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Identification and Characterization of Interleukin-13 Receptor in Human Medulloblastoma and Targeting These Receptors with Interleukin-13-Pseudomonas Exotoxin Fusion Protein

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Aim. To identify and characterize the subunit structure of interleukin-13 receptor (IL-13R) in human medulloblastoma cell lines and target them with a chimeric fusion protein consisting of interleukin-13 and Pseudomonas exotoxin (termed as IL-13 cytotoxin).

Methods. Five human medulloblastoma cell lines were examined for the expression of IL-13R subunits at the mRNA and protein levels by reverse transcriptase-polymerase chain reaction (RT-PCR) and indirect immunofluorescence studies, respectively. In addition, IL-13 cytotoxin-induced cytotoxicity was examined in these medulloblastoma cell lines by measuring protein synthesis inhibition.

Results. All five medulloblastoma cell lines expressed mRNA and proteins for IL-4Rα and IL-13Rα1 chains, whereas three of the cell lines also showed the presence of IL-13Rα2. mRNA or protein for IL-2γc chain was not detected in any of the cell lines. Consistent with the expression of IL-13Rα2 chain, IL-13 cytotoxin was highly and specifically cytotoxic to three of five medulloblastoma cell lines. The sensitivity of medulloblastoma cell lines to IL-13 cytotoxin could be completely eliminated by concurrent incubation with excess of IL-13, but not with IL-2 or IL-4.

Conclusion. These studies establish IL-13R, in particular IL-13Rα2, as a medulloblastoma-associated target for IL-13 cytotoxin therapy. IL-13 cytotoxin may be useful for medulloblastoma therapy. Alternatively, IL-13Rα2 may serve as a tumor-specific antigen for active immunotherapy.

Key words: *bacterial toxins; fluorescent antibody technique; interleukin-13; medulloblastoma; Pseudomonas aeruginosa; receptors, interleukin; reverse transcriptase polymerase chain reaction*

Medulloblastomas are mostly small-cell embryonal tumors of the cerebellum and are highly malignant. They primarily occur in children within the first decade of life and carry a poor prognosis in the advanced stage of the disease (1). Increase in anaplasia, mitosis, apoptosis, and necrosis are considered to be associated with poor prognosis in patients with medulloblastoma (2,3). New therapeutic anticancer agents are needed to improve survival and enhance the quality of life of these patients.

Several biological and immunological markers, including overexpressed growth factors or cytokine receptors, have been associated with central nervous system malignancies that may potentially act as tumor-associated targets or specific antigens, such as fibroblast growth factor-1β (FGFR) (4), insulin-like growth factor receptor (IGFR) (5-7), epidermal growth factor receptor (EGFR) (8,9), transferrin receptor (10), urokinase receptor (11), or substance P receptor (12-14). We have previously identified abundant expression of the receptors for IL-4 (IL-4R) and IL-13 (IL-13R), and two Th2-lymphocyte-derived and related immunoregulatory cytokines on various human brain tumor cell lines (15-23). Normal human brain tissues or primary explants did not express detectable IL-4R and IL-13R (16,18,21). The structure of IL-4 and IL-13 receptors has been extensively studied. The IL-4 receptor complex exists as three different types. Type I IL-4 receptors are composed of IL-4Rα and IL-2Rγ subunits (γc), type II, IL-4Rα and IL-13Rα1 subunits (24,25), whereas Type III IL-4R are composed of all three chains. IL-13 receptors are also known to exist as at least three different types. Type I IL-13R are comprised of IL-13Rα1, IL-13Rα2, and IL-4Rα chains, whereas type II IL-13R consists of IL-4Rα and IL-13Rα1 chains (25-30). The type III IL-13R is similar to type II IL-13R, except that these cells also express γc chain (29). The role of γc chain in the formation of the IL-13R complex is not clear. It has been shown that in-

troduction of γc can decrease IL-13 and IL-4 binding and interfere in functioning of both receptors in cells that do not normally express this chain (30-32). These and other studies have shown that IL-4Rα and IL-13α1 chains are shared between IL-4 and IL-13R complexes (25-29). Furthermore, both chains are required for signal transduction through type II IL-4R and both type I/II IL-13R (25). Although both IL-4 and IL-13R are overexpressed on tumor cells, the significance of expression of these receptors on tumor cells is still not known. It is also not known which chains of these receptors are present on medulloblastoma tumor cells. Different configurations of these subunits on tumor cells may contribute to a specific or peculiar biological function.

