

 Advances in Brief

In Situ Expression of Interleukin-4 (IL-4) Receptors in Human Brain Tumors and Cytotoxicity of a Recombinant IL-4 Cytotoxin in Primary Glioblastoma Cell Cultures

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Abstract

We have reported that human malignant glioma cell lines express high levels of plasma membrane interleukin-4 receptors (IL-4R). We have also reported that biopsy/surgical samples or primary explant cell cultures from brain tumors express mRNA and protein for the IL-4Rα chain, a primary IL-4-binding protein. However, whether IL-4R are expressed in brain tumors in situ has not been resolved. In addition, expression of IL-4R on the cell surface of various normal brain tissues is not known. We examined the expression of IL-4R by using a monoclonal antibody to the IL-4Rα chain (also known as IL-4Rβ) in surgical/biopsy samples of brain tumor tissues by immunohistochemistry. Our data indicate that 15 of 18 glioblastoma multiforme (GBMs) tumors obtained from two different institutions and 12 other brain tumor samples are moderately to intensely positive for IL-4Rα. In contrast, although IL-4Rα mRNA was expressed, no IL-4R protein was detectable in two adult and one pediatric brain tissue specimens. In addition, a commercially available human neural tissue grid containing fixed tissues from various areas of brain showed no positive staining for the IL-4Rα chain. IL-4Rα expression was also demonstrated in astrocytomas grades I, II, and III. Because IL-4 cytotoxin comprised of a circularly permuted IL-4 and a mutated form of Pseudomonas exotoxin [termed IL4-(38-37)-PE38KDEL; Refs. 7, 8]. This cytotoxin (cpiL4-PE) is highly and specifically cytotoxic to brain tumor cells in vitro and in vivo (9, 10). In immunodeficient xenograft models of human GBM tumors, IL-4 cytotoxin has been shown to mediate remarkable antitumor activity (10). In addition, complete regression of established human tumors, including malignant glioma, has been demonstrated at the maximum tolerated dose (10–12). On the basis of preclinical studies, we initiated a Phase I clinical trial using IL-4 cytotoxin for the therapy of recurrent malignant glioma. Our initial results suggest that IL-4-cytotoxin can mediate extensive necrosis of the glioma without much evidence of significant toxicity to normal adjoining brain tissues (13). Currently, additional patients are being treated with this novel agent to determine the maximum tolerated dose for a Phase II clinical trial for efficacy.

Introduction

Previously we have identified overexpression of high-affinity receptors for immune regulatory cytokine IL-4 in human brain tumor cell lines (1). Structural studies have demonstrated that IL-4R in brain tumor cell lines are comprised of type II IL-4R in that the IL-4Rα (also known as IL-4Rβ) chain forms a complex with IL-13Rα1 (also known as IL-13Rα2; Refs. 2–5). The expression of both these chains has also been demonstrated in biopsy/surgical samples and primary glioma cultures (6).

Materials and Methods

Tissue Specimens and Primary Cell Cultures. Tumor specimens were obtained from patients who underwent surgical resection or core biopsy for newly diagnosed GBM (n = 18), astrocytoma (n = 7); low-grade astrocytoma, malignant astrocytoma, anaplastic astrocytoma, anaplastic oligoastrocytoma, subependymal giant cell astrocytoma, and pilocytic astrocytoma), meningioma (n = 2), oligodendroglioma (n = 3), schwannoma (n = 1), and pituitary adenoma (n = 1). Three specimens from normal human brain as well as a neural grid of eight normal brain tissue specimens (Novagen, Madison, WI) were included in the study. Some normal control brain tissue specimens were also obtained from Dr. Wallace W. Tourtellotte, National Neurological Research Specimen Bank, Veterans Affairs Medical Center, West Los Angeles, CA, which is sponsored by National Institute of Neurological Disorders and Stroke/National Institute of Mental Health, the National Multiple Sclerosis Society, the Hereditary Diseases Foundation, and the Veteran Health Services and Research Administration, Department of
Veterans Affairs. All frozen sections (5-μm thickness) were prepared from quick-frozen tumors and stored at −80°C until analysis. Mostly, tissue sections were prepared from 10% formalin-fixed, paraffin-embedded tumors. Two replicate experiments were carried out with each tissue specimen.

To generate primary cell cultures, tumor samples were minced and suspended in HBSS containing 4 mg of DNase, 40 mg of collagenase type IV, and 100 units of hyaluronidase type V (all from Sigma Chemical Co., St. Louis MO) at room temperature for 3 h. The single-cell suspensions were passed through No. 100 nylon mesh, washed twice in HBSS, and added to fibronectin-coated tissue culture flasks. Cells were cultured in media that consisted of DMEM (84%), X-VIVO 15 (10%; Bio-Whittaker, Walkersville, MD), human AB serum (5%; Sigma Chemical Co.), G5 (1%; Life Cell Technologies, Grand Island, NY), and hydrocortisone (10 μg/ml; Ref. 15). The cells were maintained in astrocyte growth medium (AGM bullet kit; Clonetics-BioWhittaker, Walkersville, MD) after first passage. NHAs were also obtained and maintained in an AGM bullet kit (both from Clonetics-BioWhittaker, Walkersville, MD). NT2 (human neuronal cell line) was kindly provided by Dr. Kathy Carbone of Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, MD.

