20,027 ± 1,022, and 20,649 ± 4,292 cpm ± SD, respectively. Control PHA-activated T cells, H9 cells, and EBV-B cells incorporated 58,007 ± 3,897 cpm ± SD, 64,592 ± 6,034 cpm ± SD, and 24,966 ± 1,579 cpm ± SD, respectively. U937 cells and bone marrow cells incorporated 49,660 ± 2,277 cpm ± SD and 301 ± 107 cpm ± SD, respectively, whereas BL-41 and JD-38 cell lines incorporated 42,585 ± 744 cpm ± SD and 44,769 ± 1,463 cpm ± SD, respectively.

As shown previously, PHA-activated T cells, H9 cells, and U937 cells did not express detectable numbers of IL-13R. Consistent with these observations, IL13-PE38QQR was not cytotoxic to any of these cell types (Fig 2). EBV-transformed B-cell line did express about 300 IL13-binding sites/cell; however, IL13-PE38QQR was not cytotoxic to them at concentrations up to 1,000 ng/mL. Although IL-13R expression was not determined on human bone marrow cells or Burkitt’s lymphoma cell lines, based on their insensitivity to IL13-PE38QQR, it is expected that these cells do not express IL-13R either or express a low number of these receptors.

Clonogenic assay. The antitumor activity of IL13-PE38QQR against human RCC was also evaluated in a colony-forming clonogenic assay. Human PM-RCC and HL-RCC cells formed colonies when 500 to 600 cells were cultured in petri dishes. Using this number of cells, both PM-RCC and HL-RCC cells formed 175 and 348 colonies, respectively, with a clonogenic efficiency between 35% and 58%. When PM-RCC cells were treated with 50 ng/mL
IL13-PE38QQR for 10 days, only 32 colonies were formed (Table 2). However, 123 or 175 colonies were formed when cells were treated with recombinant IL-13 or media alone, respectively. Similar to cytotoxicity data (Table 1), HL-RCC were much more sensitive to cytotoxic effect of IL13-PE38QQR. A concentration as low as 5 ng/mL of IL13-PE38QQR almost completely inhibited colony formation (Table 2).

IL-4 does not block the cytotoxic activity of IL13-PE38QQR on RCC cells. We have previously reported that in RCC cells IL-13R may share a component with the IL-4R.13 In addition, IL-13 competed for the binding sites of IL-4, whereas IL-4 did not compete for the binding site of IL-13. However, in other cancer cell types, IL-4 neutralized the cytotoxicity mediated by IL13-PE38QQR.16 We therefore examined whether IL-4 can neutralize the cytotoxicity of IL13-PE38QQR on RCC cells. As shown in Fig 1, only IL-13 blocked the cytotoxicity of IL13-PE38QQR, whereas IL-4 did not block this cytotoxicity in all 3 RCC cell lines tested.

Binding affinity of IL13-PE38QQR on human RCC cells. We next examined the binding affinity of IL13-PE38QQR to IL-13R. HL-RCC or PM-RCC cells were used for this purpose. These cells were incubated with a saturating concentration of radiolabeled IL-13 in the absence or presence of various concentrations of IL-13 or IL13-PE38QQR. As shown in Fig 3, in HL-RCC cells the IC50 (the protein concentration at which 50% displacement of [125I]-IL-13 binding is observed) for native IL-13 was ~20 x 10^-9 mol/L, compared with ~180 x 10^-9 mol/L with IL13-PE38QQR. Thus, IL13-toxin bound to IL-13R with about eightfold to 10-fold lower affinity compared with IL-13 on this cell line.

DISCUSSION

In this study, we show that an IL-13–based cytotoxin, IL13-PE38QQR, is highly cytotoxic to human RCC cells. The IC50 in RCC cell lines ranged from less than 0.03 ng/mL to 350 ng/mL. The cytotoxicity of the IL13-PE38QQR was specific and mediated through IL-13R because excess IL-13 neutralized the cell killing activity of IL13-PE38QQR. These results corroborate with the data obtained in a clonogenic assay that show a significant inhibition of colony formation by IL13-PE38QQR.

Resting human cells, including nonactivated T-cell line (H9), EBV-transformed B-cell line, and promonocytic (U937) cell lines were not sensitive to the cytotoxic effect of IL13-PE38QQR. Similarly, PHA-activated human T cells and cells obtained from normal bone marrow biopsy were also insensitive to the cytotoxic effect of IL13-PE38QQR. It has previously been reported that hematologic progenitor cell lines and fresh human bone marrow cells express low numbers of IL-4 receptors.21,22 However, IL-13R expression on these cells has not been determined. A recent study reported that IL-13 has a direct regulatory role in the proliferation and differentiation of primitive murine hematopoietic progenitor cells,22 indicating expression of some level of IL-13R on these cells. However, in the present study, we show that IL13-PE38QQR was not cytotoxic to fresh bone marrow-derived cells, indicating that progenitor cells probably express insufficient amounts of IL-13R or that IL-13R on these cells is not susceptible to the cytotoxic action of IL13-PE38QQR.