In the present study, we investigated medulloblastoma cell lines for the expression of mRNA for various receptor subunits of IL-13 receptor by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. In addition, surface expression of these receptor proteins on medulloblastoma cell lines has been examined by indirect immunofluorescence assays (IFA). Finally, we examined the functional property of IL-13R chains by performing protein synthesis inhibition assays, using IL-13 cytotoxin, to evaluate the specificity of binding and cytotoxicity on various medulloblastoma cell lines.

Materials and Methods

Medulloblastoma Cell Lines

UW-228-1, UW-228-2, and UW-228-3 cell lines were derived from a medulloblastoma tumor and provided by Dr. John Silber of Washington University, Seattle, WA, USA (33,34). D283 and D341 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Three of 5 cell lines (UW 228-1, UW228-2, and UW228-3) grew as monolayers, D283 grows mostly in suspension and some monolayer, whereas D341 cell line grows as a suspension cell line. Renal cell carcinoma cell line PM-RCC was derived in our laboratory (35) and H-9 T cells were purchased from ATCC. All of these cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (Biowhittaker, Walkersville, MD, USA), 1 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mmol/L non-essential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin (Biowhittaker).

Recombinant cytokines and toxins

Recombinant chimeric fusion protein IL-13PE38QQR (IL-13 cytotoxin) was expressed in *E. coli* and purified to >95% homogeneity in our laboratory as described elsewhere (36-38). Recombinant IL-4 and IL-13 were also produced by us as described elsewhere (39,40).

RNA Extraction

Total RNA was extracted from medulloblastoma cell lines by using Trizol reagent (Gibco, Gaithersburg, MD, USA) according to manufacturer's instructions. Briefly, 5-10x10^6 cells were pelleted, lysed, and centrifuged after adding chloroform. RNA from the aqueous phase was separated and precipitated with cold isopropanol after addition of 1 μg of glycogen as a co-precipitant. The pellet was washed with 70% ethanol twice, dried, and reconstituted with RNase-free water. RNA was quantitated after measuring the optical densities at 260 and 280 nm in a spectrophotometer and stored at -70 °C.

Reverse Transcriptase-Polymerase Chain Reaction

Levels of IL-13R subunit mRNA were determined by RT-PCR using primers and conditions as described previously (26). Electrophoresis of amplified products was performed on a 2% agarose gel. The products were then stained with ethidium bromide, visualized on a transilluminator, and photographed. The band intensities of RT-PCR products for IL-13Rα2 and β-actin were evaluated with a FluoroImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The relative fluorescence intensity of IL-13Rα2 chain mRNA was determined by dividing the intensity by density of the β-actin band and expressed as a ratio of relative fluorescence units (RFU). The ratio of amplified products for these two genes were determined by performing two independent experiments in duplicate and expressed as mean±SD. Further, the data were analyzed with Student's t test for their significance within each IL-13R positive cell line.

Immunofluorescence Assay

Twenty thousand cells from different medulloblastoma cell lines were cultured in chambered glass slides (Lab Tek- Nalge Nunc International, Naperville, IL) for 48 h as described elsewhere (21). The cells were washed with phosphate-buffered saline (PBS) and fixed in cold methanol:acetone (1:1) and incubated at -20 °C for 2 h. The slides were then washed and rehydrated with PBS and used for indirect immunofluorescence analysis. Monoclonal antibodies against IL-13Rα1 and IL-13Rα2 were purchased from Diaclone, Besancon, France. Mouse monoclonal antibody (M57) against IL-4Rα chain was a kind gift from Immunex Corporation, (Seattle, WA, USA) and polyclonal rabbit anti-γc antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rehydrated cells in the chambered slide were incubated with 1% bovine serum albumin, and either goat or horse serum (5%) in PBS buffer to block non-specific binding of antibody. The slides were washed and incubated either with the specified primary antibody (1:1500 for mAb IL-13Rα1, IL-13Rα2, IL-4Rα and rabbit polyclonal IL-2Rγc) or appropriate isotype control (mouse IgG1 or rabbit IgG) at room temperature for 2 h. Slides were then washed three times with PBS at room temperature for five minutes and stained with secondary antibodies conjugated to either rhodamine (TRICT) or fluorescein isothiocyanate (FITC), which were diluted in PBS containing 0.1% bovine serum albumin (BSA) according to the manufacturer's recommendation. After three washes with PBS, slides were dried and layered with Vectashield anti-fluorescence fading mounting medium (Vector Laboratories, Burlingame, CA, USA) and cover slipped. The slides were viewed in a Nikon epifluorescence microscope by using appropriate filters.

