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ORIGINAL ARTICLE

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Interleukin-13 sensitivity and receptor phenotypes of human glial cell lines: non-neoplastic glia and low-grade astrocytoma differ from malignant glioma

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Abstract Many of the actions and receptor components of interleukin-13 (IL-13), a pleiotrophic cytokine with immunotherapeutic potential, are shared with IL-4. Because human low-grade astrocytoma cells express IL-4 receptors and their growth is arrested by IL-4, we speculated that IL-13 sensitivity and receptor expression might also be present. The purpose of the current study was to investigate IL-13 receptor components and sensitivity in a series of glial cell lines derived from adult human non-neoplastic cerebral cortex, low-grade astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme. Unlike peripheral blood lymphocytes (PBL), glial cells did not express IL-2 receptor γ chain. IL-13 receptor α-1 (IL-13Rα1), however, was present in 11/13 glial lines and PBL. Deficient cell lines were all glioblastoma-derived. All anaplastic astrocytoma and glioblastoma but not other glial lines or PBL expressed IL-13 receptor α-2 (IL-13Rα2). In non-neoplastic glia, low-grade, and anaplastic astrocytoma, IL-13 decreased DNA synthesis, an effect reversible with antibody to IL-4Rα. Results indicate that low-grade astrocytoma cells resemble non-neoplastic glia in terms of IL-13 sensitivity and IL-4Rα/IL-13Rα1 receptor profile but alterations occur with malignant progression. Glioblastoma cells were uniformly insensitive to IL-13 and, unlike other glia, failed to phosphorylate STAT6 after IL-13 challenge. Data suggest that IL-13 and analysis of IL-13 receptors may have clinical application in glial tumors.

Key words Interleukin 13 · Astrocytoma · Receptors · Astrocytes · STAT6

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Introduction

We reported previously that interleukin-4 (IL-4) mediates growth arrest at the G1/S interface in human astroglial cell lines derived from non-neoplastic adult cortex and low-grade neoplasia (astrocytoma) [2, 10]. More recent data indicate that IL-4 achieves this effect by utilizing a dual-inhibitor mechanism in which both p21 (waf1/cip1) and p27 (kip1) are essential to the cell-cycle blockade [15, 16]. The entire process has been shown to depend upon astrocyte expression of the IL-4 receptor chain (IL-4Rα) [2].

IL-4Rα, however, represents only a portion of the IL-4 receptor complex, which contains other cytokine-binding polypeptides as well [19, 24]. The phenotype of the IL-4 receptor complex appears to vary in a cell-type-specific fashion. In lymphocytes, the interleukin-2 (IL-2R) γ chain is dominant and forms a heterodimer with IL-4Rα [18, 21]. In many other cell types, IL-4Rα associates with IL-13-binding components in place of IL-2R γ chain [12–14, 20]. At least two different IL-13-binding components have been cloned and designated as IL-13 receptor α-1 (IL-13Rα1) and α-2 (IL-13Rα2) [1, 3, 12, 13, 17].

Because IL-13 and IL-4 share receptor components, many of the actions of IL-13 closely resemble those of IL-4. Both cytokines stimulate IgE production in B lymphocytes and both reduce production of pro-inflammatory cytokines by macrophages [25]. Currently there are no data on IL-4/IL-13 receptor phenotypes, IL-13 signaling or IL-13 sensitivity of adult human non-neoplastic glia or low-grade astrocytoma cells. In highly malignant glioblastoma multiforme lines and tissues, an

unusual IL-13-binding component has been described and IL-13 sensitivity does not appear to be present [4–8]. The purpose of the current study was to profile IL-13 sensitivity and receptor expression in a series of adult human glial cell lines derived from non-neoplastic cortex, low-grade astrocytoma, anaplastic astrocytoma and glioblastoma multiforme. Data suggest that expression of IL-13Rα1 and IL-4Rα together with IL-13 sensitivity characterize non-neoplastic astroglia and persist in low-grade astrocytoma. With malignant progression, however, this "normal" glial profile gradually becomes altered, reflecting loss of IL-13 sensitivity, sporadic loss of IL-13Rα1 or IL-4Rα, and acquisition of IL-13Rα2 expression. These findings suggest that IL-13 may have immunotherapeutic potential and that IL-13 receptor profiles may have clinical application for glial tumor evaluation.