It has been previously shown that IL-13 competed for the binding of IL-4, whereas IL-4 did not compete for the binding of IL-13 on RCC cells.13 Similar to these results, our data in the current study show that IL-4 does not neutralize the cytotoxic effect of IL13-PE38QQR. We have previously shown that IL-4–based cytotoxin is highly cytotoxic to human RCC cells.23 A comparison could not be made between IL13-PE38QQR and IL4-PE4E because the PE portion in these two chimeric proteins is different. However, both IL-13 and IL-4 competed with the cytotoxicity of IL4-toxin (Obiri et al, manuscript in preparation). These data suggest that the receptors for IL-13 and IL-4 share a component. However, the subunit that is shared between these two receptors is not known. Additional studies are underway to identify specifically common subunits that are shared between receptors for IL-4 and IL-13 (Puri et al, unpublished data).

It is of interest to note that IL13-PE38QQR binds to IL-13 receptor with a lower affinity compared with that of IL-13. These results are similar to that reported for many toxin-cytokine fusion proteins.24,25 We have recently reported that IL-4–based PE fusion protein (IL4-PE4E) bound to IL-4R with a lower avidity compared with IL-4.26 The mechanism of decrease in binding affinity of cytokine-based toxins to their respective receptors is not completely clear. It is possible that steric hindrance due to bulky toxin component, incorrect refolding of the cytokine component, or toxin occupation of receptor binding domain of the cytokine may be responsible for this decrease in binding affinity. We have recently reported that the binding affinity of IL4-PE chimeric protein can be improved by freeing the native C-terminus of the IL-4 molecule and attaching the truncated PE portion to newly created C-terminus within the IL-4 molecule.27,28 Because truncated PE molecule was attached to the native

**Table 2. Effects of IL-13 and IL-13-PE38QQR on PM-RCC and HL-RCC Cells by Clonogenic Assay**

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<th>PM-RCC</th>
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<td>No. of Colonies ± SD</td>
<td>% Surviving Fraction</td>
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<td>IL-13</td>
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<tr>
<td>Control</td>
<td>175 ± 5</td>
<td>100</td>
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<tr>
<td>IL13-PE38QQR</td>
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<td>IL-13</td>
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<td>IL-13</td>
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<td>Control</td>
<td>348 ± 9</td>
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<tr>
<td>IL13-PE38QQR</td>
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<td>5 ng/mL</td>
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<td>50 ng/mL</td>
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PM-RCC or HL-RCC cells (500 to 600) were plated in 100-mm petri dishes and exposed in triplicate to either medium control, IL-13 (20 ng/mL), or IL13-PE38QQR (5 and 50 ng/mL). Cells were cultured in the presence of drug for 10 days, medium was removed, and colonies were fixed and stained in crystal violet. Colonies containing 50 or more cells were scored. The percentage of the surviving fraction was calculated by the ratio of the number of colonies formed in treated and untreated cells.
are larger than the size of the point symbols. In addition, our data also suggest that a chimeric IL-13 toxin molecule in which toxin moiety is attached at a site away from the C-terminus residues may be more cytotoxic to cancer cells which toxin moiety is attached at a site away from the C-terminus of the IL-13 molecule, these data suggest that, similar to IL-4,\textsuperscript{20-31} IL-13 may interact with its receptor predominantly through C-terminal end residues. In addition, our data also suggest that a chimeric IL-13 toxin molecule in which toxin moiety is attached at a site away from the C-terminus residues may be more cytotoxic to cancer cells (Debinski et al, unpublished data).

In summary, our results indicate that IL13-toxin IL13-PE38QQR is highly cytotoxic to human RCC cells that express high numbers of IL-13R. Because resting or activated immune cells or bone marrow cells are not sensitive to IL13-toxin, our data suggest that this toxin may be a useful drug for the treatment of RCC without being cytotoxic to normal immune cells. Further studies are ongoing to develop an appropriate RCC model to evaluate IL13-PE38QQR in vivo.

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Targeting of interleukin-13 receptor on human renal cell carcinoma cells by a recombinant chimeric protein composed of interleukin-13 and a truncated form of Pseudomonas exotoxin A (PE38QQR)

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