Protein synthesis inhibition assays

The cytotoxic activity of IL-13 cytotoxin was determined by protein synthesis inhibition assay as previously described (41). Protein synthesis inhibition directly correlates with cell death (42). Typically, 104 medulloblastoma cells were cultured in leucine-free medium with or without various concentrations of IL-13 cytotoxin at 37 °C for 20-22 h. For competition studies, cells were pre-incubated with IL-4 or IL-13 or IL-2 (2 μg/mL) at 37 °C for 30 min prior to the addition of IL-13 cytotoxin to the cells. Then 1 μCi of 3H-leucine (NEN Research Products, Wilmington, DE, USA) was added, cells incubated for an additional 4 h, and harvested on filtermats. Radio labeled leucine incorporation into cells was measured by a Betaplate counter (Wallac, Gaithersburg, MD, USA).

Cell proliferation assay

Proliferation assays were performed as described previously (43). Briefly, the cells were washed, and resuspended in complete medium in which the fetal bovine serum (FBS) content was reduced to 2%. For each cell line, 2x10^4 cells were plated in 96 well plates and cultured at 37 °C in a 5% CO_2 incubator for 6 h. IL-13 (0.1 to 1,000 ng/mL) was added to the cultures and incubated for an additional 16 h. The cells were then pulsed with [3H]- thymidine [1μCi/well] for additional 8 h and frozen at –70 °C. The plates were thawed, harvested on filtermats, and radiolabeled thymidine uptake was measured by a Betaplate counter (Wallac, Gaithersburg, MD, USA).

Results

IL-13R Expression in Medulloblastoma Cell Lines

RT-PCR analyses revealed that three of five cell lines, UW 228-1, UW228-2, and UW 228-3, showed

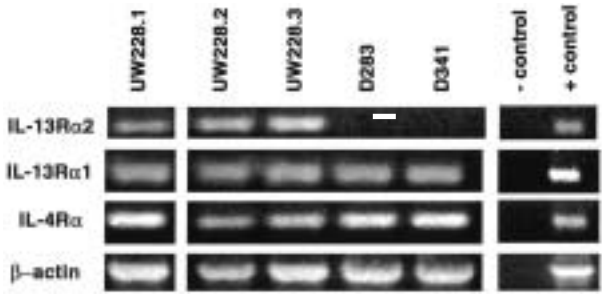

Figure 1. Interleukin-13 receptor (IL-13R) subunit expression analysis. Reverse transcriptase-polymerase chain reaction (RT-PCR) products underwent electrophoresis in 2% agarose gel and were visualized by Ethidium bromide staining. RNA from renal cancer cells (PM-RCC RNA) served as positive control for IL-13Rα2, IL-13Rα1, and IL-4Rα chains.

a strong positive band for IL-13Rα2 chain. However, all five medulloblastoma cell lines expressed similar levels of mRNA for IL-4Rα and IL-13Rα1 chains, except D283 cell line, which showed moderate expression of IL-13Rα1 (Table 1, Fig. 1). In contrast, mRNA for IL-2R γ chain (γc) was not expressed in any of the five cell lines. PM-RCC cells were used as a positive control, which expressed mRNA for IL-4Rα, IL-13Rα1, and IL-13Rα2 chains (29). We also compared the ratio of relative fluorescence units of IL-13Rα2 and ß-actin bands in the three IL-13Rα2 receptor-positive cell lines. The mean values for this ratio ranged from 0.53 to 0.62, indicating that these cell lines expressed similar levels of IL-13Rα2 chain relative to β-actin ($p > 0.08$).

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for mRNA expression of interleukin-13 receptor (IL-13R) chains in human medulloblastoma cell lines

	Receptor subunit mRNA*				
Cell Line	2	1	IL-4R	IL-2R c	Ratio of RFU(α2/β-actin)†
UW228-1	+ +	+ +	+ + +	–	0.62 ± 0.08
UW228-2	+ +	+ +	+ +	–	0.54 ± 0.11
UW228-3	+ +	+ +	+ + +	–	0.53 ± 0.2
D283	–	+	+ + +	–	–
D341	–	+ +	+ + +	–	–

*Symbols: – for no expression; + for moderate expression; + + for strong expression; and + + + for very strong expression.
†Relative fluorescence unit (RFU) was determined by fluorescence densitometric analysis of RT-PCR bands. The RFU values for α2 were divided by those for β-actin and expressed as mean±SD of two experiments performed in duplicate.