Materials and methods

Reagents and cell cultures

Recombinant human IL-4 was a gift from Dr. Satwant Narula, Schering-Plough Research Institute (Kenilworth, N.J.). Recombinant human IL-13 was obtained from R & D (Minneapolis, Minn.) and epidermal growth factor (EGF) from Gibco-BRL (Grand Island, N.Y.). Antibodies specific for STAT6 and phosphotyrosine were purchased from Santa Cruz (Santa Cruz, Calif.). Non-neoplastic astrocytic cultures (P2N, G4N and T5N) were established from resected temporal lobe tissue of patients with intractable epilepsy by methods previously described [2]. Cultures derived from low-grade astrocytoma (RTLGA and FRLGA) and glioblastoma multiforme (WITG3, 9C, CRTG2, RUTG4 and STTG1) have been described [2]. Similar procedures were used to establish cultures ALA1, YTA2, and YWA3 from anaplastic astrocytoma tissues. Both non-neoplastic astrocytic lines and low-grade astrocytoma lines were non-immortal and cryogenically preserved aliquots of primary passage were used for study. Peripheral blood lymphocytes (PBL) were prepared and stimulated with mitogen as previously described [2].

DNA synthesis and proliferation

DNA synthesis was determined by incorporation of 5-bromo-2′-deoxyuridine (BrdU) in a colorimetric assay according to the manufacturer's (Boehringer-Mannheim, Indianapolis, Ind.) instructions. Glial cells (5000/well) were cultured in microtiter plates in RPMI-1640 medium as previously described [15]. Cultures were exposed to EGF (10 ng/ml), IL-13 (2.5–25 ng/ml) or a combination thereof for 24 h. Some experiments used IL-4 (10 ng/ml). BrdU was included during the last 4 h of culture and the absorbance (A_{450}) of lysates was determined by enzyme-linked immunosorbent assay reader. Proliferation assays were carried out as previously described and the absorbance (A_{550}) was determined [10]. Data were analyzed by the Tukey-Kramer multiple-comparisons test using Graph Pad software as previously described [15].

Immunoprecipitation and immunoblotting

For STAT6 immunoprecipitation, cells were challenged with EGF, IL-13 (10 ng/ml), or IL-4 (10 ng/ml) for 5 min, washed and lysed in a modified NP-40 lysis buffer as previously described [16]. Lysates (500 μl) were incubated with 20 μl antibody against STAT6 for 1 h on ice prior to admixture with protein-A–Sepharose beads (3 mg/tube in 30 μl) (50% v/v) for 1 h at 4 °C. The beads were centrifuged and washed, and immunoprecipitated samples were solubilized as described [15]. Samples were electrophoresed through 10% sodium dodecyl sulfate/polyacrylamide gels, and proteins were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, Calif.) for immunoblotting. Blots were revealed by chemiluminescence after exposure of the membranes to Hyperfilm (Amersham) for 0.5–5 min [15].

RNA analysis

Total cellular RNA (0.5 μg) was diluted and transcribed (42 °C for 15 min, 99 °C for 5 min, 5 °C for 5 min) using reverse transcription/polymerase chain reaction (RT-PCR; Perkin Elmer-Cetus, Norwalk, Conn.) as described [2]. Primers were specific for human IL-13Rα1 (0.52 kb) [1], IL-13Rα2 (0.48 kb) [7], IL-4Rα (0.53 kb) (Clontech, Palo Alto, Calif.), IL-2Rγc (0.56 kb) (Clontech), and β-actin (0.84 kb) (Clontech). Products were amplified by PCR using 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and primer extension at 72 °C for 2 min. Negative controls consisted of primers without RNA. PCR product sizes were determined by comparison with molecular mass markers after electrophoresis in 2% agarose gels and ethidium bromide staining.

