Retargeting Interleukin 13 for Radioimmunodetection and Radioimmunotherapy of Human High-grade Gliomas

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Abstract
A vast majority of patients with glioblastoma multiforme (GBM), a high-grade glioma, overexpress abundant amounts of a receptor for interleukin (IL)-13 in situ. This receptor is more restrictive because it is IL-4-independent and therefore differs from the IL-13/4 signaling receptor of normal tissue that is shared with IL-4. We previously identified one of the sites on the human IL (hIL)-13 molecule that is important for its interaction with the IL-13/4 receptor, a residue of glutamic acid at position 13. In this study, we mutated the cytokine and produced hIL-13.E13Y, in which the glutamic acid was substituted by tyrosine. This additional tyrosine residue was therefore strategically located within the region of IL-13 interaction with the signaling physiological receptor. hIL-13.E13Y did not transduce signals through the IL-13/4 receptor, whereas its interaction with the more restrictive, GBM-associated receptor remained intact. The mutated hIL-13 could be readily radiolabeled. Radiolabeled hIL-13.E13Y produced specific autoradiographic images of human GBM specimens. We demonstrate an effective way to redirect hIL-13 to its more restrictive receptor found in high-grade gliomas by mutagenizing the cytokine, and, concomitantly, we equipped hIL-13 with an additional tyrosine residue for higher specificity of radiolabeling.

Introduction
Maria Sklodowska-Curie discovered radium 100 years ago. This event enabled the treatment of cancer patients in a different and often efficacious way. A new field of designer antitumor drugs is intended to deliver cytotoxic energy specifically to cancer cells. Much work in the field is described in this volume of Clinical Cancer Research. Clinical evaluation of radiolabeled antibodies that recognize tumor-associated antigens in the treatment of cancer is well under way (1). Radiotherapy and surgery or combinations of the two represent the first line of treatment for high-grade gliomas. GBM is a high-grade glioma for which no curative treatment is available, and the incidence of brain tumors is on the rise (2, 3). We recently found large amounts of a receptor for IL-13 on a majority of the studied established human high-grade glioma cell lines (4) and cells cultured from freshly resected GBM (5). IL-13 regulates immune responses in a manner similar to that of its homologue, IL-4 (6, 7), and shares a functional IL-13/4 receptor that is present on selected normal tissues and overexpressed on some adenocarcinomas (8-10).

The homology between IL-13 and IL-4 was the sole reason to produce cytotoxic agents based on IL-13, because it had previously been shown that the IL-4 receptor is present in a wide range of malignancies and has the potential to be pharmacologically tractable for antitumor therapies under certain conditions (11, 12). Surprisingly, the receptor for IL-13 in human glioma cells is not shared with IL-4, because IL-4 did not compete for the IL-13 binding sites (4, 5, 13, 14). More recently, we discovered an extraordinarily rich and frequent presence of the IL-13 receptor in human GBM but not in normal brain in situ. In fact, all necrotic sections of 23 GBM tissue samples were positive for the IL-13 receptor. The GBM-associated receptor for IL-13 detected in situ is also IL-4 independent. Based on structural and functional homology to IL-4 (Fig. 1), we mutagenized IL-13 to make hIL-13.E13K and found that this mutant lost its interaction with the signaling IL-13/4 receptor but retained all of the reactivity with the GBM-associated IL-4-independent receptor (13). This demonstrated the feasibility of retargeting the molecule of IL-13 from its physiological receptor, thus making it a new, more specific vehicle for the delivery of anti-GBM drug candidates.

hIL-13 has one tyrosine residue available for radiolabeling with 125I or 131I-Na. Because we had identified one of the regions in the molecule of hIL-13 that is important for the interaction with its receptor shared with IL-4 (13), we attempted to make IL-13 mutants carrying more bulky tyrosine residues in this particular region of the molecule. This would allow higher specific radiolabeling and the use of such compounds for the diagnosis, imaging, and treatment of GBM.

Materials and Methods
Materials. Restriction endonucleases and DNA ligase were obtained from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), and

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3 The abbreviations used are: GBM, glioblastoma multiforme; CTX, cytotoxin; IL, interleukin; hIL, human IL; MTS/PMS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate.