We next examined the expression of various IL-13R receptor chains by indirect immunofluorescence assays in the five medulloblastoma cells lines. Immunofluorescence staining for IL-13Rα2, IL-4Rα,

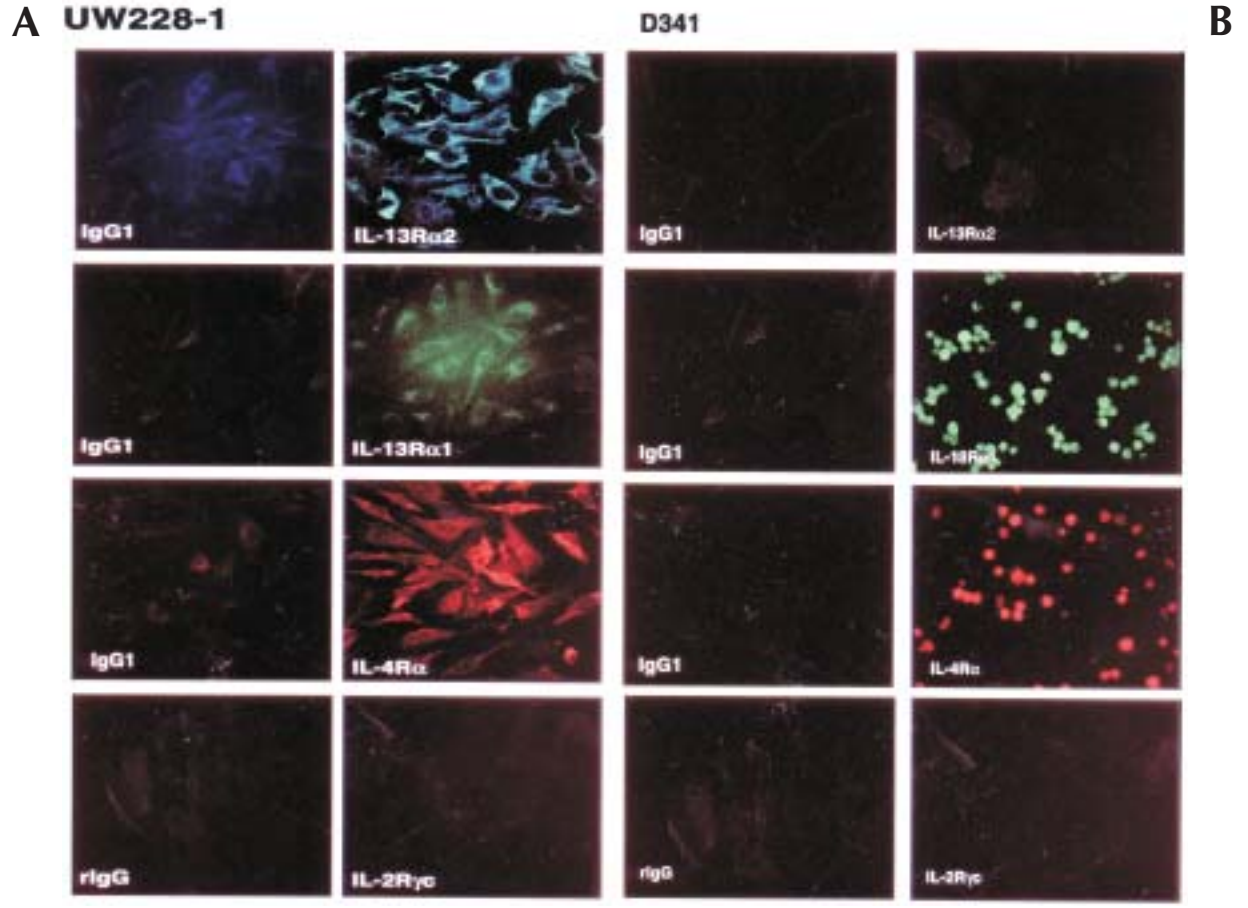

Figure 2. Indirect Immunofluorescence analysis of interleukin-13 receptor (IL-13R) chains on medulloblastoma cell lines. **A.** UW228-1 and **B.** D341medulloblastoma cell lines were stained with either murine IgG1 isotype (IgG1) or rabbit IgG (rIgG) control or mouse monoclonal antibody to IL-13Rα2, IL-13Rα1, and IL-4Rα chain, or rabbit polyclonal antibody to IL-2R γc chains. D341 cell line grew as a suspension culture showing few cells positive in the microscopic field at x400 magnification.