Southern blotting

PCR products were analyzed by Southern Blotting [22]. cDNA probes used consisted of: IL-2R γ chain and IL-13Rα1 (provided by Dr. Warren J. Leonard, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Md.), IL-13Rα2 (provided by Dr. Patrick Laurent, Sanofi Recherche, Labege Cedex, France), IL-4Rα (provided by Dr. Karlheinz Friedrich, The Theodor-Boveri-Institut fur Biowissenschaften (Biozentrum), Universitat Wurzburg, Wurzburg, Germany), and β-actin [2].

Results

Expression of IL-4/IL-13 receptor components

Southern blotting of RT-PCR products indicated that IL-13Rα1 was detectable in 3/3 non-neoplastic glial lines, 2/2 low-grade astrocytoma and 3/5 glioblastoma lines (Fig. 1A). IL-13Rα2 was selectively expressed in all five glioblastoma lines but not in low-grade astrocytoma (0/2) or in non-neoplastic glia (0/3) (Fig. 1A). Because of the marked disparity in IL-13Rα2 expression between low-grade astrocytoma and glioblastoma, it was decided to investigate anaplastic astrocytoma, which is histologically intermediate between the two. All anaplastic astrocytoma lines examined (3/3) expressed both IL-13Rα2 and IL-13Rα1 (Fig. 1B). IL-2R γ chain was not detectable in any glial line (0/13) but was strongly expressed, as anticipated, by mitogen-stimulated PBL. PBL also expressed IL-4Rα and IL-13Rα1, but not IL-13Rα2. Almost all glial lines (with the exception of glioblastoma 9C) expressed IL-4Rα.

Biological effects of IL-13

IL-13 treatment of non-neoplastic astrocytes or low-grade astrocytoma cells significantly reduced growth-factor(EGF)-stimulated BrdU incorporation in a dose-dependent manner with 2.5–5.0 ng/ml providing