Clinical Cancer Research 3143s
Boehringer Mannheim (Indianapolis, IN). Fast protein liquid chromatography columns and media were purchased from Pharmacia (Piscataway, NJ). Oligonucleotide primers were synthesized at the Macromolecular Core Laboratory at the Penn State College of Medicine. The PCR kit was from Perkin-Elmer Corp. (Norwalk, CT). The kit for mutagenesis was obtained from Boehringer Mannheim (Indianapolis, IN). Procedures for recombinant proteins and IL-4 ending at the first α-helix, α-helix A. Amino acids that form α-helix A are underlined. The conserved residue of glutamic acid (E) is in bold.

Production and Purification of Recombinant Proteins.

E. coli BL21 (DE3) cells were transformed with pET-19b (DE3) cells were transformed with plasmids of interest and cultured in Terrific Broth (Difco Laboratories, Detroit, MI). Procedures for recombinant proteins and purification have been described previously (4, 11).

Mutagenesis.

Mutations of the IL-13 gene were made using a unique site elimination mutagenesis procedure developed by Deng and Nickoloff (15). The sequence of the mutagenizing primer was 5'-TTTGTGTGTCATATGTCCCCAG-GCCCTGTGCCCTCCCTCTACAGCCCTCAGGTACCTCAT-TGAGGAG-3'.

Autoradiography. Recombinant hIL-13.E13Y was labeled with 125I by using Iodo-Gen reagent (Pierce) according to the manufacturer’s instructions. The specific activity of 125I-hIL-13.E13Y was ~300 μCi/μg protein. All studies involving human specimens were approved by the respective Human Subjects Protection Offices at the Penn State College of Medicine (Protocol No. IRB 96-123EP).

Serial tissue sections were cut (10 μm) on a cryostat, thaw-mounted on chrome-alum coated slides, and stored at 4°C until analyzed. To observe the binding distribution of 125I-hIL-13.E13Y, sections were incubated (1 h, 22°C) with 1.0 nm labeled IL in binding buffer (200 mM sucrose, 50 mM HEPES, 1% BSA, and 10 mM EDTA). Adjacent serial sections were incubated with radiolabeled recombinant hIL-13.E13Y after a 30-min preincubation at 22°C in the presence of binding buffer alone; a 100–500-fold molar excess of unlabeled hIL-13, hIL-13.E13Y, or hIL-4; or a monoclonal antibody against human transferrin receptor. To dissociate nonspecifically bound radioligand, sections were rinsed in four consecutive changes (5 min each) of ice-cold 0.1 M PBS. At least two sections of each of the tissue specimens were assayed for the evaluation of 125I-hIL-13.E13Y binding specificity. After drying, the labeled sections were exposed to Kodak autoradiography film at −65°C for 8 h to 11 days.

Cell Proliferation Assay.

Cell killing by cytotoxins was tested as follows: usually, 5 × 10^4 cells/well were plated in a 96-well tissue culture plate in 150 μl of media. Various concentrations of cytotoxins were diluted in 0.1% BSA/PBS, and 25 μl of each dilution were added to cells 18–24 h after cell plating. Cells were incubated at 37°C for another 48 h. The cytotoxicity was then determined using a colorimetric MTS/PMS cell proliferation assay. MTS/PMS was added at a half final concentration as recommended by the manufacturer. The cells were incubated with the dye for 4 h, and then the absorbance was measured at 490 nm for each well using a microplate reader (Cambridge Technology, Inc., Watertown, MA). The wells containing cells treated with cycloheximide (10 mM) or wells with no viable cells left served as a background for the assay. For blocking studies, ILs at a concentration of 1.0 μg/ml were added to cells for 60 min before the addition of cytotoxins.

Cell proliferation studies using TF-1 cells were performed essentially as described previously (13). TF-1 cells are human promyelocytic erythrophagocytic leukemic cells, which do express the shared IL-13/4 receptor (10). Maximal proliferative activities of the ILs were obtained at a concentration of 100 ng/ml. The values were expressed as the difference between the background and the maximal MTS conversion that was recorded at A_{590 nm}. Data were obtained from the average of triplicates, and the assays were repeated several times.

Results

To determine whether the E13Y mutation in hIL-13 alters its reactivity with the shared IL-13/4 receptor, we measured proliferative responses to ILs in TF-1 cells. We treated TF-1 cells with hIL-13, hIL-13.E13Y, and hIL-13.E13K (Fig. 2). As expected, hIL-13 was very potent in stimulating the growth of TF-1 cells (Fig. 2). In contrast, hIL-13.E13Y was not active on those cells, and hIL-13.E13K did exhibit very weak proliferative activity, as previously observed (13).