and IL-13Rα1 chains was very intense in the UW 228-1 cell line (Fig. 2A). D283 and D341 cell lines did not show any immunostaining for IL-13Rα2, but stained positively for two other chains, IL-4Rα and IL-13Rα1 (Fig. 2B). The immunofluorescence staining of cells with matched isotype control antibody did not reveal any staining at all. Similar to UW228-1, two other cell lines UW228-2 and UW2 28-3, also showed strong immunoreactivity for IL-13Rα2, IL-4Rα, and IL-13Rα1, confirming our RT-PCR analysis (Fig. 2A). Similar to RT-PCR results, IL-2Rγc protein was also not detected in any of the five-medulloblastoma cell lines tested (Fig. 2A, 2B, and data not shown).

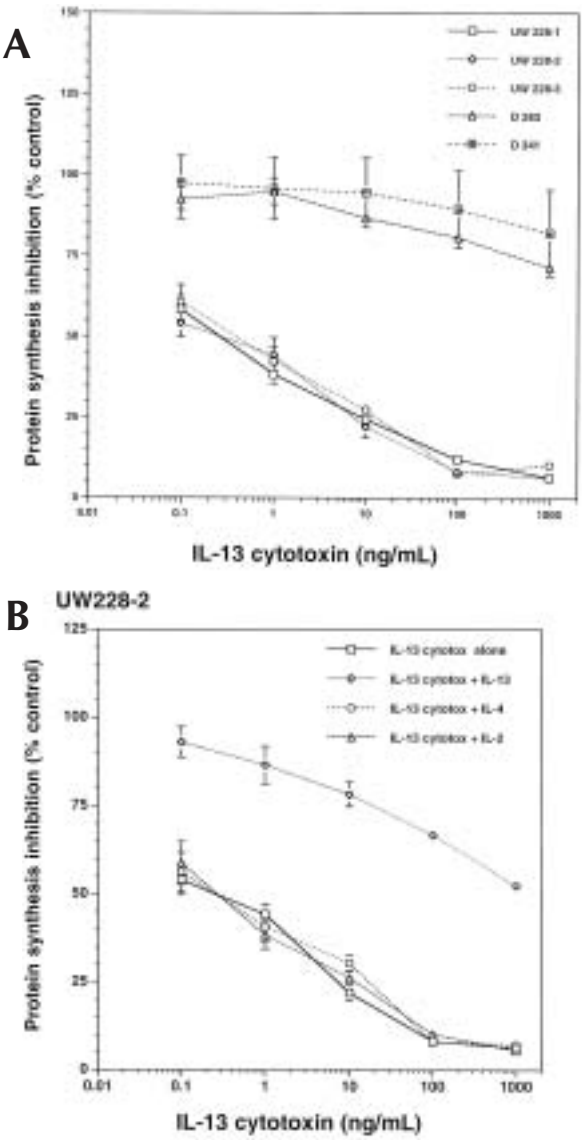

Figure 3. Cytotoxic activity of interleukin-13 (IL-13) cytotoxin against medulloblastoma cell lines. **A.** Ten thousand cells were cultured with various concentrations of IL-13 cytotoxin. Cells were then pulsed with 1 μCi [3H]-Leucine and cell-associated radioactivity was measured with a Betaplate Counter. The results are shown as mean±SD of quadruplicate determinations and the experiment was repeated two times with similar results. **B.** UW-228-2 cells were cultured with various concentrations of IL-13 cytotoxin. For blocking experiments, cells were pre-incubated with IL-2, IL-4, or IL-13 (2 μg/mL) for 30 minutes prior to the addition of IL-13 cytotoxin. Data are shown as mean±SD of quadruplicate determinations.

Cytotoxicity of IL-13 Cytotoxin to Medulloblastoma Cell Lines

Since in vitro sensitivity of tumor cells to IL-13 cytotoxin may correlate with IL-13R expression and *in vivo* antitumor activity, we tested whether these medulloblastoma cell lines were sensitive to IL-13 cytotoxin.