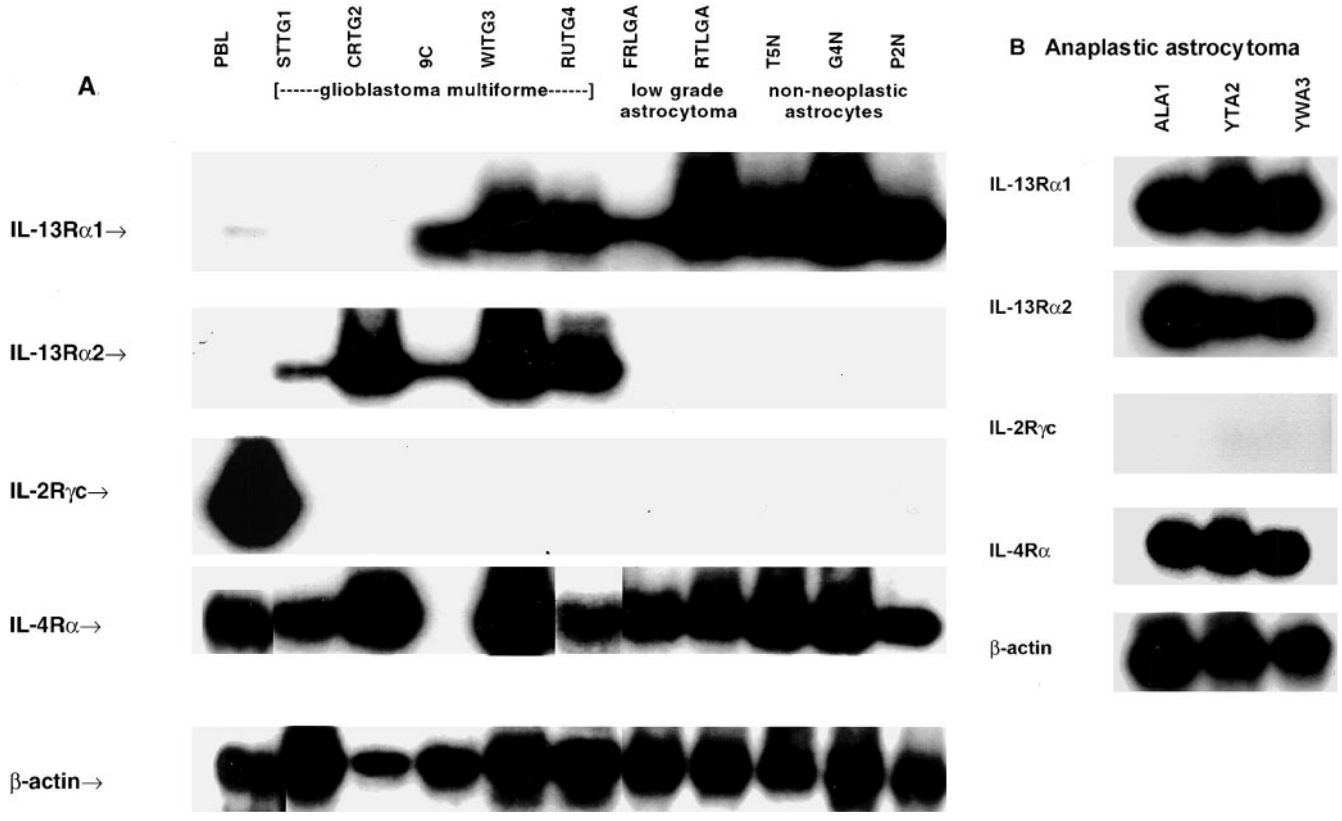

Fig. 1A, B Distribution of interleukin-13 (*IL-13*) receptor (*R*) expression in glial cell lines. Southern blot analysis of RT-PCR products from glial cell lines and peripheral blood lymphocytes (*PBL*) using cDNA probes to IL-13Rα1, IL-13Rα2, IL-2R γ chain (*IL-2Rγc*), IL-4Rα, and β-actin. **A** Non-neoplastic astroglial lines (P2N, T4N, G5N), low-grade astrocytoma (RTLGA, FRLGA), glioblastoma multiforme (WITG3, 9C, RUTG4, CRTG1, STTG1). **B** Anaplastic astrocytoma lines (ALA1, YTA2, YWA3)

maximal reduction (Fig. 2A). This inhibitory effect of IL-13 was reversed by concurrent treatment with antibody to IL-4Rα but not by irrelevant IgG, indicating that expression of IL-4Rα was essential for IL-13 action (Fig. 2B). Antibody to IL-2R γ chain had no effect (data not shown). Overall, sensitivity to IL-13-mediated reduction of BrdU incorporation was present in 3/3 non-neoplastic glia, 2/2 low-grade astrocytoma, 2/3 anaplastic astrocytoma and 0/5 glioblastoma lines (Fig. 2C). BrdU incorporation in glioblastoma cells remained unaffected by IL-13 at concentrations up to 25 ng/ml, as noted previously [4, 5]. RTLGA low-grade astrocytoma cells were also evaluated for the effect of IL-13 on proliferation of 7-day cultures. Proliferation of cultures containing EGF (10 ng/ml) together with IL-13 (10 ng/ml) was significantly less (21%, $P < 0.01$) than that of EGF alone.

IL-13 signal transduction

To investigate whether lack of IL-13 sensitivity in glioblastoma cells could be related to anomalies in signal transduction, STAT6 phosphorylation was examined. Cells were challenged separately with IL-13 and IL-4 to determine whether responses differed. In all non-neoplastic glia (P2N, T5N), low-grade astrocytoma (RTLGA), and anaplastic astrocytoma (ALA1, YTA2, YWA3) lines studied, STAT6 phosphorylation was observed after challenge with either IL-13 or IL-4 (Fig. 3). In contrast, 0/4 glioblastoma (STTG1, CRTG2, WITG3, 9C) lines showed evidence of STAT6 phosphorylation after IL-13 challenge although constitutive STAT6 activation was apparent in 9C (Fig. 3). In glioblastoma lines, IL-4 elicited STAT6 activation only in WITG3 (data not shown), as reported previously [16]. The presence of EGF did not interfere with STAT6 activation by either IL-13 or IL-4 (Fig. 3).