Immunohistochemical Analysis. The frozen sections were incubated, washed, and preincubated with diluted blocking serum before antibody application. Paraffin-embedded sections were deparaffinized by xylene treatment and then washing with gradient alcohol (95–50%) and PBS. Monoclonal antihuman IL-4R antibody (M-57, IgG2b isotype) was kindly supplied by Immunex Corporation, Seattle, WA. This antibody was raised in mice against recombinant human IL-4R p140 and has been shown to bind human IL-4R as determined by immunoprecipitation and immunohistochemistry (16, 17). Immunohistochemistry was performed using the Vector ABC peroxidase kit according to the manufacturer’s instructions. Primary antibody incubation was done for 18 h at 4°C. The chromogen used was 3,3′-diaminobenzidine purchased from Vector Lab (Burlingame, CA). The sections were counterstained lightly with hematoxylin. In general, the concentration of antibodies used for immunohistochemistry was 20 μg/ml. For control, samples were stained with isotype control IgG2b (Bio-Whittaker, Walkersville, MD) or no primary antibody. A field was defined as a field viewed at ×200. The percentage of positive fields were counted by viewing the tumor section under the same magnification. Tissue sections were evaluated and graded for IL-4R staining twice at different time points by one of the authors (F. V.) in the blinded fashion. In general, positive samples were read positive both times.

Protein Synthesis Inhibition Assay. The cytotoxic activity of IL-4 toxin was determined by protein synthesis inhibition assay as previously described (18). Protein synthesis inhibition directly correlates with cell death (8). Typically, 10⁶ GBM cells were cultured in leucine-free medium with or without concentrations of cpIL4-PE for 20–22 h at 37°C. For competition studies, cells were preincubated with IL-4 (2 μg/ml) for 40 min at 37°C before the addition of cpIL4-PE to the cells. Then 1 μCi of [3H]-leucine (NEN Research Products, Wilmington, DE) was added to each well, and cells were incubated for an additional 4 h. Cells were harvested and radioactivity incorporated into cells was measured by a β plate counter (Wallac, Gaithersburg, MD).

Results and Discussion

In Situ Expression of IL-4Rα Chain. A total of 32 brain tumor sections were subjected to immunohistochemical analysis. Eighteen GBM specimens were examined along with 14 other specimens representing other central nervous system tumors. Tissue sections from three specimens of normal brain and a human neural grid from eight normal brain specimens were also stained in parallel with tumor sections. Fifteen of 18 (83%) GBM samples expressed IL-4Rα in situ with varying degrees of immunostaining that ranged from modest to intense staining (1+–4+). Six of seven (86%) astrocytoma specimens (including low-grade astrocytoma, malignant astrocytoma, subependymal giant cell astrocytoma, anaplastic oligoastrocytoma, and pilocytic astrocytoma) constituted the second major group of samples that demonstrated modest to intense (1+–4+) in situ positivity for this receptor. Two meningioma and three oligodendroglioma specimens also showed positivity (1+–4+). One pituitary adenoma sample showed positivity, whereas one schwannoma specimen did not show positive immunostaining for IL-4R (not shown). All nonneoplastic brain specimens (three normal brain tissue sections and eight neural tissue grid specimens) did not show detectable IL-4R staining.

A representative stain of normal brain and a GBM sample is shown in Fig. 1. The section from normal brain did not show immunostaining with or without anti-IL-4R antibody (Fig. 1, panels 1 and 2). However, immunostaining of the sections from GBM specimens showed an intense staining with anti-IL-4R antibody (Fig. 1, panel 4). A higher magnification of the immunohistochemically stained section demonstrated that staining was localized intracellularly and at the cell surface (Fig. 1, panel 6). Similar results were obtained for IL-4R immunostaining of sections from the single subependymal giant cell astrocytoma (Fig. 2A, panels 2 and 3) and oligodendroglioma (Fig. 2B, panel 2 and 3). Again, normal brain cortex (Fig. 2B, panel 1) and normal cerebral cortex (Fig. 2A, panel 1) did not show immunostaining for IL-4Rα chain. In some cases, isotype IgG2b was also used as a control, which showed no staining (data not shown). The positive staining for IL-4 receptor in brain tumor samples was evaluated on a scale of 1+–4+, where 4+ indicated intense expression. Thus, a comparative evaluation of in situ expression of IL-4R within malignant brain tumors and normal brain specimens revealed that IL-4R protein seemed to be overexpressed in the majority of brain tumor specimens. Single anaplastic oligoastrocytoma and schwannoma samples were negative for the IL-4R expression. Additional samples of these two tumors need to be tested to confirm these preliminary observations.