Three of five medulloblastoma cell lines were highly sensitive to the cytotoxic activity of IL-13 cytotoxin (Fig. 3A, Table 2). The IC_{50} was less than 1 ng/mL. On the other hand, two other cell lines (D341 and D283) were not sensitive to IL-13 cytotoxin, even at concentrations up to 1,000 ng/mL. These results indicated that IL-13 cytotoxin was cytotoxic to cells that expressed IL-13Rα2 chain but not to IL-13Rα2 negative medulloblastoma cell lines.

The cytotoxic activity of IL-13 cytotoxin was neutralized by the addition of excess IL-13 in UW228-2 cell line (Fig. 3B and data not shown for UW228-1 and UW228-3). As excess of IL-2 or IL-4 did not neutralize the cytotoxicity of IL-13 cytotoxin, these results

Table 2. Cytotoxic activity of interleukin-13 (IL-13) cytotoxin on human medulloblastoma cell lines

Tumor cells	IC_{50} (ng/mL)*
UW-228-1	0.2±0.012
UW-228-2	0.3±0.01
UW-228-3	0.4±0.03
D283	>1,000
D341	>1,000

*Concentration of IL-13 cytotoxin at which 50% inhibition of protein synthesis is observed compared to untreated cells. The results are shown as mean±SD of 2 independent experiments.

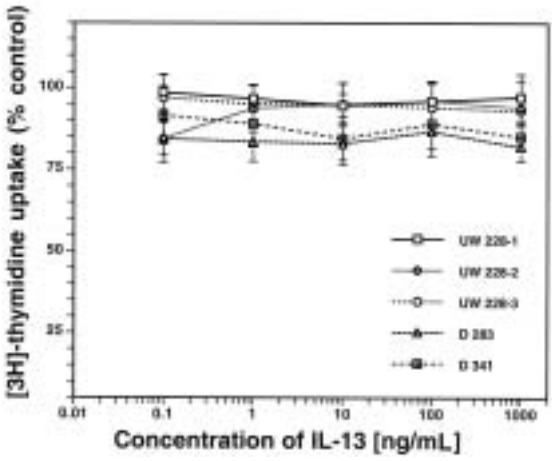

Figure 4. Inerleukin-13 (IL-13) does not stimulate the growth of medulloblastoma cells. $2x10^4$ cells of five medulloblastoma cell lines were grown in the absence or presence of 0.1 ng to 1,000 ng/mL IL-13. The cells were pulsed with 1 μCi of [3H]-thymidine and harvested after 8 h. The incorporated radiolabeled thymidine was measured and shown as percentage of control. Data are shown as mean ± SD of quadruplicate determinations.

indicate that IL-13 cytotoxin mediated cytotoxicity exclusively through IL-13R (Fig. 3B).

Lack of Growth Modulation in Medulloblastoma Cell Lines

To study the function of IL-13R, we next determined whether IL-13 modulated growth of human medulloblastoma cell lines. IL-13 did not alter [3H]-thymidine incorporation in any of the five cell lines, even up to a concentration of 1,000 ng/mL (Fig. 4). These results indicated that IL-13 did not modulate the growth of medulloblastoma cell lines.

Discussion

In this study, we demonstrated that all five human medulloblastoma cell lines examined expressed IL-13R at mRNA and protein levels. However, only three cell lines derived from a single medulloblastoma tumor expressed the primary IL-13R binding protein, IL-13Rα2 chain. Since IL-13Rα1 and IL-4Rα chains are required for IL-4 or IL-13 induced signal transduction (24-28,44,45), our data suggested that medulloblastoma cell lines expressed functional IL-13R. These results also indicated that medulloblastoma cell lines expressed two types of IL-13R: 60% of cell lines (3/5) expressed type I IL-13R, whereas 40% expressed predominantly type II IL-13R. As none of the medulloblastoma cell lines expressed γc chain, no type III IL-13R were observed. Thus IL-13Rα2 chain may define the phenotypic heterogeneity of medulloblastoma tumors.