Discussion

These data suggest that low-grade astrocytoma cells resemble non-neoplastic astroglia in terms of IL-13 sensitivity and IL-4/IL-13 receptor profile (summarized in Table 1). The findings also imply that the receptor phenotype of non-neoplastic astroglia and low-grade astrocytoma cells resembles that of endothelial cells and other non-hematopoietic cells, which utilize a receptor complex composed of IL-4Rα and IL-13Rα1, but not the IL-2R γ chain subunit [14, 20]. With the acquisition of malignant status, as illustrated by anaplastic astrocytoma, the receptor phenotype appears to become altered to include new expression of IL-13Rα2. This new

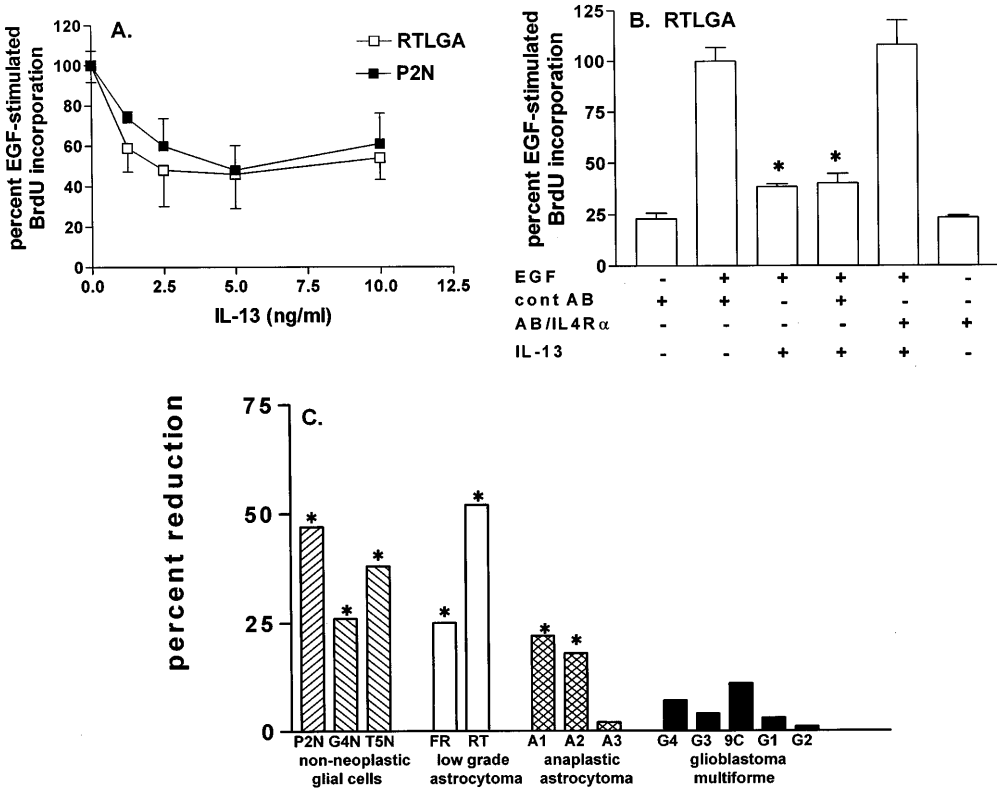

Fig. 2A–C IL-13 reduction of BrdU incorporation. DNA synthesis of untreated and epidermal-growth-factor(*EGF*)-stimulated glial cell lines with and without IL-13 exposure was analyzed by BrdU incorporation and measurement of absorbance at 450 nm. **A** Dose/response study of effects of IL-13 on EGF-stimulated BrdU incorporation of non-neoplastic astroglia (P2N) and low-grade astrocytoma (RTLGA) cells. **B** Antibody to IL-4Rα (AB/IL4Rα) but not irrelevant IgG (*cont AB*) abrogates IL-13 reduction of EGF stimulation in RTLGA cells. * Significant reduction by IL-13 compared to EGF alone ($P < 0.01$). **C** Percentage reduction of BrdU incorporation in EGF-stimulated glial cell lines by IL-13 exposure. * Significant reduction by IL-13 compared to EGF alone ($P < 0.05$)