To demonstrate the ability of hIL-13.E13Y to compete for the hIL-13 binding sites in clinical specimens of GBM in situ, we performed autoradiographic studies (Fig. 3). The two GBM tissues studied labeled densely for 125I-hIL-13.E13Y binding sites (Fig. 3), as they did previously for the labeled wild-type hIL-13.4 The binding was specific because both hIL-13.E13Y...
Fig. 3 Autoradiographic demonstration of the binding sites for $^{125}$I-hIL-13.E13Y on human GBM and normal human endothelial cells (HUVEC). Films were exposed for 3 days. Tf, transferrin.

We previously failed to show any significant and specific interaction of $^{125}$I-hIL-13 with normal brain or normal human cells. We found that $^{125}$I-hIL-13.E13Y does not interact with normal human cells, such as human umbilical vein endothelial cells, because it did not label those cells in vitro (Fig. 3). Thus, the binding of hIL-13.E13Y to GBM is very specific.

In another test of hIL-13.E13Y binding specificity to the GBM-associated receptor for IL-13, we analyzed the blocking activity of the mutant on the action of the hIL-13-based cytotoxin (5) on two different human malignant glioma cell lines (Fig. 4). We pretreated glioma cells with either hIL-13, hIL-13.E13Y, or hIL-13.E13K before the addition of the cytotoxic fusion protein (Fig. 4). We found previously that the cytotoxic action of hIL-13-CTX is hIL-13 receptor specific because an excess of hIL-13 on all tested glioma cells blocked it (5), and we reproduced this effect (Fig. 4). Importantly, hIL-13.E13Y neutralized the cytotoxicity of hIL-13-CTX in a manner similar to that of hIL-13 and its hIL-13.E13K mutant (Fig. 4).

Discussion

The main finding of this study is that a mutant of hIL-13, hIL-13.E13Y, lost its ability to interact with the signaling shared IL-13/4 receptor of normal tissue, whereas its affinity toward the more restrictive, GBM-associated hIL-13 receptor remained unchanged. This preserved affinity toward the GBM-associated receptor was detected on the basis of the neutralizing potency of the killing activity of a hIL-13 cytotoxin, as well as autoradiographic studies on GBM sections using radiolabeled hIL-13.E13Y in situ. At the same time, hIL-13.E13Y spares the IL-13/4 receptor and becomes a nonsignaling molecule through its physiological receptor. These data indicate an effective way to amplify the targeting specificity of a tumor-associated receptor.

The GBM-associated receptor for hIL-13 is frequently overexpressed with negligible, if any, binding of hIL-13 to normal brain using autoradiography (4, 5). However, an analysis of gene expression suggests that discrete regions of the brain possess elements of the shared signaling IL-13/4 receptor (5). Therefore, very low levels of the signaling IL-13/4 receptor proteins may be present in the central nervous system. It would thus be beneficial to deprive hIL-13 of any ability to

\[ A_{\text{act}} = \frac{100}{1 + \left( \frac{[\text{IL13-CTX}]}{K_d} \right)^n} \]

\[ [\text{IL13-CTX}], \text{ng/ml} \]

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Tyrosine-enriched IL-13 Specific for Glioma Cells

![Diagram of receptor signaling](image)

**NORMAL CELLS**

**CANCER CELLS**

A - IL4Rβ (p140)  
B - IL13Rα' (p50)  
C - IL4-independent IL13R

**Fig. 5** Schematic model of hIL-13.E13Y dissociation from the IL-13/4 receptor and its preservation of an affinity toward the GBM-associated IL-13 receptor. A, Mr 140,000 IL-4 receptor β-chain. B, Mr 45,000 IL-13 receptor α'-chain. A and B are the principal elements of the heterodimeric high-affinity IL-13/4 receptor. C, monomeric, GBM-associated, IL-4-independent IL-13 receptor (the primary structure of this receptor has not yet been documented). Y, additional tyrosine in hIL-13.

In summary, it is possible to divert the molecule of hIL-13 from its physiological receptor and make it a nonsignaling compound, whereas its affinity toward the GBM-associated receptor remains intact. The utility of hIL-13.E13Y described here and of other similarly designed molecules for the purpose of radioimmunodetection and radioimmunotherapy of GBM is under extensive evaluation in our laboratory.

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**References**


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