The structure of IL-13R in medulloblastoma tumor cell lines is similar to IL-13R observed in glioma cells, RCC cells, acquired immunodeficiency syndrome-Kaposi's sarcoma (AIDS-KS) cells, and some head and neck tumor cells (24-26,28-30). However, tumor cells derived from human prostate, breast, pancreas, and colon carcinoma do not seem to express IL-13Rα2 chain (46,47). Thus, the significance of IL-13Rα2 expression in some tumor type is not clear. As the extra cellular domain of IL-13Rα2 chain is present in the serum and urine of mice (48), it was suggested that this extra cellular domain of IL-13R might serve as a carrier protein for IL-13 and modulate its functions in vivo. However, the extra cellular domain of IL-13Rα2 in human plasma and urine samples has not been detected (48). It is also not known if medulloblastoma or other tumor cells that express IL-13Rα2 chain produce the extra cellular domain. It is still possible that the plasma membrane of IL-13Rα2 may serve to modulate immune response in vivo. For example, they may control tumorigenicity and immune surveillance (46). These possibilities are currently being tested in various laboratories.

The expression of IL-13Rα2 chain in medulloblastoma cell lines sensitized them to the cytotoxic effect of IL-13 cytotoxin. Sixty percent of medulloblastoma cell lines that expressed mRNA and protein for IL-13Rα2 chain were very sensitive to the cytotoxic activity of IL-13 cytotoxin. However, the cell lines which did not express IL-13Rα2 chain did not show sensitivity to IL-13 cytotoxin. The difference in the IC50 between IL-13Rα2-positive cell lines and negative cell lines ranged between 2,500-5,000 fold ($\sim$0.2-0.4 ng/mL vs $>$1,000 ng/mL). These results are similar to previous studies in which IL-13Rα2-positive solid human tumor cell lines derived from renal cell carcinoma (38), AIDS-associated Kaposi's Sarcoma (49,50), a third of squamous cell carcinoma of the head and neck (36), and malignant glioma (19,20,51) were highly sensitive to IL-13 cytotoxin. Thus, our current results support previous conclusions and extend the list of IL-13-PE38QQR responsive tumors.

Growth factor receptor-targeted agents have been tested against glioma tumors in vitro and in vivo. As EGFR is overexpressed in up to 50% of malignant glial tumors (8), fusion proteins designed to target these receptors have been found to be highly active in these tumors. Among them, DAB(389)-EGF fusion protein (made of diphtheria toxin and human EGF) and two immunoconjugates comprised of an anti-EGFR monoclonal antibody covalently linked to the type 1 ribosomal inactivating proteins from Saponaria ocymoides and Vaccaria pyramidata have been shown to exert specific inhibition of EGFR expressing target cell proliferation and growth in nude mice (52,53). Similarly, enhanced expression of transferrin receptor in glioblastoma multiforme cells in vitro has been shown to increase sensitivity to DT-Tf fusion toxin compared with normal endothelial cells that also express Tf-receptors (54). Anti-tfnR-CRM 107 was found to be effective in reducing 50% of the tumor volume in 60% of patients with malignant brain tumors (10). Our previous studies have demonstrated that human glioblastoma cell lines express elevated levels of IL-4R, and IL-4R-targeted fusion protein (IL-4PE) has been shown to be highly active in the killing of IL-4R-positive glioma cell lines in vitro or established tumors *in vivo* (17). In two phase I clinical trials, this molecule was well tolerated and mediated anti-tumor activities (55,56). We have also found that IL-4PE is highly cytotoxic to human medulloblastoma cell lines in vitro (34). In addition, malignant glioma cell lines, primary cell cultures, and tumor samples express increased concentration of IL-13R (19,21). These receptors have been shown to render them extremely sensitive to IL-13 fusion cytotoxin in vitro and in various animal models of human disease (19,20, 47). Based on these studies, IL-13 cytotoxin is being tested against recurrent adult glioma tumors in three different clinical trials in the United States, Germany, and Israel (57,62). The sensitivity of medulloblastoma tumor cell lines to IL-13 cytotoxin will generate interest of testing this agent for pediatric tumors.

Finally, to delineate the functional significance of IL-13R on medulloblastoma tumors, we tested the growth modulating activity of IL-13. IL-13 did not seem to modulate proliferation of any of the five medulloblastoma cell lines in vitro. Similarly, in our previous studies, we did not observe any effect of IL-4 on these cell lines even though they expressed all components of the IL-4R (34). These studies suggested that other biological pathways might be activated as a result of IL-13 treatment. Since IL-13 induce a VCAM-1 expression in malignant glioma cell lines (22), it is possible that IL-13 may also induce adhesion

molecules in medulloblastoma cell lines. These studies are currently being pursued in our laboratory.

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