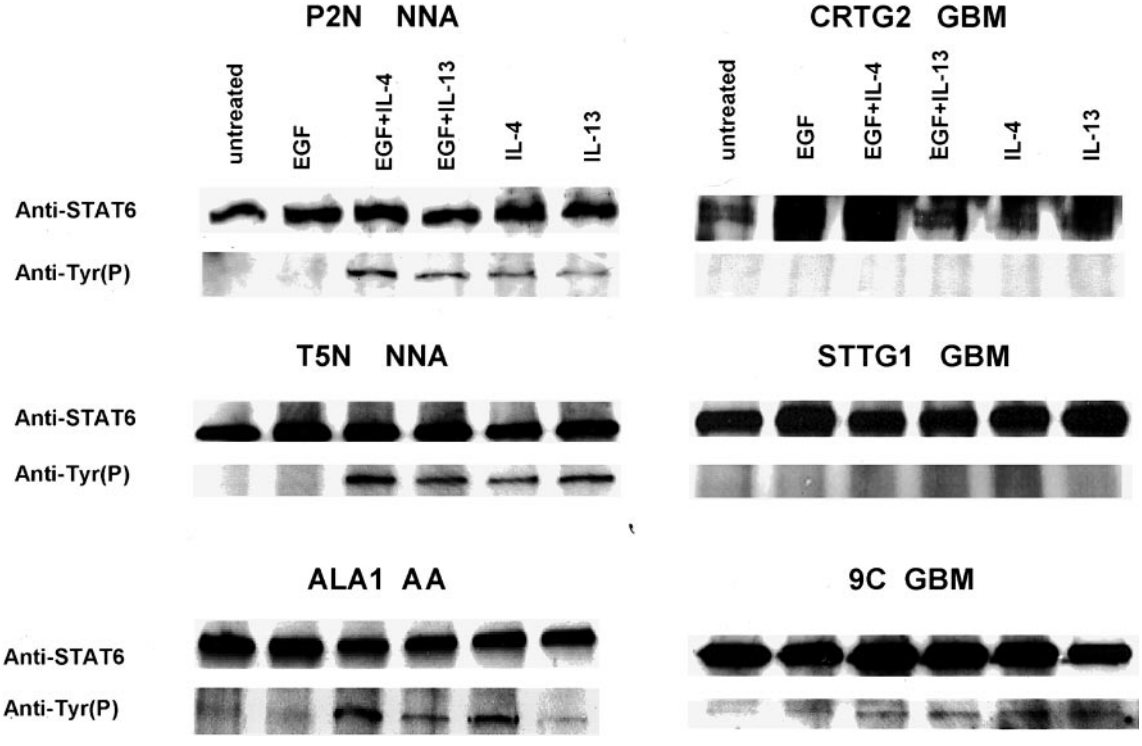

Fig. 3 STAT6 phosphorylation. Glial cells were treated as shown for 5 min. Solubilized STAT6 immunoprecipates were immunoblotted with antibodies to phosphotyrosine [*Anti-Tyr(P)*] and STAT6. Representative results from non-neoplastic astrocytes (P2N, T5N NNA), anaplastic astrocytoma (ALA1 AA) and glioblastoma (CRTG2, STTG1, 9C GBM) are shown

Table 1 Interleukin-13(IL-13)-related profiles of human glial cell lines

Histopathological diagnosis of tissue from which cell line was derived	IL-4/IL-13 receptor component expressed			Sensitivity to IL-13 action	
	IL-13Rα1	IL-4Rα	IL-13Rα2	Reduction of DNA synthesis	Activation of STAT6
Non-neoplastic cortex[a]	3/3	3/3	0/3	3/3	2/2
Low-grade astrocytoma	2/2	2/2	0/2	2/2	1/1
Anaplastic astrocytoma	3/3	3/3	3/3	2/3	3/3
Glioblastoma	3/5	4/5	5/5	0/5	0/4

[a] Resected for treatment of intractible epilepsy

marker persisted in all of the glioblastoma lines examined whereas the IL-4Rα or IL-13Rα1 components were occasionally lost (summarized in Table 1). These data are intriguing because they suggest that acquisition of malignancy in glial cells somehow activates pathways regulating IL-13Rα2 expression. Mechanisms responsible for such regulation are currently unknown.