![Fig. 1. Immunostaining of normal brain and GBM sections with IL-4R antibody](image-url)
Primary GBM Cell Cultures Are Highly Sensitive to Cytotoxic Effects of cpIL4-PE. Because previous studies have demonstrated that overexpression of IL-4R on tumor cell lines sensitizes these cells to the cytotoxic effects of cpIL4-PE, we investigated whether GBM tumors that express IL-4R in situ were also sensitive to cpIL4-PE (9, 10, 17, 18). Resolution of this issue is critical for the additional development of this targeted agent for brain tumor therapy. We tested the cytotoxic activity of cpIL4-PE in vitro on 15 primary explants obtained from patients with GBM. Cytotoxic activity of the toxin was evaluated by measuring the inhibition of protein synthesis determined by the incorporation of [3H]-leucine. As shown in Table 2, 13 of 15 primary explant (GBM) cell cultures were highly sensitive to the cytotoxic effect of cpIL4-PE. Protein synthesis was inhibited in a concentration-dependent manner in these samples (not shown). The IC50 (concentration of cpIL4-PE causing 50% protein synthesis inhibition) in six cell cultures was <6 ng/ml and <13 ng/ml in seven additional cultures. However, two GBM explant cells showed a poor response to cpIL4-PE toxin, with IC50s of 200 and 750 ng/ml. One available normal brain cell line (NHA) and the NT-2 neuronal cell line showed limited sensitivity to cpIL4-PE with IC50s of 350 and >1000 ng/ml, respectively. To determine the specificity of the toxin, GBM explant cells were incubated with cpIL4-PE in the presence of recombinant IL-4 (2 μg/ml). IL-4 could block the cytotoxic activity of cpIL4-PE on both glioma and NHAs, indicating that the action of the toxin is mediated through IL-4R (not shown). Because inhibition of protein synthesis directly correlates with cell death (1), these results suggest that IL-4R on glioma tissues will sensitize tumor cells to the cytotoxic effect of cpIL4-PE.

It is of interest to note that the majority of GBM tissue sections

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<th>Specimen no.</th>
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* A field was defined as a field viewed under ×200 magnification. The percentage of positive fields were counted in a blinded manner by viewing the entire tumor section under the same magnification.

b ~b, negative.
(n = 7) showed only 10–40% fields positive for IL-4Rα expression; only two samples showed >50% fields positive. These results indicate existence of heterogeneity within the histologically proven GBM samples. Thus, the expression of IL-4R within the GBM samples may define a unique population of cells otherwise not detectable by conventional H&E (H&E) or glial fibrillary acidic protein (GFAP) staining. Our results also suggest that cPL4-PE may not be effective in eliminating all cells in a GBM tumor. Because cPL4-PE causes apoptotic cell death, it is possible that the dying 10–60% tumor cells may cause a bystander effect on the remainder of GBM cells that express undetectable levels of IL-4R (20). Alternatively, dying tumor cells may recruit inflammatory cells that may cause necrosis of remaining tumor cells. These possibilities are currently being tested. Nevertheless, a majority of GBM primary cell cultures were extremely sensitive to the cytotoxic effects of cPL4-PE.

It is also of interest that 11 different normal brain tissue sections did not show detectable staining with IL-4R antibody, although 7 of 11 normal brain tissue samples were positive for IL-4Rα chain mRNA by RT-PCR analysis (6). Lower sensitivity of immunohistochemical staining is most likely the reason for these results. A recent study has demonstrated expression of IL-4R in astrocytes of epileptic cerebral cortex (14). We have also demonstrated expression of IL-4R protein in one normal astrocytic cell line (6). Consistent with these results, this cell line was minimally sensitive (IC_{50} = 350 ng/ml) to the cytotoxic effect of cPL4-PE. Thus, it is possible that normal brain tissue also may be susceptible to cPL4-PE at high doses. However, most normal brain sections showed undetectable immunohistochemical staining. These results suggest that IL-4R are overexpressed in glioma tumors, and thus it is most likely that glioma tumors can be selectively targeted. These conclusions are supported by our initial clinical trial in which extensive glioma necrosis was observed in the absence of detectable damage of normal brain tissues (13).

The significance of overexpression of IL-4R on tumor cells is not known. It is possible that overexpression of IL-4R is associated with uncontrolled growth of tumor cells. Although many normal cells and brain tumor cells seem to express the IL-4Rα chain, the level of IL-4R expression seems to be higher on tumor cells, including tumors derived from nonmalignant meningioma and pituitary adenoma. This is because these cells are dividing rapidly compared with quiescent normal cells. Additional studies are ongoing to determine the significance of overexpression of IL-4R in brain tumors.

In conclusion, we demonstrate that human brain tumors with different histopathological subtypes express IL-4R in situ. In addition, the majority of GBM primary cell cultures, but not normal astrocytes or a neuronal cell line, are found to be highly sensitive to cPL4-PE. Thus, differential expression of IL-4R may offer a therapeutic window for the targeting of IL-4 cytokinin for brain tumor therapy. Ongoing clinical trials will establish further the clinical activity of IL-4 cytokinin.

Acknowledgments

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References