Activation of the STAT6 pathway has been shown to be important to signal transduction by both IL-13 and IL-4 [18, 23, 25]. Current results indicate that, overall, STAT6 activation correlates with glial sensitivity to IL-13-mediated growth arrest. Strikingly, glioblastoma cells were uniformly insensitive and failed to show STAT6 activation after IL-13 challenge. IL-13 had no effect on STAT6 even in the WITG3 glioblastoma line, which activated STAT6 in response to IL-4 [16]. These results suggest that glioblastoma cells, even those with functional IL-4 signalling, may have a specific deficiency in IL-13 signal pathways. STAT6 activation in response to IL-13, however, may not be sufficient to confer IL-13 sensitivity, as illustrated by the YWA3 anaplastic astrocytoma line where IL-13-mediated growth arrest was absent despite STAT6 activation. Similar findings were observed previously in studies of IL-4 effects on cell cycling in low-grade astrocytoma and glioblastoma [16]. The WITG3 glioblastoma line was not growth-arrested by IL-4 although IL-4 challenge elicited STAT6 activation [16]. Sensitivity to IL-4-mediated growth arrest was shown to require up-regulation of p21 (waf1/cip1) which occurred in cells with wild-type p53 but not in WITG3, which carries mutated, non-functional p53 [16]. The possible roles of p53 and p21 (waf1/cip1) in the actions of IL-13 have yet to be addressed.

IL-13Rα2 receptors have been shown to bind IL-13 with higher affinity than IL-13Rα1 but not to transduce signals for STAT6 activation [11]. This has led to speculation that IL-13Rα2 may act as an inhibitory or "decoy" silent receptor [11]. In our studies, all glioblastoma cells exhibited IL-13Rα2 and were refractory to the biological effects of IL-13, even those expressing IL-13Rα1 and IL-4Rα. All anaplastic astrocytoma cells, however, responded to IL-13 with STAT6 activation, and 2/3 lines showed significant growth inhibition even though all expressed IL-13Rα2 in addition to IL-13Rα1 and IL-4Rα. These results suggest that anaplastic astrocytoma lines may contain fewer cells expressing IL-13Rα2 and/or a lower density of IL-13Rα2 receptors than glioblastoma lines. Our studies clearly showed, however, that glial sensitivity to IL-13 depended upon expression of IL-4Rα as reported for other cell types [14, 26].

Malignant glioma cells and glioblastoma tissues have been found to display large numbers of high-affinity binding sites for radiolabelled IL-13 [4, 7, 8]. These binding sites have been shown to differ from those present on normal cell types in that the glioma sites are not blocked by IL-4 [5]. Although the molecular nature of these binding sites has not been defined, the high concentration of unusual IL-13 binding sites in malignant glioma has led to the development of IL-13-conjugated toxins for targeting such cells [4, 6]. Our molecular data demonstrating distinctly different IL-4/IL-13 receptor phenotypes in non-neoplastic compared to malignant glial cells may lend support to this therapeutic approach. It should be noted, however, that IL-13Rα2 expression has been detected in non-neoplastic cells, for example fibroblasts derived from synovial and lung tissues [9, 11]. Currently, there are no data on IL-13Rα2 expression in other types of non-neoplastic CNS cells.

In summary, our data suggest that IL-13 and IL-4/IL-13 receptor analysis may have some clinical relevance for glial tumors. It should be noted, however, that IL-13, like IL-4, exhibited anti-mitogenic properties primarily against non-neoplastic astrocytes and low-grade astrocytoma rather than malignant glioma. Such results may imply the existence of pathways regulating the cell cycle of normal astrocytes that are inactivated with malignant progression. The putative physiological roles of IL-13 and IL-4 in the CNS have yet to be defined.

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